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GENETICS AND GENOMICS STUDIES OF RICE DISEASE RESISTANCE AND DEVELOPMENT OF ALTERNATIVE DISEASE MANAGEMENT METHODS FOR BACTERIAL PANICLE BLIGHT AND SHEATH BLIGHT

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Plant Pathology and Crop Physiology

by
Bishnu Kumar Shrestha
B. S., IAAS, Tribhuvan University, Nepal, 2005
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December 2014

This dissertation is dedicated to my late mother Shanta Laxmi Shrestha and my family for their love, support and wishes throughout my life.

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ABSTRACT

Bacterial panicle blight (BPB) and sheath blight (SB), caused by the bacteria *Burkholderia glumae* and *B. gladioli*, and the fungus *Rhizoctonia solani*, respectively, are two major rice diseases in southern rice growing regions of US. No completely resistant rice cultivars have been identified for these diseases. However, a medium-grain cultivar, Jupiter, showed partial resistance to BPB. In order to understand the mechanisms of rice resistance against BPB and SB, rice genetics and genomics studies have been conducted. Alternative methods to suppress BPB and SB were also studied. Broad-sense heritability and correlations were calculated for the traits, BPB and SB disease ratings, days to heading, and plant height, with recombinant inbred lines generated from a cross between Trenasse and Jupiter in replicated trials for two years. Days to heading and plant height had high heritability, and were negatively correlated with BPB and SB disease ratings. The traits with high heritability will not have environmental influence, and can be used as indirect selection tools.

Study on genomic characteristics of five rice genotypes grown in Louisiana using their whole genome sequence data provides genome-wide DNA polymorphisms among them. These information will enable us to understand genetic elements for phenotypic variations among these genotypes, which will help to enhance the genetic studies of US rice cultivars. The sequence data were also used to develop microsatellites and single nucleotide polymorphism markers, which can be used for genetic mapping studies.

Previous microarray studies showed that the gene encoding a NAC4-like transcription factor, named **b**acterial **p**anicle **b**light **r**esponse gene **1** (*BPR1*), was highly up-regulated in Jupiter upon *B. glumae* inoculation. Expression of *BPR1* in response to *B. glumae* was not detected in both Jupiter and Trenasse at seedling and tillering stages. However, rapid induction

of *BPR1* expression was observed in Jupiter, but not in Trenasse, when it was treated with *B. glumae* or chemicals, such as ascorbic acid and jasmonic acid, at its heading stage, suggesting that *BPR1* expression is tissue-specific, and might be involved in rice defense response against *B. gluame*.

Several rice-associated bacteria (RAB) isolated from healthy rice leaves were tested for their ability to suppress BPB and SB in rice. Those RAB were able to suppress bacterial cell growth and sclerotia germination *in vitro*, and were able to reduce the BPB and SB symptoms in rice in the field. Based on the 16S rDNA sequencing analysis, those RABs were identified as *Bacillus* and *Lysinibacillus* spp., and are potential candidates for biological control agents.

CHAPTER I: GENERAL INTRODUCTION

1.1 INTRODUCTION

Rice belongs to the grass family Poaceae, and to the genus *Oryza*. Rice is a major staple for about 50% of world population, and 90% of which are from Asian countries (Mohanty, 2013). It provides about 35- 60 % of the calories intake to the people from developing countries. It comes after wheat in terms of area and production. It can be grown in wide ranges of soil moisture regimes and growing environments, including irrigated and rain-fed lowlands, wetlands, and uplands. It is grown in more than one hundred countries around the world with acreage of 158 million hectares of cultivated area (http://ricepedia.org/rice-as-a-crop/rice-productivity). Two species of rice are commonly grown: *Oryza sativa*, known as Asian rice, which is grown worldwide, and *O. glaberrima*, known as African rice, which is grown in some parts of west Africa. *O. sativa* has two subspecies, *indica* and *japonica*. *Indica* rice has light green leaves, long grains, and tends to shatter more easily. It is grown in the tropical and subtropical regions, whereas *japonica* rice is grown in cooler regions of subtropical and in temperate regions. *Japonica* rice is characterized with short plant height with narrow, dark green leaves, short and round grains, and does not shatter easily compared to *indica* rice (http://ricepedia.org/rice-as-a-plant/rice-species/cultivated-rice-species).

Domestication of rice was supposed to be started before 9000 years ago (Huang et al., 2012; Molina et al., 2011). *Indica* rice was grown in Indian subcontinent and was brought to Madagascar and East Africa, and then to West Africa, whereas *japonica* rice was supposedly domesticated in southeast Asia, and brought to northern regions including Korea and Japan as early as first century. Migrants of Indonesia introduced *japonica* rice in Madagascar in 5th century. Similarly, Portuguese introduced *indica* and tropical *japonica* to Brazil from Indonesia, and later Spanish people brought it to Latin Americas. Rice was introduced in the United States,

in South Carolina, in 1685 from Madagascar (Khush, 1997). From South Carolina, rice was brought to the southern regions of US in 19th century (www.lsuagcenter.com).

In the United States, about 40 commercial rice varieties are grown in six states including Arkansas, California, Louisiana, Mississippi, Missouri, and Texas (USA rice Federation, http://riceinfo.com/media-resources/usa-rice-federation-fact-sheet). USA is one of the largest rice exporters after India, Vietnam, Thailand, and Pakistan (Production, Supply and Distribution Online; Foreign Agriculture Service, USDA). It produced 8,613,094 tones of rice from 998,765 hectares of harvested area in 2013 (http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor).

Growth of the world population is increasing in geometric proportion and the food production in an arithmetic proportion that is creating a gap between demand and supply of food. At the same time cultivable agricultural land is also decreasing due to overgrowing population. It is becoming a challenge to produce surplus food to fulfill the demand of ever increasing population of the world with limited cultivable agricultural land resources. The demand for rice is growing day by day due to rapid growth of population mainly in rice feeding countries. It is necessary to increase the grain production by 50% by 2025 to meet the food demand. The development of modern tools of biotechnology and its use in crop production will be useful to improve crop yield with higher level of micronutrients (Khush, 2001). However, about 10% of the crop production is reduced due to various plant diseases caused by various plant pathogens including bacteria, fungi, and viruses (James, 1998).

Rice production around the world is threatened due to various rice diseases including fungal diseases, such as sheath blight (SB), brown spot, and blast caused by *Rhizoctonia solani*, *Cochliobolus miyabeanu*, and *Pyricularia grisea*, respectively; bacterial diseases, such as

bacterial leaf blight and bacterial panicle blight caused by *Xanthomonas oryzae* pv. *oryzae* and *Burkholderia glumae* and *B. gladioli*, respectively; and several other seedling diseases. In the rice-growing areas of United States including Louisiana, these diseases cause about 7 to 15% annual yield loss (Groth, 2008; Latif et al., 2011). Bacterial panicle blight (BPB) caused by bacteria, *B. glumae* and *B. gladioli*, is an economically important rice disease in the southern United States (Nandakumar et al., 2007). However*, B. gladioli* is less virulent and found less frequently compared to *B. glumae* from naturally infected rice plants (Nandakumar et al., 2009). *B. glumae* was previously described as a causal agent of grain rot, seedling blight, and seedling rot of rice in Japan in 1956 (Goto & Ohata, 1956). BPB became an emerging rice disease around the rice growing areas of the world including Korea, Vietnam, USA, the Philippines, China, South Africa, and recently in Ecuador (Cottyn, B. et al., 1996; Cottyn, B et al., 1996; Luo et al., 2007; Riera et al., 2014; Shahjahan et al., 2000; Trung et al., 1993; Zhou, 2014). BPB is characterized by discoloration of panicles with sterile florets that causes reduction in yield. A typical BPB symptom in rice is shown in Figure 1.1. High humidity and high night temperature, above 90°F during the growing season favors the epidemics of BPB (Kurita et al., 1964; Tsushima et al., 1995). Flowering time of rice plants grown in late season coincides with high temperature environment, resulting in more disease development in Louisiana. So, BPB development occur more frequently in late season rice plants compared to early season plants. Severe outbreaks of BPB have been reported to have occurred in Louisiana, and causing about 40% of yield loses in severely infected fields in 1995, 1998, and 2000 (Nandakumar et al., 2009; Shahjahan et al., 2000). As high temperature favors this disease, it will be one of the major rice diseases around the rice growing regions of the world due to global warming (Ham et al., 2011; Schaad, 2008).

B. glumae, a rod-shaped, Gram-negative bacteria with four polar flagella is one of the two causal agents of BPB. It produces water-soluble yellow pigment in nutrient rich media including King'B, potato dextrose agar, and Luria Broth media, when incubated at 37°C, but toxoflavin production is much lower at the temperatures below 30°C (Matsuda & Sato, 1988). The yellow phytotoxin, toxoflavin, is a major virulence factor and regulated by quorum-sensing of *B. glumae* (Kim et al., 2004). Toxoflavin acts as an active electron carrier between NADH and oxygen that helps to generate peroxides, and this peroxides can be poisonous to plants (Latuasan & Berends, 1961). *B. glumae* is a seed-borne pathogen with wide host ranges causing bacterial wilt in tomato, sesame, perilla, and eggplant (Jeong et al., 2003). Infected rice seeds act as a primary source of inoculum for the following year (Tsushima, 1996). As rice plants develop this pathogen moves from lower leaves to upper leaves and ultimately colonizes flag leaf, and that colonization is essential for disease development (Tsushima, 1996; Tsushima et al., 1991). It enters through stomatal openings in the lemma and palea of rice seed, multiplies in the intercellular space, and uses vascular system of the plant for the long distance movement (Tabei et al., 1989; Yuan, 2004).

Sheath blight (SB), a major rice disease worldwide, is caused by a fungal pathogen *R. solani*. This disease is an economically important disease in the southern United States (Damicone JP, 1993; Lee & Rush, 1983). Yield loss was estimated to be between 1 to 10% in lowland rice in tropical regions of Asia (Savary et al., 2000). High nitrogenous fertilizers and plant densities create conducive microclimates for the development of sheath blight. Early maturing rice cultivars are more prone to this disease susceptibility compared to late maturing due to the more favorable environmental conditions during the early cultivation of rice. Since it is a soil-borne disease, soil-borne sclerotia act as a primary source of inoculum that infect the

water-line sheath area of rice plant (Belmar et al., 1987; Lee, 1980). Water-soaked, circular to ellipsoid lesions during late tillering stage on sheath of lower leaves of rice characterize sheath blight in rice. A typical symptom of sheath blight in rice is shown in Figure 1.2. High temperature of 80 to 90°F with relative humidity of 95% favors those lesions to spread rapidly toward upper parts of rice plants using hyphae. In this case, hyphae act as a secondary source of inoculum. Those lesions merge together covering whole plant parts. The lodging of plants ultimately cause reduced yield and grain quality. Losses in grain yield and reduced milling quality caused by sheath blight in the United States were estimated up to 42% and 20%, respectively (Marchetti, 1983). Upon maturity sclerotia will form near the infected tissues, and those sclerotia are prone to separate from the plant after maturity (Lee & Rush, 1983). These sclerotia overwinter on soil and serve as a primary source of inoculum for the following year.

Various types of cultural practices including crop rotation and clean cultivation, biological control, judicious use of pesticides and fungicides, and exploitation of genetic diversities of several landraces and germplasm are the common practices used for plant disease management. In addition, use of biotechnology techniques is an emerging field for crop improvement in recent years.

Oxolinic acid, a quinolone derivative, was used for rice seed treatment before sowing in the rice field to manage BPB. This chemical inhibits DNA synthesis in bacterial cell by inhibiting supercoiling activity of the DNA gyrase (Drlica & Zhao, 1997; Nandakumar et al., 2005). However, this chemical agent is not allowed for rice treatment in the US (Nandakumar et al., 2009). Moreover, some strain of *B. glumae* develops resistance to oxolinic acid (Maeda et al., 2004). Host resistance is a desirable and durable control measure for most of the plant diseases. However, most of the rice cultivars commercially grown in Louisiana are susceptible to BPB

(Nandakumar et al., 2005; Shahjahan et al., 2000) and SB. None of the rice cultivars are completely resistant to both diseases. However, a medium-grained rice cultivar, Jupiter and a long-grained, gamma-radiated mutant-derivative of Lemont, LM-1, developed by LSU AgCenter showed high levels of partial resistance against BPB (Groth et al., 2007; Sha et al., 2006). Better understanding of the molecular mechanisms of rice resistance will give an insight to develop disease resistant lines and cultivars. Application of biocontrol agents including avirulent strains of *B. glumae* and bacteriophages that lyse *B. glumae* have also been introduced to suppress seedling rot and seedling blight caused by *B. glumae*, which can be more efficient than chemical control to manage BPB in rice (Adachi et al., 2012; Furuya et al., 1991). In addition, genetically modified strain of *Burkholderia* sp. has been used as a biocontrol agent to reduce seedling rot in rice caused by *B. glumae*. This genetically modified biocontrol agent contains an N-acyl-homoserine lactonase (*aiiA*) gene derived from *Bacillus thuringiensis* that inhibits the production of quorum-sensing signal in plant pathogenic *B. glumae* (Cho et al., 2007).

Unlike BPB, various types of fungicide trials have been conducted to manage sheath blight in rice (Araki & Yabutani, 1993; Groth, 2005; Miah et al., 1994). Use of these fungicides, however, increases the cost of cultivation, leaves a residual effect to the environments, and increases the risk of development of fungicide-resistant fungi (Bennett, 2012). Use of biological control agents can be an alternative method to fungicide usage to manage and suppress sheath blight in rice. Various epiphytic, endophytic, and rhizospheric bacteria have been isolated either from plant parts or from soil, and used to manage and suppress sheath blight in rice (De Costa et al., 2008; Kanjanamaneesathian et al., 1998; Nagendran et al., 2014; Padaria & Singh, 2009; Wang et al., 2013). Those types of biocontrol agents were used either by spraying on plant parts or by soil treatments (Soe & De Costa, 2012). Various bacterial species including *Pseudomonas*

fluorescens, *Trichoderma* sp., *Aspergillus niger*, and hyphal colonizing *Burkholderia vietnamiensis* have been reported as biocontrol agents to manage sheath blight in rice (Cuong et al., 2011; Devi et al., 1989; Naeimi et al., 2010). These biocontrol agents produce different kinds of secondary metabolites including *β*- 1,3-glucanase, hydrogen cyanide, chitinase by *P. fluorescens*, 2,4-diacetylchloroglucinol by *P. putida*, and pyrrolnitrin by *B. cenpacia* that suppress mycelial growth of *R. solani*. These metabolites, when sprayed in pure form or in the form of culture filtrates, can reduce growth and development of disease in rice. (Nagarajkumar et al., 2004; Rosales et al., 1995). In addition, these bacterial agents not only suppress disease symptoms but also help to induce plant resistance, and stimulate growth of plant and enhance yield (Niranjan Raj et al., 2006). Different formulations of biocontrol agents either by using two or more biological agents together or by integration with different fungicides and fertilizers have increased effectiveness of its usage (Datnoff et al., 1995; Duffy & Weller, 1995; Kanjanamaneesathian et al., 1998).

Similar to BPB, there is no completely resistant rice cultivars for sheath blight, but it has been reported that partial or horizontal resistant rice cultivars are available (Lee & Rush, 1983; Liu et al., 2009). Various trials have been conducted in rice growing areas to identify sources of resistance for sheath blight from wild relatives of rice. However, those wild relatives of rice also lack complete resistance. Several moderately resistant rice cultivars for sheath blight have been described from several different areas in the world. In the US, medium-grained rice cultivars have higher level of resistance compared to long-grained rice cultivars (Lee & Rush, 1983).

Figure 1.1 Typical symptom of bacterial panicle blight in rice.

Figure 1.2 Typical symptom of sheath blight in rice.

Plants are continuously facing various stresses including biotic and abiotic stresses, which reduce plant performance in terms of yield and quality. Regarding biotic stresses, various plant pathogens including bacteria, fungi, viruses, and nematodes cause huge crop losses every year. Plants are evolving with efficient mechanisms to confront the challenges from external stresses. Physical as well as chemical barriers are developed as plant defense mechanisms that will hinder the plant pathogen infection. In addition, some other defense mechanisms are activated by external signal associated with pathogen infection, which is called microbe-associated molecular patterns (Bittel & Robatzek, 2007). It involves signal transduction after pathogen infection that induces expression of various genes associated with plant defense (Yang et al., 1997). Gene-for-gene interaction, also known as vertical resistance, in which a single avirulence gene of a pathogen is recognized by its corresponding resistance (*R*) gene in host plant (Flor, 1971). Various *R* genes conferring complete resistance from several plant hosts have been characterized, and these *R* genes share conserved motifs. Most of the *R* genes encode nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins, which occur in a clusters at specific loci following gene amplifications and duplications (Marone et al., 2013).

Various studies report involvement of several *R* genes for major rice diseases, including blast and bacterial blight, caused by *Pyricularia grisea* and *Xanthomonas oryzae oryzae*, respectively. Some of the *R* genes were well characterized and cloned, and most of them showed race-specific resistance and have dominant trait. *R* genes for *X. oryzae oryzae*, including *Xa21*, encoding leucine-rich repeat/ kinase receptor protein, and *Xa1* nucleotide binding site- leucine-rich repeat were identified by map-based cloning (Song et al., 1995; Yoshimura et al., 1998). However, unlike bacterial blight of rice, no known dominant *R* gene(s) has been reported yet for BPB and sheath blight. It has been reported that most *R* genes are strain-specific and are not durable because it will lose its effectiveness easily during time course (McDonald & Linde, 2002). So, concept of non-host resistance evolved, in which all plant species show resistant to specific pathogen (Heath, 2000). Vertical resistance governed by a single *R* gene is monogenic, while horizontal disease resistance govern by many genes with cumulative effects is polygenic.

Various agronomically important traits, including plant height, yield, abiotic and biotic stress resistance are controlled by polygenes. The polygenic resistance is durable, non-race specific and are quantitative in nature. Study of these traits controlled by multiple genes is complex and can be performed with the help of molecular markers by quantitative trait loci (QTL) mapping (Tanksley, 1993). The QTL mapping was first described by Sax in 1923 (Sax, 1923). With the help of QTL mapping, genetic architecture of a trait, its relationship with biological function can be determined. QTL mapping of any quantitative trait is highly dependent on the level of effect on specific phenotype. Large population is required to detect the QTL for a trait with small effects. In addition, large populations with large number of molecular markers help to detect recombination effects resulting in increased mapping resolution (Lander & Botstein, 1989). Unlike other rice diseases, very few reports were available for partial

resistance to BPB and bacterial grain rot in rice caused by *B. glumae* (Mizobuchi et al., 2013; Pinson et al., 2010). In these studies, heading date of rice was correlated to BPB resistance. So, little is known about the QTL mapping of BPB in rice. However, several studies were conducted for the QTL mapping of sheath blight in rice (Channamallikarjuna et al., 2010; Sharma et al., 2009; Zou et al., 2000).

QTL mapping utilized DNA-based molecular markers including, random-amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), and simple sequence repeats (SSR), which facilitate isolation of loci along the chromosomes associated with agronomically important traits such as disease and insect resistance (McCouch et al., 1988). In recent years, single nucleotide polymorphisms are used for genetic mapping and association mapping (Kumar et al., 2012; Rafalski, 2002). Each type of molecular marker has its own advantages and disadvantages. Choice of these molecular markers depends on the structure of population, availability of marker, technological complexity and cost (Staub et al., 1996).

Analysis of genome sequence of cereals including corn and rice has been conducted since 1990 to identify genes associated with biotic and abiotic stress tolerance, and agronomically important traits (Sasaki et al., 2008). However, the advent of next-generation sequencing technology is making large-scale genomic studies possible with large amount of genomic data. Several next generation sequencing platforms are available for whole genome sequencing. Each platform has its specific characteristics, which includes pyrosequencing-based 454 (Margulies et al., 2005), sequencing-by-synthesis-based Illumina (Bentley, 2006), sequencing-by-ligation-based SOLiD (Valouev et al., 2008), hydrogen-ion detecton-based Ion-torrent (Rusk, 2010), and Pacific Bioscience (Brakmann, 2010) and Oxford Nanopore (Clarke et al., 2009). These

advances in platforms helped to reduce the cost of sequencing and time drastically (Bentley, 2006).

Genome sequencing of economically important cereal crops has provided a comprehensive analysis of the gene structure, function, and gene-trait relationships. Identification of variants and development of various molecular markers can be implemented using sequence information from next-generation sequencing technology including SSR and SNPs (Qu & Liu, 2013; Zhang et al., 2007; Zou et al., 2013). This leads to formation of bridge between the genotype and phenotype. In addition to its implications for a trait of an individual, next-generation sequencing technology provides an opportunity for population genomic and evolutionary genomic studies (Fumagalli et al., 2013).

Early domestication and genetic diversity of rice, relatedness between cultivated and wild relatives of rice, and association studies of agronomically important rice traits have been conducted with the help of whole genome sequence data. Along with bulk-segregant analysis techniques, whole genome sequencing data was used for QTL mapping, host pathogen interaction, and identification of candidate and/or specific genes for a particular trait, including sheath blight resistance in rice (Abe et al., 2012; Silva et al., 2012; Takagi et al., 2013; Terauchi et al., 2011).

Furthermore, RNA-Seq and ChIP-Seq were used to analyse genome-wide expression of genes, and DNA-binding protein activity, respectively (Nagalakshmi et al., 2010; Park, 2009). In recent years, sequencing of organisms of several ecological niches, known as metagenomics, is providing the genetic information of the particular environment. Those information can be used to manipulate environment for beneficial uses.

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CHAPTER II: PHENOTYPIC CHARACTERIZATION OF RECOMBINANT INBRED LINES (RILS) GENERATED FROM THE CROSS BETWEEN TRENASSE AND JUPITER

2.1 INTRODUCTION

Rice is a major staple throughout the world. It is a major source of calories in diet for people in many Asian countries. Rice is grown in more than 158 million hectares of cultivated area around the world. In the United States, rice industry is a multi-billion dollar industry. It produces approximately 19 billion pounds of rice on more than 2 million acres of land. The United States covers 2% of world rice market and ranks among the top five rice-exporting nations (USA Rice Federation, 2007). Rice is grown in six states, including Arkansas, California, Louisiana, Mississippi, Missouri, and Texas in the United States. Planted and harvested area of rice in 2013 was estimated to be 2.49 and 2.47 million acres, respectively, which is 8% lower than 2012. However, average yield throughout the United States was estimated 7,694 pounds per acre, which is 245 pounds higher than in 2012. Favorable environmental conditions with new varieties and dry weather during harvesting helped to increase the yield in the southern United States (USDA, 2013). Among these states, Louisiana is the third-largest rice producer, and mostly grown long-grain rice. In 2013, rice has been cultivated on about 410 thousands acres of land with 31 millions cwt. of rice with gross value of more than $494 million.

Various abiotic and biotic stresses on rice plants are reducing the rice production each year worldwide. Bacterial and fungal diseases are major threats for rice industry. Bacterial panicle blight (BPB), caused by *Burkholderia glumae* and *B. gladioli*, and sheath blight, caused by *Rhizoctonia solani* are the two economically important diseases in the southern rice growing states of the United States including Louisiana (LSUAgCenter, 2013). The environmental conditions in Louisiana during rice growing season favors the development of both diseases. Prolonged high temperature and high humidity favor BPB development (Cha et al., 2001). BPB

causes panicle sterility, thereby reducing total rice yield, whereas sheath blight causes dark-brown ellipsoid lesions on the sheath causing rice plant prone to lodging (Lee & Rush, 1983; Shahjahan et al., 2000). About 10 to 20% of yield loss has been reported each year due to BPB and sheath blight in rice (Groth, 2008; Latif et al., 2011; Savary et al., 2000; Shahjahan et al., 2000).

Despite its economic importance, few control measures have been developed so far for BPB, and no chemical pesticide has been registered to control BPB in the US. For controlling sheath blight, few fungicide treatments are available (Groth, 2005; Miah et al., 1994). Various cultural management practices including proper use of inputs such as disease resistant varieties, optimum seed rate, recommended doses of fertilizers, and changing planting dates help to reduce BPB as well as sheath blight (LSUAgCenter, 2013). However, there are few rice cultivars that are resistant to either BPB or sheath blight. Most of the commercially grown rice cultivars in Louisiana are susceptible to BPB and sheath blight, and only some of the cultivars are partially resistant to these diseases (Lee & Rush, 1983; Sha et al., 2006; Shahjahan et al., 2000). However, genetic and molecular mechanisms of the partial resistance to BPB and/or sheath blight are still unknown. Better understanding of disease resistance at both phenotypic and genotypic levels is important for the rice breeders and geneticists to develop strategies for effective selection. In rice, late flowering trait was reported to be associated with BPB resistance, and similarly, plant height and days to heading were shown to be associated with sheath blight resistance (Pinson et al., 2010; Sharma et al., 2009).

Various genetic parameters for agronomically important traits have been studied. A commonly used parameter for quantitative traits, like disease resistance is heritability, which measures the proportion of phenotypic variance that is due to genetic factors. Estimation of

heritability of traits provides power to the breeders for the effective selection (Bernardo, 2002). There are various ways to estimates heritability from various populations including double haploids population, backcross population, and recombinant inbred lines (RILs). Use of recombinant inbred line (RIL) population is appropriate to estimate heritability of a trait (Dudley & Moll, 1969). In addition, these RILs are useful assets for mapping quantitative trait loci (QTL) fro target traits. RILs are developed by crossing two lines with contrasting agronomic traits of interest, and hybrids resulting from the cross are self-pollinated to develop F_2 plants. Individual F_2 plants are further advanced using single seed descent method until F_7 to F_9 by selfing (Brini, 1966). Several studies on the heritability of agronomical traits of rice including sheath blight and bacterial blight resistance, days to heading, plant height, and yield have been conducted, which showed medium to high heritabilities. Development of rice breeding line with desirable features depends heritability of traits (Aung, 1990; Mazid et al., 2013; Nelson et al., 2012). The focus of this study was to estimate heritability and calculate correlations among phenotypic traits including bacterial blight resistance, sheath blight resistance, days to heading, and plant height, using RILs derived from the cross between Trenasse and Jupiter.

2.2 OBJECTIVES

i. To estimate heritability of phenotypic traits, including bacterial panicle blight reistance, sheath blight resistance, plant height, and days to heading, and
ii. To study correlations among those phenotypic traits.

2.3 MATERIALS AND METHODS

A mapping population was developed from a cross, between a partially resistant medium-grained cultivar Jupiter (Sha et al., 2006), and a susceptible long-grained cultivar Trenasse (Linscombe et al., 2006; Nandakumar et al., 2007). A total of 300 F_2 plants were chosen and

grown to develop recombinant inbred lines (RILs) following single seed descent method. Briefly, individual panicles harvested from each F_2 plant were grown, and progeny from F_2 individual plants were developed as separate lines in each generation by harvesting a single panicle per plant. Seeds from F_5, F_6 and F_7 generations were used to grow in 2012, 2013 and 2014, respectively, in this study to represent the RILs.

RILs were grown at the Rice Research Station, Crowley, with two replicates. Each replication includes rows for parental lines, and each row contains approximately 15 to 20 plants. Phenotypic evaluation for two economically important rice diseases, BPB and SB, and other important agronomic traits, plant height and days to heading, was conducted in 2012 and 2013. In 2014, only sheath blight was assessed.

2.3.1 Inoculation of RILs with *Burkholderia glumae* 336gr-1 inoculum

Each replication of RILs was inoculated with 1×10^8 CFU/ml of bacterial inoculum prepared in deionized water with the help of hand sprayer in 2012 and 2013. Inoculation was done until the inoculum run off at 30% heading stage of rice plants. Since there was no synchronization of heading in a population to reduce the chance of escapes, at least four inoculations were done in 2-4 days interval.

2.3.2 Inoculation of RILs with *Rhizoctonia solani* inoculum

Both replications of RILs were inoculated with the inoculum of *R. solani* at an active tillering stage of rice plants in 2012 and 2013. *R. solani* inoculum was prepared in rice grain:hull (1:2 v/v) mixture. Briefly, 600 gm of the mixture with 500 ml of water was sterilized at 121°C for 30 min. The sterilized mixture in a flask was inoculated with ~16 cm^2 of PDA plugs containing 5 to 7 days old *R. solani* mycelia, and incubated at 25°C for 10 days. After 10 days, inoculum was prepared and mixed with larger volume of the sterilized rice grain:hull mixture at

1:2 ratio and sterilized to increase the inoculum volume. After proper mixing the mixture was spread uniformly on a clean brown paper sheet and covered with a clean plastic sheet at room temperature. Inoculum for application was ready in about 24 h of incubation.

2.3.3 Field evaluation

Data on major agronomic traits, including days to heading, disease score of both BPB and sheath blight, plant height, were recorded for all experiments.

1. Days to 50% heading was recorded in each row of two replications in 2012 and 2013 as the number of days from planting until 50% of the plants in each row have fully visible panicles.
2. BPB symptoms on panicles were observed and recorded 10 days after last inoculation. Disease scores were rated visually identifying the percentage of discoloration of panicles on a 0 to 9 scale; 0 means no symptoms and 9 means more than 90% of panicles area were infected with BPB (Shahjahan et al., 2000).
3. The symptoms for sheath blight were observed and recorded during the milk stage of rice. Disease scores were rated visually identifying the percentage of leaf sheath showing sheath blight symptoms. Disease scores were rated from 0 to 9 scale (IRRI, 1996).
4. Plant heights for RILs were taken during maturity on randomly selected three plants in each row measuring from the base of the plant to the tip of panicle.

2.4 DATA ANALYSIS

Means of the traits for individual parents and populations in individual year as well as across years were calculated. Analysis of variance and broad sense heritability were calculated using PROC GLM and SAS procedures, respectively, in SAS 9.4 (Holland et al., 2003; SAS

Institute, 2013). Furthermore, correlations among the phenotypic traits were calculated using Pearson's correlation coefficients for each of the individual year. Broad sense heritability of each trait for single year was calculated by using the formula:

$$\text{Broad sense heritability } (h^2) = \frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_e}{r}}$$

Similarly, estimation of heritability of each trait for multiple years with several replications per year was calculated by using the formula:

$$\text{Broad sense heritability } (h^2) = \frac{\sigma^2_g}{\sigma^2_g + \frac{\sigma^2_{ge}}{n} + \frac{\sigma^2_e}{(n \times r)}}$$

where, 'σ^2_g' is the genotypic variance, 'σ^2_{ge}' is the genotype × environment variance, 'σ^2_e' is the error variance, and 'n' and 'r' are the number of years and replications, respectively (Holland et al., 2003).

Heritability is defined as the proportion of observed phenotypic variations due to genetic differences. Higher heritability value in a population suggests that selection will be effective on an individual basis while low heritability in a population suggests that selection on an individual basis will not be effective. Hence, heritability values will be useful to determine the probable success of transferring trait of interest among varieties.

2.5 RESULTS

2.5.1 Days to heading

A long-grain cultivar, Trenasse, showed earlier heading than the medium-grain cultivar Jupiter in both years. However, both cultivars had earlier heading in 2012 than in 2013 (Table

2.1, Figure 2.1 [a, b]). Average days to 50% heading of Trenasse and Jupiter across years were 94 days and 102 days, respectively, whereas RILs population took 104 days for heading across years. In 2012, average heading days of RILs was higher than both of the parents, and in between two parents in the year of 2013. Significant differences were obtained in the number of days to 50% heading from seeding day in RILs population in both years. Genotype by environment interactions was highly significant for this trait (Table 2.2). Heritability for days to heading was higher, 0.89, 0.84, and 0.71 in 2012, 2013, and across years respectively (Table 2.2).

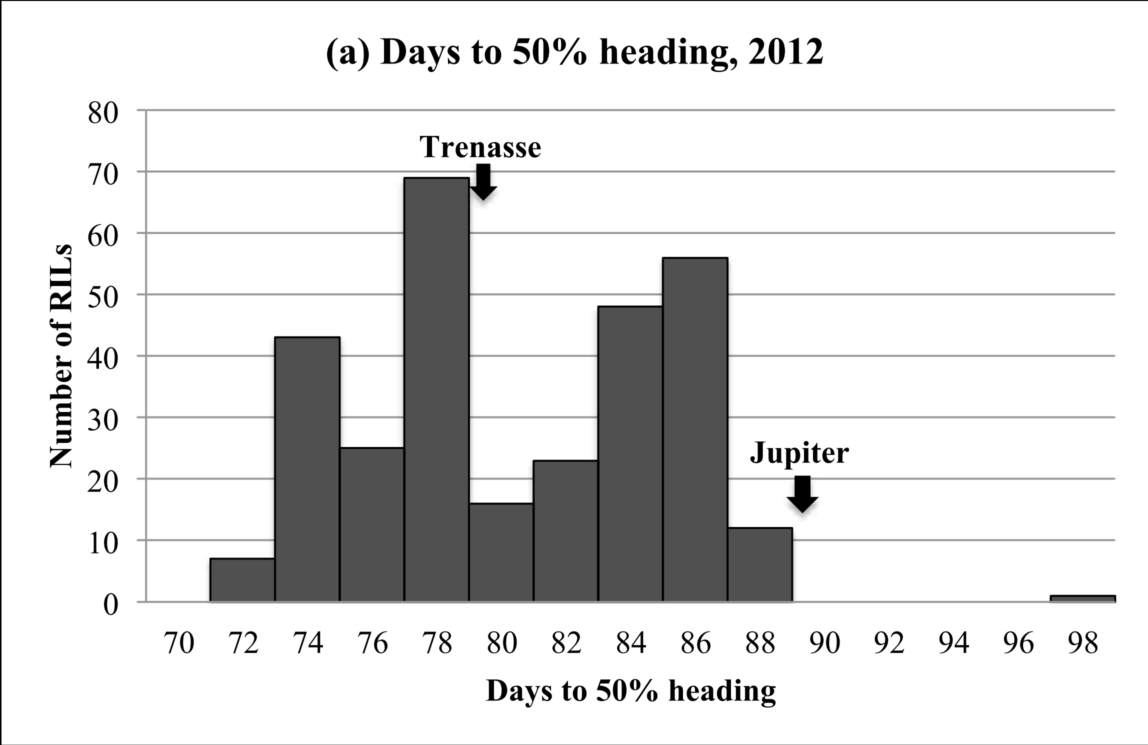
Table 2.1 Mean days to 50% heading of Trenasse, Jupiter, and RILs, derived from Trenasse × Jupiter, taken in 2012, 2013, and across years.

	2012	2013	Across years
(Mean) Trenasse	79	109	94
(Mean) Jupiter	89	114	102
(Mean) RILs	81.79	112.4	97.12
Range	74-102	105-124	74-124
Standard deviation	4.79	3.33	15.87

Table 2.2 Sources of variation and their *F* values, and heritability estimates for days to 50% heading of RILs, generated from Trenasse × Jupiter, taken in 2012, 2013, and across years.

	2012	2013	Across years
Replication	1.60*[a]	5.14*	3.06*
Genotype (RILs)	9.79**	6.45**	13.03**
Environment (Years)	-	-	77745.1**
Genotype (RILs) x environment (Years) interaction	-	-	3.79**
Broad sense heritability	0.89	0.84	0.71

[a] * and ** = Significant at the 0.05 and 0.01 level, respectively



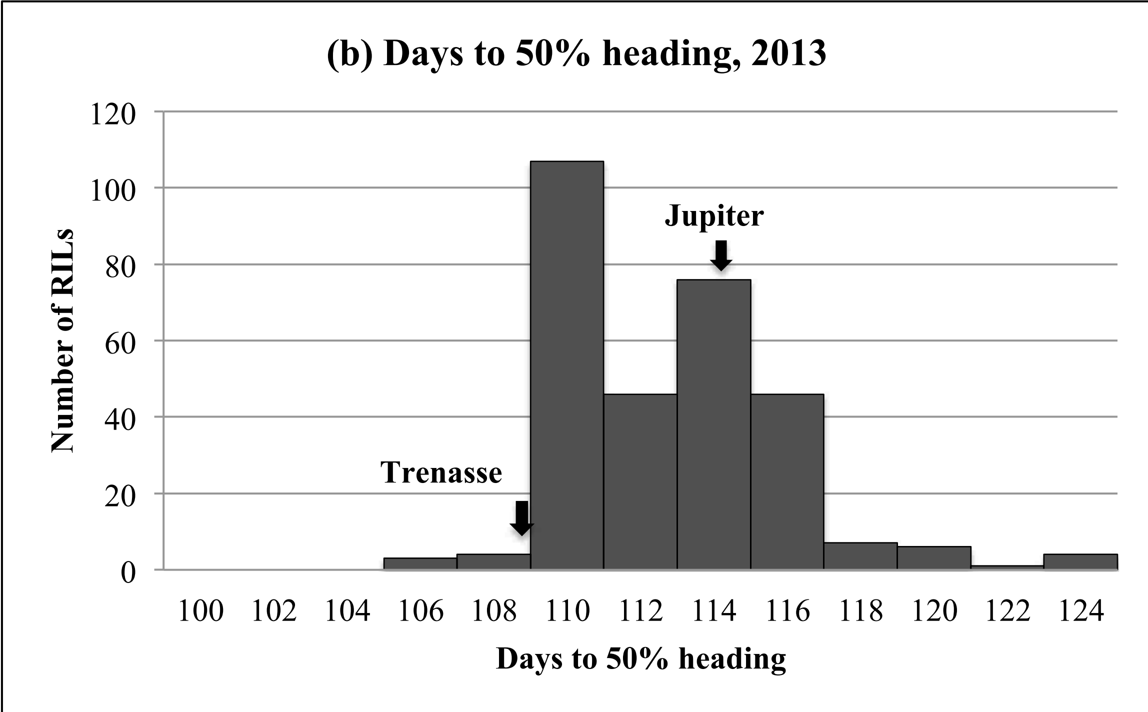


Figure 2.1 Frequency distribution of RIL population for days to 50% heading in the Rice Research Station, Crowley in 2012 (a) and in 2013 (b). Arrows indicate mean values for the parental cultivars, Trenasse and Jupiter.

2.5.2 Disease score for bacterial panicle blight

Trenasse had higher BPB score than Jupiter in 2012, 2013, and across years. Average BPB score of RILs was 6.71 in 2012, 5.43 in 2013, and 6.07 in across years, which was in between disease scores of two parental cultivars (Table 2.3 and Figure 2.2 [a, b]). Significant variation was observed between the genotypes in 2012, 2013, and across years. Significant effect of genotype by environment on BPB score was observed (Table 2.4). Heritability estimates was medium, 0.57, in 2012, higher, 0.84 in 2013 and lower, 0.25 in across years (Table 2.4).

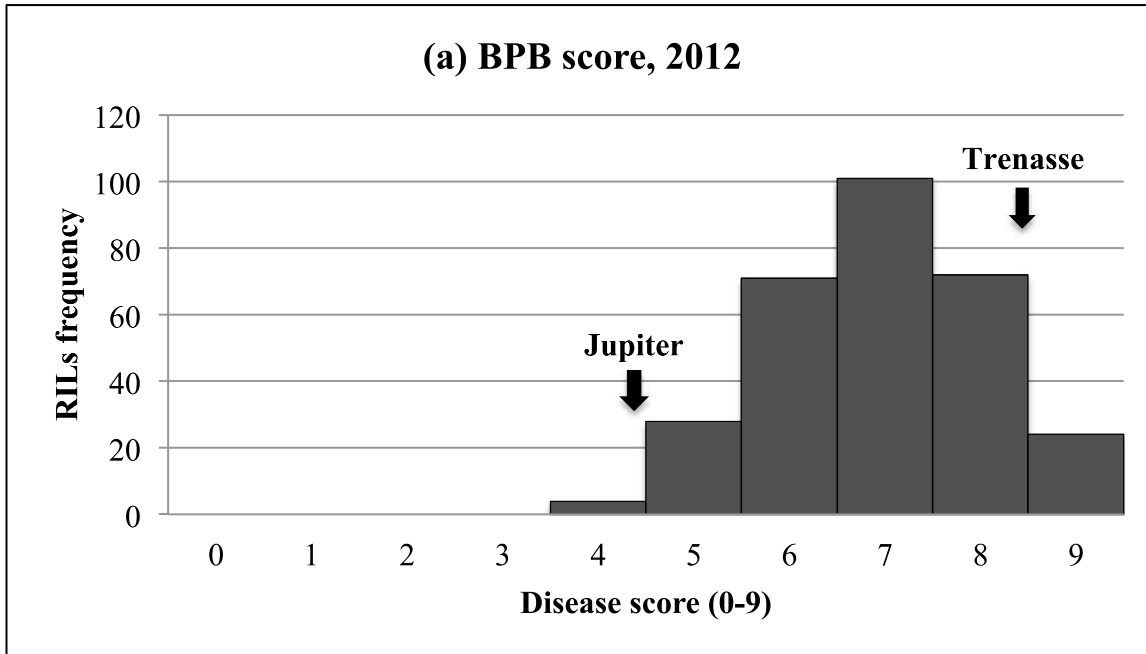
Table 2.3 Mean bacterial panicle blight score on Trenasse, Jupiter, and RILs, derived from Trenasse × Jupiter, taken in 2012, 2013, and across years.

	2012	2013	Across years
(Mean) Trenasse	8.75	8.7	8.65
(Mean) Jupiter	4.4	1.9	3.15
(Mean) RILs	6.71	5.43	6.07
Range	4-9	0-9	0-9
Standard deviation	1.38	1.85	1.75

Table 2.4 Sources of variation and their *F* values, and heritability estimates for bacterial panicle blight score on RILs, generated from Trenasse × Jupiter, taken in 2012, 2013, and across years.

	2012	2013	Across years
Replication	5.43*[a]	18.89**	11.50**
Genotype (RILs)	2.31**	6.29**	4.68**
Environment (Years)	-	-	473.23**
Genotype (RILs) x environment (Years) interaction	-	-	3.52**
Broad sense heritability	0.57	0.84	0.25

[a] * and ** = Significant at the 0.05 and 0.01 level, respectively



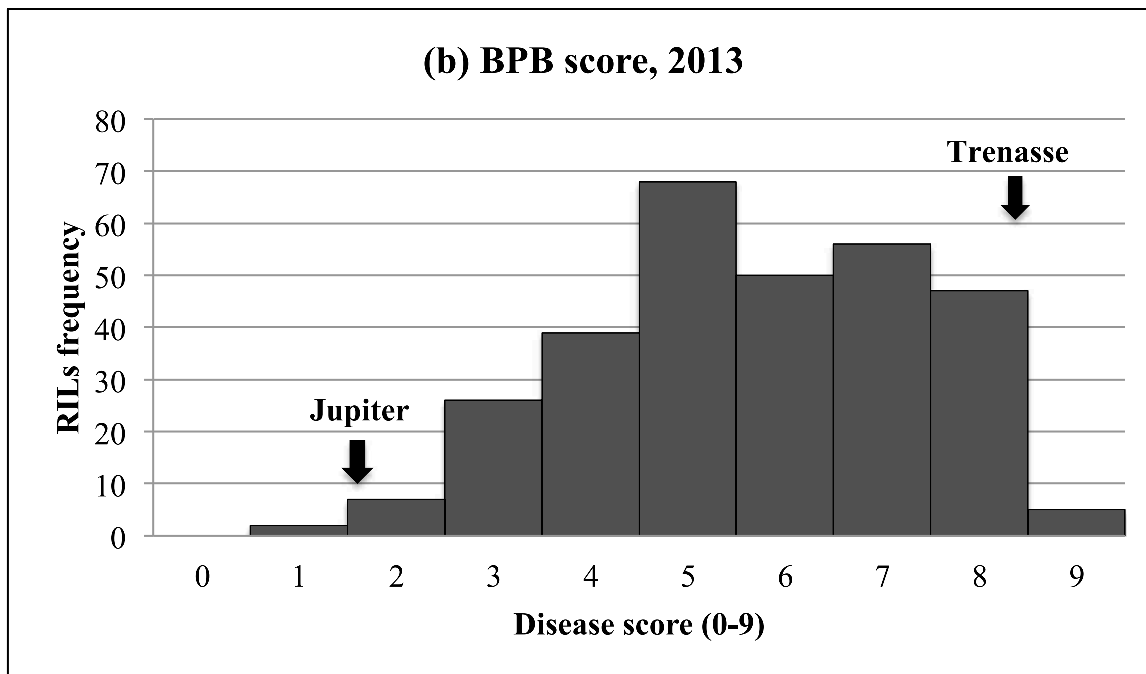


Figure 2.2 Frequency distribution of RIL population for bacterial panicle blight score at Rice Research Station, Crowley, in 2012 (a) and in 2013 (b). Disease score 0 to 9 were taken 10 days after *B. glumae* 336gr-1 inoculations. Inoculation of *B. glumae* inoculum (~1X 10^8 cfu/ml @ $OD_{600} = 0.1$) was done at ~30% heading stage of rice plants. Arrows indicate mean values for parental cultivars Jupiter and Trenasse.

2.5.3 Disease score for sheath blight

Sheath blight disease score was higher in Trenasse than in Jupiter in 2012 and 2014 (Table 2.5 and Figure 2.3 [a, b]). Sheath blight score in the RILs were skewed in both years (Figure 2.3 [a, b]). Average sheath blight disease score of RILs was between the score of parental lines in both years (Table 2.5). Significant variation among the RILs was observed in both years and across years (Table 2.6). Similarly, significant effect of genotype by environment interaction was observed. Heritability was higher 0.91, in 2012 and medium 0.63, in 2014, but lower 0.48 in across years (Table 2.6).

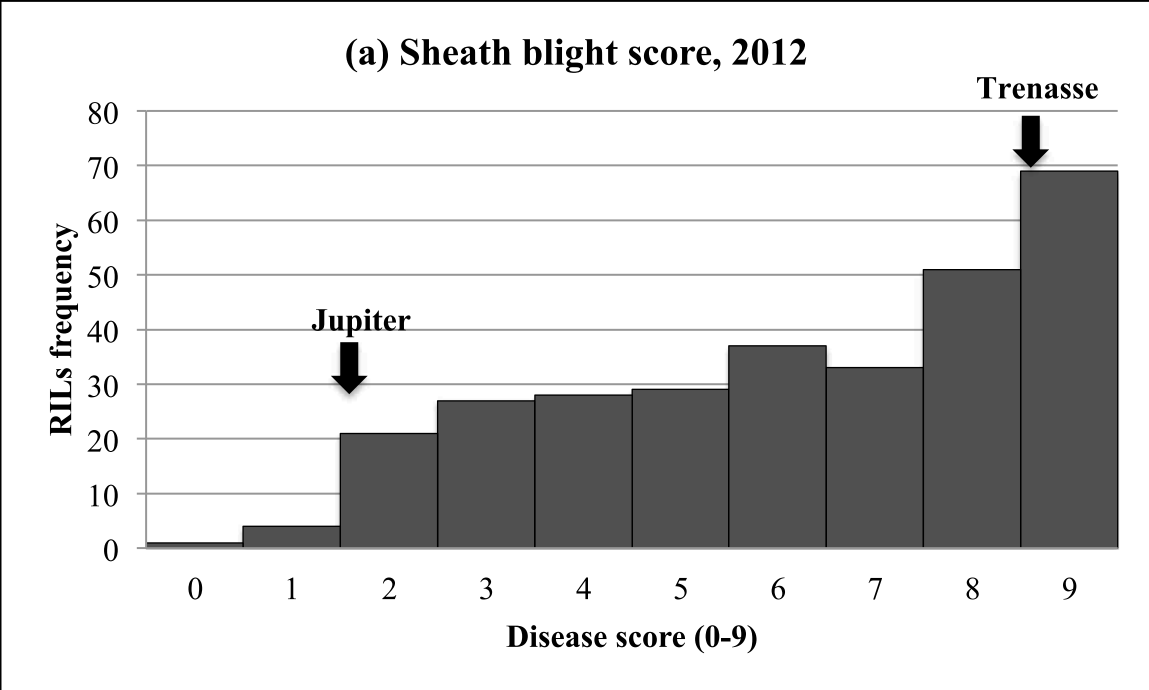
Table 2.5 Mean sheath blight score on Trenasse, Jupiter, and RILs, derived from Trenasse × Jupiter, taken in 2012, 2013, and across years.

	2012	2014	Across years
(Mean) Trenasse	9.00	8.00	8.45
(Mean) Jupiter	2.00	3.00	2.30
(Mean) RILs	5.98	6.64	6.31
Range	0-9	0-9	0-9
Standard deviation	2.52	1.76	2.2

Table 2.6 Sources of variation and their *F* values, and heritability estimates for sheath blight score on RILs, generated from Trenasse × Jupiter, taken in 2012, 2013, and across years.

	2012	2014	Across years
Replication	58.63**[a]	2.00*	23.43**
Genotype (RILs)	11.30**	2.67**	7.81**
Environment (Years)	-	-	94.91**
Genotype (RILs) x environment (Years) interaction	-	-	4.07**
Broad sense heritability	0.91	0.63	0.48

[a] * and ** = Significant at the 0.05 and 0.01 level, respectively



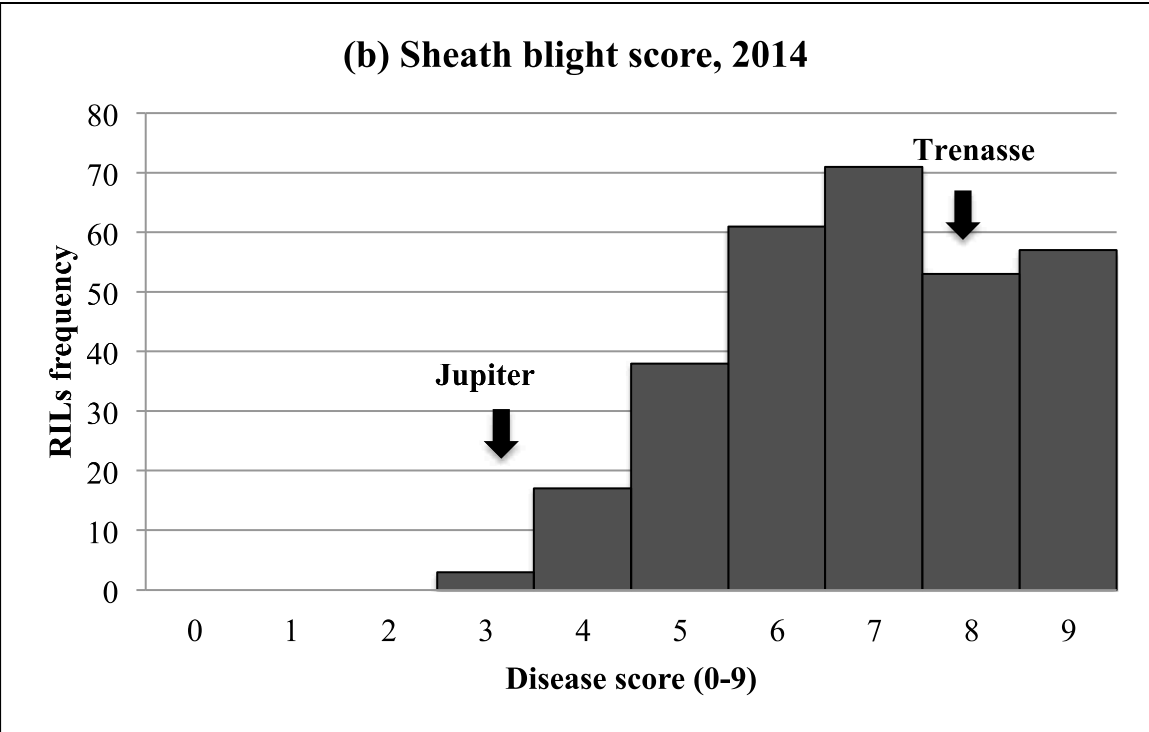


Figure 2.3 Frequency distribution of RIL population for sheath blight score at Rice Research Station, Crowley, in 2012 (a) and in2014 (b). Disease score 0 to 9 were taken during dough stage of rice plants. Inoculation was done with *R. solani* inoculum at active tillering stage of rice. Arrows indicate mean values for parental cultivars Jupiter and Trenasse.

2.5.4 Plant height

Jupiter grew taller than Trenasse in 2013 and 2014, but in 2012 it grew shorter (Table 2.7 and Figure 2.4 [a, b]). Average plant height of RILs was 96, 94, and 102 cm in 2012, 2013 and 2014, respectively. There was a significant difference in plant height among the populations in all three years and across years. Gentoype by environment interaction was significant for plant height (Table 2.8). Estimation of heritability for plant height was medium in all three years, but low in across years (Table 2.8).

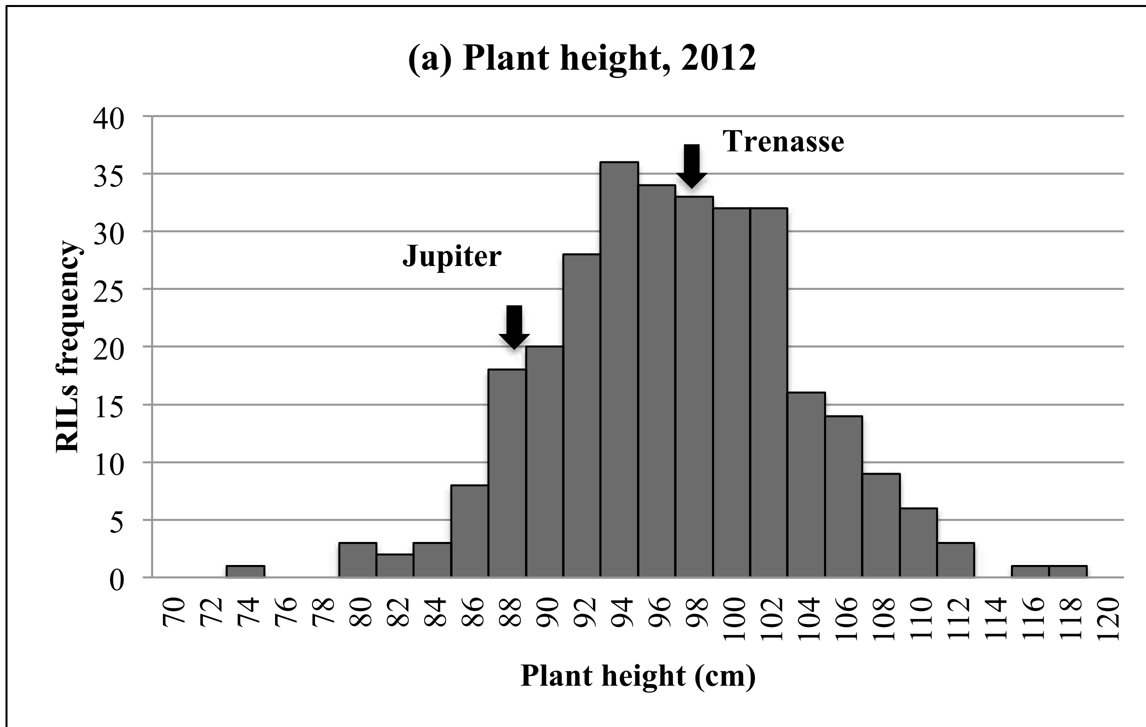
Table 2.7 Mean plant height of Trenasse, Jupiter, and RILs, derived from Trenasse × Jupiter, taken in 2012, 2013, 2014 and across years.

Plant height	2012	2013	2014	Across years
(Mean) Trenasse	97.67	93	94	94.89
(Mean) Jupiter	88.33	95	98	93.78
(Mean) RILs	96.06	93.6	101.5	97
Range	70-120	70-115	80-125	70-125
Standard deviation	7.53	7.28	8.11	8.32

Table 2.8 Sources of variation and their *F* values, and heritability estimates for plant height of RILs, generated from Trenasse × Jupiter, taken in 2012, 2013, 2014 and across years.

	2012	2013	2014	Across years
Replication	4.14*[a]	3.42*	2.28*	3.4**
Genotype (RILs)	7.72**	9.71**	14.45**	20.24**
Environment (Years)	-	-	-	954.55**
Genotype (RILs) x environment (Years) interaction	-	-	-	5.04**
Broad-sense heritability	0.69	0.74	0.82	0.75

[a] * and ** = Significant at the 0.05 and 0.01 level, respectively



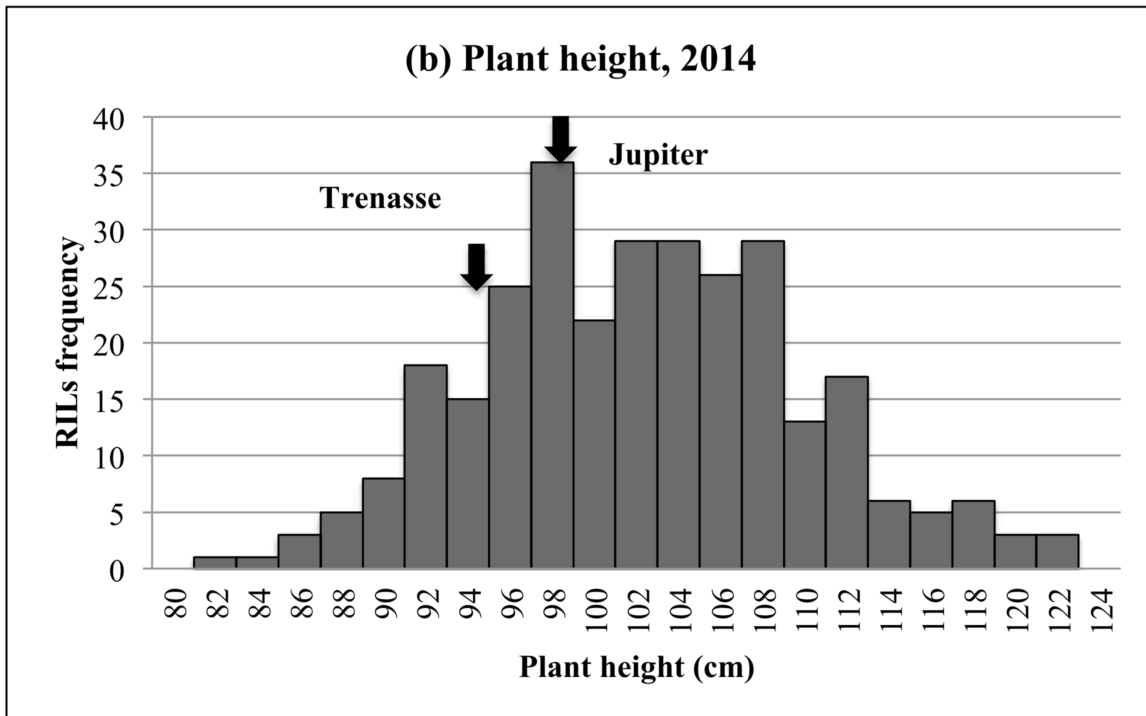


Figure 2.4 Frequency distribution of RIL population for plant height at Rice Research Station, Crowley, in 2012 (a) and in 2014 (b). Plant height was taken during maturity. Arrows indicate mean values for parental cultivars Jupiter and Trenasse.

2.5.5 Correlation among the traits

Pearson correlation coefficient was used to calculate correlation between the traits in individual years. In 2012, days to 50% heading was significantly negatively correlated with BPB disease score (r = -0.24) and sheath blight (r = -0.77). Similarly, trait for plant height was significantly negatively correlated with BPB and sheath blight score (r = -0.18) and (r = -0.21), respectively. Disease score of BPB and sheath blight was positively correlated (r = 0.31) to each other at $\alpha = 0.001$ (Table 2.7).

Similarly, in 2013, days to 50% heading was significantly negatively correlated with BPB score (r = -0.48). Correlation was negative, between plant height, and bacterial panicle blight score (r = -0.095), but was not significantly correlated (Table 2.7). Furthermore, plant height of RILs was significantly negatively correlated with sheath blight disease score (r = -0.15) in 2014 (Table 2.7).

Table 2.7 Pearson correlation coefficients among phenotypic traits for RILs developed from Trenasse and Jupiter cross in 2012

Year		Days to 50% heading (days)	BPB score (0-9)	Plant height (cm)	SB score (0-9)
2012					
	Days to heading (days)	1			
	BPB score (0-9)	-0.24**[a]	1		
	Plant height (cm)	0.18**	-0.18**	1	
	SB score (0-9)	-0.77**	0.31**	-0.21**	1
2013					
	Days to heading (days)	1			
	BPB score (0-9)	-0.48**	1		
	Plant height (cm)	-0.12*	-0.095	1	
2014					
	Plant height (cm)			1	
	SB score (0-9)			-0.15**	1

[a] * and ** = Significant at the 0.05 and 0.01 level, respectively

2.6 DISCUSSION

In this study a medium-grained rice cultivar, Jupiter, which is partially resistant to BPB was crossed with a long-grain rice cultivar, Trenasse, which is very susceptible to BPB and sheath blight, and 300 RILs were generated at Rice Research Station, Crowley, Louisiana. Phenotypic evaluation including, disease scoring for BPB was done for each generation (data not shown). In 2012, 2013 and 2014, observation was taken on four phenotypic traits (BPB disease score, disease score for sheath blight, plant height and days to 50% heading) and statistical analysis was conducted.

Significant variations among the RILs for all four traits were observed in each year suggesting the presence of genetic variability within the population. Broad-sense of heritability estimates for days to 50% heading were high in 2012 and 2013. Similarly, heritability for the plant height was also high in 2012, 2013, and 2014. Higher heritability indicated higher genetic variance contributing toward phenotypic expression and less influence of environment. On the other hand, BPB and sheath blight score in rice was significantly negatively correlated with plant height and days to 50% heading. Although the correlations were not strong, resistant cultivars for BPB and sheath blight could be selected among late-matured and short cultivars. It was reported that QTLs for heading days in rice is associated with the BPB resistance (Pinson et al., 2010). Similarly, it is known that QTLs for sheath blight coincides with the QTLs of plant height and heading time, so plants with late heading days showed higher level of resistance than the plants with early heading dates (Nelson et al., 2012; Park et al., 2008; Sharma et al., 2009).

Since heritability estimate is an important parameter for any breeder for the selection of plants to use in any breeding programs, the traits including plant height and days to heading with high heritability estimates could be used in the selection process. These traits can be used as

indirect selection criteria to reduce the disease incidence for both BPB and sheath blight. Narrow window period for artificial inoculation of bacterial inoculum for BPB disease assay made us difficult to obtain proper disease development. In this situation, indirect selection of those traits with high heritability values and significantly negatively correlated with disease ratings could solve the problem.

During the estimation of heritability of disease ratings for BPB and sheath blight, we were unable to obtain consistent values. Estimation of heritability of disease rating for BPB was medium (0.57) in 2012, and high in 2013 (0.84), similarly, heritability estimates for sheath blight disease was high in 2012 (0.91), and medium in 2014 (0.63). These variations in heritability estimates might be due to the variability in the environment in two different years. In 2013, the environment was not favorable for BPB development thus reducing the occurrence of BPB symptoms. Similarly, there was poor growth of the lines due to the poor field condition in 2014. Poor stand of RILs reduced the chance of occurring sheath blight in rice. Aung (1990) described similar result, where heritability estimates for sheath blight were observed higher due to inconsistency of environmental conditions.

In conclusion, traits with higher heritability having correlation with the BPB and sheath blight resistance can be used for selecting germplasm in future breeding programs.

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CHAPTER III: COMPARATIVE ANALYSIS OF GENOMIC CHARACTERISTICS OF FIVE RICE CULTIVARS COMMERCIALLY GROWN IN LOUISIANA

3.1 INTRODUCTION

Rice is one the major staples around the world because it contributed 35-60 % of total calorie in intake. Two major types of rice, *Oryza sativa japonica* and *O. sativa indica* are grown for food. Various types of external stresses restrict the grain quality and yield of rice. In order to provide food security for the overgrowing population, it is essential to improve the rice yield and its quality. Various innovative researches have been continuously conducted to improve rice productivity. Recent advances in the next generation sequencing technology increased new opportunities to understand and tackle the problems in crop improvement. Genome sequencing of economically important cereal crops has provided a thorough analysis of the genetic elements, and also led to correlate to corresponding phenotypic traits. Analysis of genome sequence of cereals including corn and rice has been carried out since 1990 to identify genes related to yield, biotic and abiotic stress tolerance, plant height, and days to heading, which are agronomically important (Sasaki et al., 2008).

Rice has compact and small genome among cereals. Also, wide genetic diversity and abundant genomic resources made it a model plant for the study of other important cereals. With innovative efforts of various nations, whole genome sequences have generated for *O. sativa japonica* and *O. sativa indica* (Goff et al., 2002; International Rice Genome Sequencing Project, 2005; Yu et al., 2002). Comprehensive studies on whole genome genotyping and genome-wide association studies on rice have been possible with the availability of high-quality reference genome sequences of rice (Caicedo et al., 2007; McNally et al., 2006; McNally et al., 2009). Whole genome resequencing provides genome-wide genetic polymorphisms, and facilitates the identification of structural and functional variation. Origin of cultivated rice, genome-wide

association studies of several agronomically important traits, and study on relatedness between wild and domesticated rice varieties have been conducted based on whole genome resequencing of more than 1500 wild and domesticated modern rice varieties (Huang et al., 2012; Huang et al., 2010; McNally et al., 2009; Xu et al., 2012).

Genetic polymorphisms including, single nucleotide polymorphism (SNP), insertions, and deletions (indels) are key factors for expression of a trait in an individual. Those genetic variations are the basis for developing DNA-based markers for genotyping and genetic mapping study. However, types of markers have been evolved with the progressive development in molecular biology. In recent years, SNPs have been widely used as molecular markers for genotyping complex-traits and genome-wide association studies because of its abundance and easier detection in the genome compared to other molecular markers (Huang et al., 2009; Lee et al., 2008; Rafalski, 2002). McNally et al., (2009), developed OryzaSNP project, and discovered genetic variations and relationships within 20 rice varieties and landraces using 160, 000 SNPs within 100 Mb of reference genome. They also found the shared SNPs in some regions were associated with agronomic traits. Similarly, insertions and deletions, which contribute to genetic variation, are being for alternative molecular marker development (Väli et al., 2008). In *Arabidopsis*, array of insertions and deletions has been used for mapping recessive mutations (Salathia et al., 2007). A large number of databases for the rice variants have been developed already. However, those information is being used mainly for the study of traits including yield and yield attributing traits. Thus it is imperative to genotype rice varieties by resequencing for genome-wide association mapping studies and gene-trait relationships of other important quantitative traits such as disease resistance.

In the southern United States, most of the commercially available cultivars are susceptible to major rice diseases, including bacterial panicle blight and sheath blight. These diseases cause 30 to 40 % yield reduction (Marchetti, 1983; Nandakumar et al., 2005; Nandakumar et al., 2009; Shahjahan et al., 2000). Completely resistant rice cultivars for those diseases are not known. In addition, use of chemical control measures for the bacterial disease is not available in the US, but some fungicides are available for the management of sheath blight (Groth, 2005; Nandakumar et al., 2009). However, a medium-grain cultivar, Jupiter, and a mutant line, LM-1, showed partial resistance to both diseases (Groth et al., 2007; Sha et al., 2006). Defense mechanisms for the diseases resistance between the two cultivar/line are still unknown. In addition, Jupiter was developed from conventional breeding approach where as LM-1 is a mutant germplasm developed form gamma radiation of the semi-dwarf, long-grain cultivar, Lemont. Along with Lemont, other commercial cultivars, Trenasse and Bengal are susceptible to bacterial panicle blight and sheath blight. Trenasse and Lemont are long-grain, and Bengal is a medium-grain cultivar (Linscombe et al., 1993; Linscombe et al., 2006).

In this study, we sequenced those five *japonica* rice genotypes to study genomic variations through comprehensive identification of genome-wide DNA polymorphisms that will provide useful information for understanding the genetic basis underlying partial disease resistance for bacterial panicle blight and sheath blight.

3.2 OBJECTIVES

1. To compare the whole genome sequences of the five rice genotypes
2. To identify variants including SNPs, insertions, and deletions among the genomes of five rice genotypes

3.3 MATERIALS AND METHODS

3.3.1 Rice genotypes and genomic DNA extractions

The five rice genotypes including Jupiter (Bengal/Rico 1/3/Bengal//Mercury/Rico 1) (Sha et al., 2006), Trenasse (Cypress//L-202/Tebonnet/3/LSBR-5) (Linscombe et al., 2006), Bengal (MARS//M-201/MARS) (Linscombe et al., 1993), Lemont (Lebonnet//CI9881/PI331581) (Bollich et al., 1985), and LM-1 (Mutant-derivative of Lemont generated by gamma radiation (250 Gy) from ^{60}Co) (Groth et al., 2007) used in this study were developed in the US and widely used for cultivation. One-week old rice seedlings of those five rice genotypes were used to isolate genomic DNA following manufacturer's instructions using DNeasy Plant Mini Kit, Qiagen, Valencia, CA 91355. The concentration of the DNA samples of Jupiter, Trenasse, Bengal, Lemont, and LM-1 was measured using Nano Spectrophotometer (Nano Drop, Wilmington, DE) and then the DNA samples were sent to Virginia Bioinformatics Institute (VBI) Genomics Lab at Virginia-Tech for DNA sequencing. The Nextera method was used to develop genomic DNA libraries and processed for paired-end sequencing to generate 100-base long reads.

3.3.2 Mapping and variants identification

Reads obtained for all five cultivars were aligned to the pseudomolecules version 7 of the reference genome of *japonica* rice cultivar, Nipponbare using Bowtie 2 with default parameters and their individual insert sizes (Langmead & Salzberg, 2012). Sequence Alignment/Map (SAM) files for individual genome obtained from Bowtie 2 procedure were used for genome wide variants discovery including single nucleotide polymorphism (SNP), and insertion and deletion (indel) using SAMtools (Li et al., 2009). Genome wide variations between the five rice

genotypes and reference genome were estimated using SnpEff v3.5e (Cingolani et al., 2012) with default parameters relative to pseudomolecules version 7 of the *japonica* reference genome.

3.3.3 Pairwise comparison with the medium-grain partially resistant cultivar Jupiter

In order to compare and find variations among five rice genotypes, including Jupiter, Trenasse, Bengal, Lemont, and LM-1, pairwise comparisons were performed using vcftools (Danecek et al., 2011) with vcf files from SnpEff, containing variants, including SNPs, and indels. Briefly, each vcf file of four rice genotypes was compared with Jupiter using vcftools. The output file from vcftools from each comparison was filtered for the common variants that were identified earlier with the Nipponbare reference genome. Each of the vcf files of four genotypes was merged with the vcf file of Jupiter, and created a new merged-vcf file. The merged-vcf file and the vcftools output file were used to create a new vcf file containing only the variants information between Jupiter and each of the other four rice genotypes. Those variants were again annotated using SnpEff v3.5e and the estimated variants were classified based on their effect on various regions in the genome and their functional type.

3.3.4 Population structure analyses

Genetic relatedness of five rice genotypes with various other rice cultivars including temperate and tropical japonica, aromatic and indica were observed by using FRAPPE (Tang et al., 2005). SNP data of 50 rice accessions from the study by Xu et al. (2012) and the five rice genotypes from this study were used to prepare population structure with different *K* values.

3.4 RESULTS

More than 40 million high quality paired-end reads with an average of 20X coverage of raw data was obtained from the Illumina GAIIx sequencer. An average of more than 92% and 9% of paired-end raw sequences were mapped with chromosomal and organelle genome of the

reference genome, respectively. Bengal and Lemont have gotten the highest and lowest alignment of 99.33% and 83.94% of chromosomal sequence, respectively, whereas Jupiter and LM-1 have the highest and the lowest alignment of 13.21% and 5.69% of organelle genome, respectively (Table 3.1).

Table 3.1 Coverage of mapped reads with reference to the Nipponbare chromosomal genome IRGSP pseudomolecule version 7

	Jupiter	Trenasse	Bengal	Lemont	LM-1
Total reads (paired-end)	78,862,636	84,341,650	49,980,244	131,158,886	75,743,584
Coverage	18X	19X	12X	30X	18X
Mapped with chromosomal genome (%)	95.78	91.02	96.33	83.94	94.81
Mapped with organelle genome (%)	13.21	11.05	9.43	9.21	5.69

3.4.1 Detection of variants, including SNPs, insertions and deletions, among the five genotypes and the reference genome sequence of Nipponbare

Various ranges in the variants including SNPs, insertions and deletions were identified genome sequences of five rice genotypes when compared with the reference genome, Nipponbare. Trenasse and Jupiter had the highest and the lowest with more than 2.1 millions and 817K of SNPs. Lemont, LM-1 and Bengal had more than 1 million SNPs (Table 3.2). Chromosome 10 in Jupiter, chromosome 11 in Trenasse and Bengal, and chromosome 1 in Lemont and LM-1 had the higher number of SNPs than other chromosome in each of the genome (Table 3.2). Similarly, chromosome 9, 2, and 3 of Jupiter, Trenasse, and Bengal, respectively, and chromosome 9 of Lemont and LM-1 has lower SNPs. Furthermore, chromosome 10 and 3 in Jupiter and Trenasse, respectively, and chromosome 1 in Bengal, Lemont and LM-1 had more

insertions and deletions, and chromosome 9 of Jupiter, Bengal, Lemont and LM-1 and chromosome 7 of Trenasse has less insertions and deletions among all chromosome of each genome (Table 3.2).

The frequency of variants discovered in every 100 Kb interval was calculated to observe the distribution of variants between the reference genome and the genomes of the five rice genotypes. Variations within the genome were observed from SNP frequency data in which higher density of variations found in chromosome 10 and lower density were found in chromosome 2. Among the five genomes, Trenasse had the highest SNP frequency in all chromosomes. Chromosome 11 and 8 of Trenasse had approximately 1000 and 800 SNPs densities per 100 Kb, respectively. Besides, chromosome 10 in Jupiter and chromosome 4, 10 and 11 in Bengal, Jupiter and Bengal had the lowest SNP frequency among all genotypes. Chromosomes 3, 9 and 12 had the lowest SNPs densities, less than 200 per 100 Kb, in both Jupiter and Bengal. Higher SNPs frequencies were found in chromosome 10 in all of the five genomes compared to other chromosomes. Lemont and LM-1 had the similar pattern of distribution of SNP frequencies (Figure 3.1a).

Distribution of insertion and deletion frequencies in the genome of each cultivar/line was similar (Figure 3.1 [b, c]). Trenasse had the highest insertions and deletion densities among all the five genomes. Within Trenasse, chromosomes 3, 8 and 11 had higher insertions and deletions frequencies, with more than 30 insertions or deletions per 100 Kb size, compared to other chromosome (Figure 3.1 [b, c]). Similar to SNPs variants, chromosome 3, 9 and 12 of Jupiter and Bengal had the lowest frequency of insertions and deletions (Figure 3.1 [b, c]). Similar to SNP frequency, Lemont and LM-1 had similar pattern of insertions and deletions distribution in the genome. Frequency of insertions and deletions were approximately similar throughout the

genome of the five genotypes. Insertion frequencies were higher than deletions except in chromosome 4, 5 and 9 of Jupiter, and chromosome 5, 6 and 9 of Trenasse (Figure 3.1 [b, c]).

3.4.2 Distribution of SNPs, insertions and deletions in the genomes of the five rice genotypes

The distribution of variants within individual chromosome showed significant variations in several regions. Trenasse, Lemont and LM-1 had higher variant frequencies distribution than Jupiter and Bengal. Among five rice genotypes, Jupiter and Bengal had the similar distribution pattern of the variants frequencies, Lemont and LM-1 have the similar distribution pattern, and Trenasse had different distribution pattern of the variants within the individual chromosome (Figures 3.2, 3.3, and 3.4). For SNPs, there were some ranges of regions within individual chromosomes in which more SNPs were identified. Particularly, the major regions containing higher SNPs densities were; 15 to 17 Mb and 18 to 22 Mb on chromosome 3, 1 to 5 Mb and 7 to 15 Mb on chromosome 4, 5 to 8 Mb and 15 to 17 Mb on chromosome 5, 1 to 3 Mb and 9 to 13 Mb on chromosome 6, 17 to 24 Mb on chromosome 7, 9 to 14 Mb and 15 to 25 Mb on chromosome 8, 1 to 13 Mb in chromosome 9, 1 to 13 Mb in chromosome 10, 9 to 19 Mb (for Trenasse and Bengal) on chromosome 11, and 11 to 20 Mb region in chromosome 12 (Figure 3.2). Similarly, distribution of insertions and deletions frequencies varied within the individual chromosome. Distribution of insertions and deletions were found to be similar with the distribution pattern of SNPs (Figures 3.2, 3.3, and 3.4).

3.4.3 Annotation of the SNPs, insertions and deletions identified between five rice genotypes and the reference genome, Nipponbare

Figure 3.5 shows the frequency of variants in different genomic regions, including upstream and downstream regions, untranslated 5’ and 3’ regions, exon, intron, and intergenic regions, of five rice genotypes. Upstream and downstream regions of genes by the SnpEff, a

Table 3.2 Number of SNPs, insertions, and deletions, on individual chromosome identified between the reference genome, Nipponbare and the five rice genotypes

	Jupiter	Trenasse	Bengal	Lemont	LM-1
SNPs					
Chromosome 1	88,362	171,060	135,180	192,199	179,793
Chromosome 2	73,313	118,974	78,248	99,451	91,454
Chromosome 3	39,733	186,792	41,286	124,820	115,626
Chromosome 4	98,462	202,335	147,305	156,189	142,943
Chromosome 5	62,823	152,115	52,613	134,000	126,457
Chromosome 6	57,508	153,493	68,284	118,993	111,417
Chromosome 7	70,094	128,665	65,303	107,851	99,801
Chromosome 8	62,114	226,527	63,626	152,692	141,889
Chromosome 9	16,854	167,467	27,344	97,367	90,075
Chromosome 10	135,193	165,413	130,505	177,872	168,225
Chromosome 11	68,711	283,002	150,632	181,171	163,973
Chromosome 12	44,717	184,048	46,968	181,450	165,381
Total	817,884	2,139,891	1,007,294	1,724,055	1,597,034
Insertions					
Chromosome 1	5,765	10,276	8,630	12,374	11,449
Chromosome 2	4,428	6,409	4,552	5,905	5,508
Chromosome 3	2,414	11,991	2,733	6,765	6,362
Chromosome 4	4,823	7,186	5,763	6,574	6,006
Chromosome 5	3,611	7,178	2,475	6,718	6,238
Chromosome 6	3,104	7,395	3,082	6,089	5,552
Chromosome 7	3,766	5,927	3,227	5,316	4,922
Chromosome 8	3,157	9,976	2,903	6,842	6,370
Chromosome 9	514	6,428	1,026	4,157	3,852
Chromosome 10	6,080	6,206	5,367	7,582	6,937
Chromosome 11	3,353	11,700	6,033	8,121	7,377
Chromosome 12	2,288	6,601	2,158	6,840	6,147
Total	43,303	97,273	47,949	83,283	76,720
Deletions					
Chromosome 1	5,636	9,661	8,448	11,385	10,795
Chromosome 2	4,275	6,193	4,302	5,738	5,409
Chromosome 3	2,339	11,070	2,594	6,519	6,145
Chromosome 4	4,824	6,943	5,678	6,314	5,804
Chromosome 5	3,629	6,881	2,594	6,478	6,062
Chromosome 6	3,088	7,138	3,085	5,798	5,355
Chromosome 7	3,749	5,776	3,192	5,261	4,843
Chromosome 8	3,063	9,319	2,743	6,273	5,768
Chromosome 9	537	6,016	1,081	3,987	3,671
Chromosome 10	5,681	6,081	5,153	7,232	6,723
Chromosome 11	3,056	10,476	5,863	7,187	6,542
Chromosome 12	2,241	6,169	2,146	6,219	5,664
Total	42,118	91,723	46,879	78,391	72,781

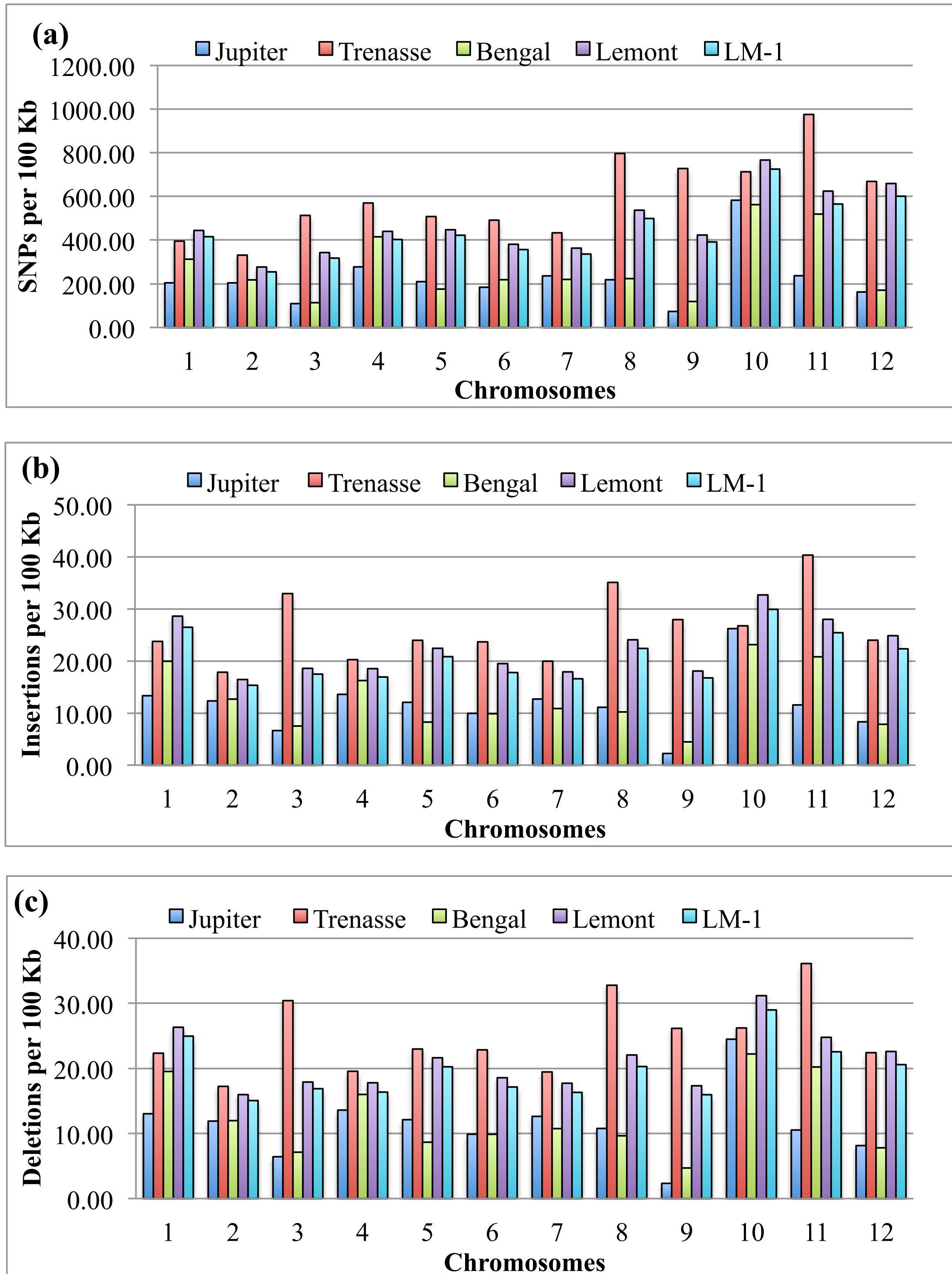


Figure 3.1 Frequency of variants on individual chromosome identified between the reference genome and the five rice genotypes, (a) SNPs densities, (b) insertions densities, and (c) deletions densities.

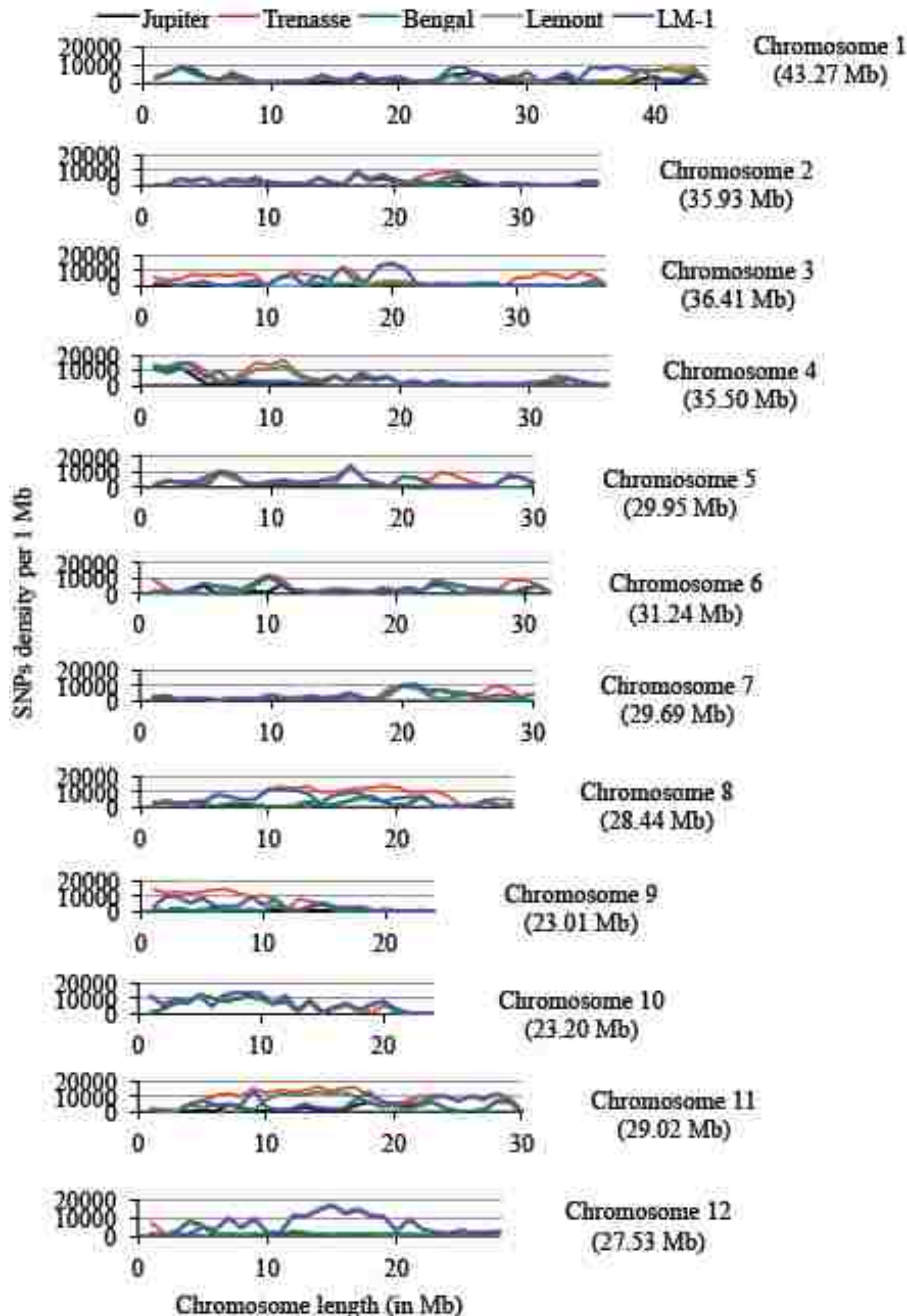


Figure 3.2 Distribution of single nucleotide polymorphisms (SNPs) between the reference genome, Nipponbare and five-rice genotypes in 12 chromosomes. X-axis represents the physical distance of each chromosome in Mb, and Y-axis represents the number of SNPs. Chromosome number and the size of each chromosome are given on the side of the graph.

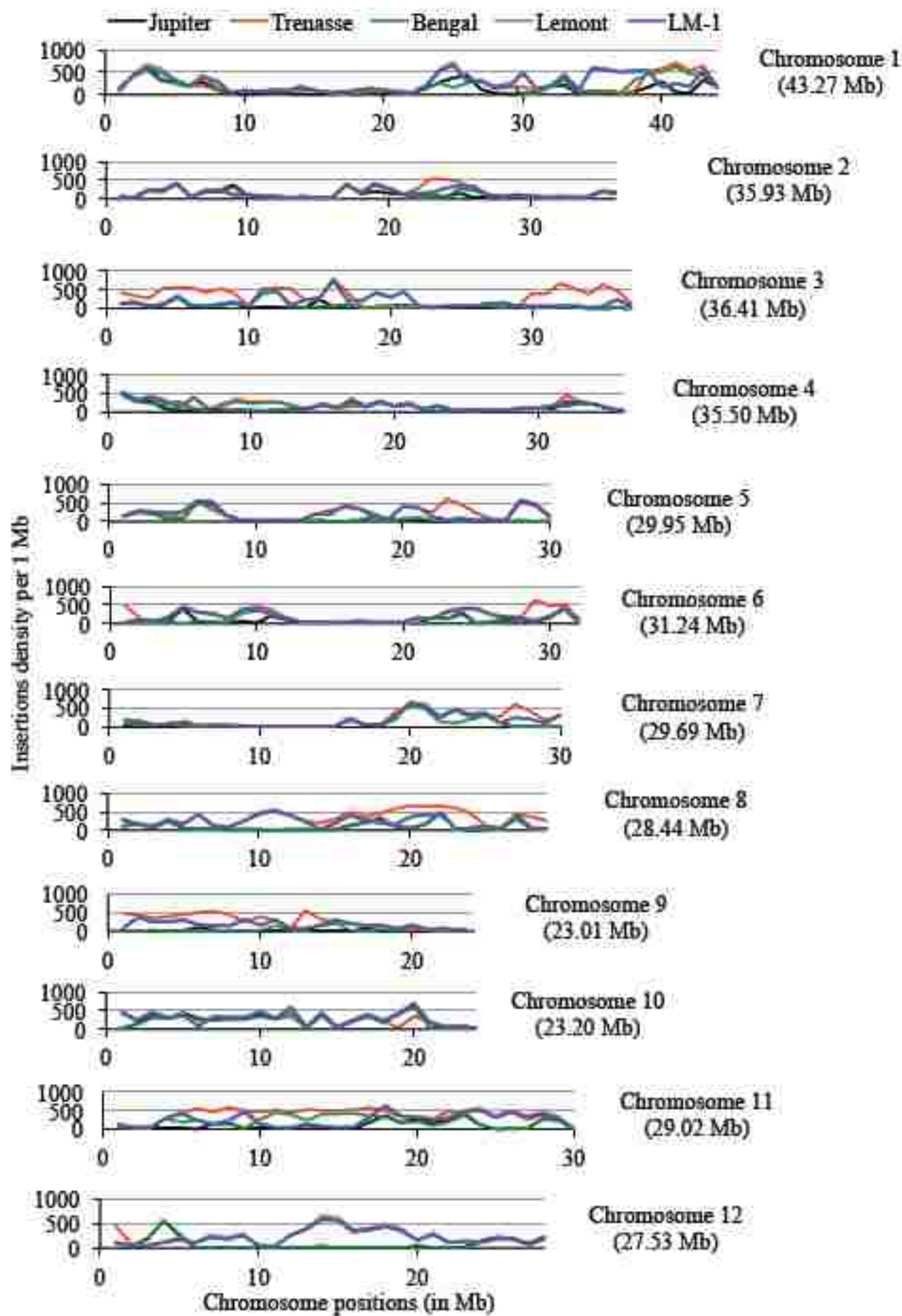


Figure 3.3 Distribution of insertions between the reference genome, Nipponbare and five-rice genotypes in 12 chromosomes. X-axis represents the physical distance of each chromosome in Mb, and Y-axis represents the number of deletions. Chromosome number and the size of each chromosome are given on the side of the graph.

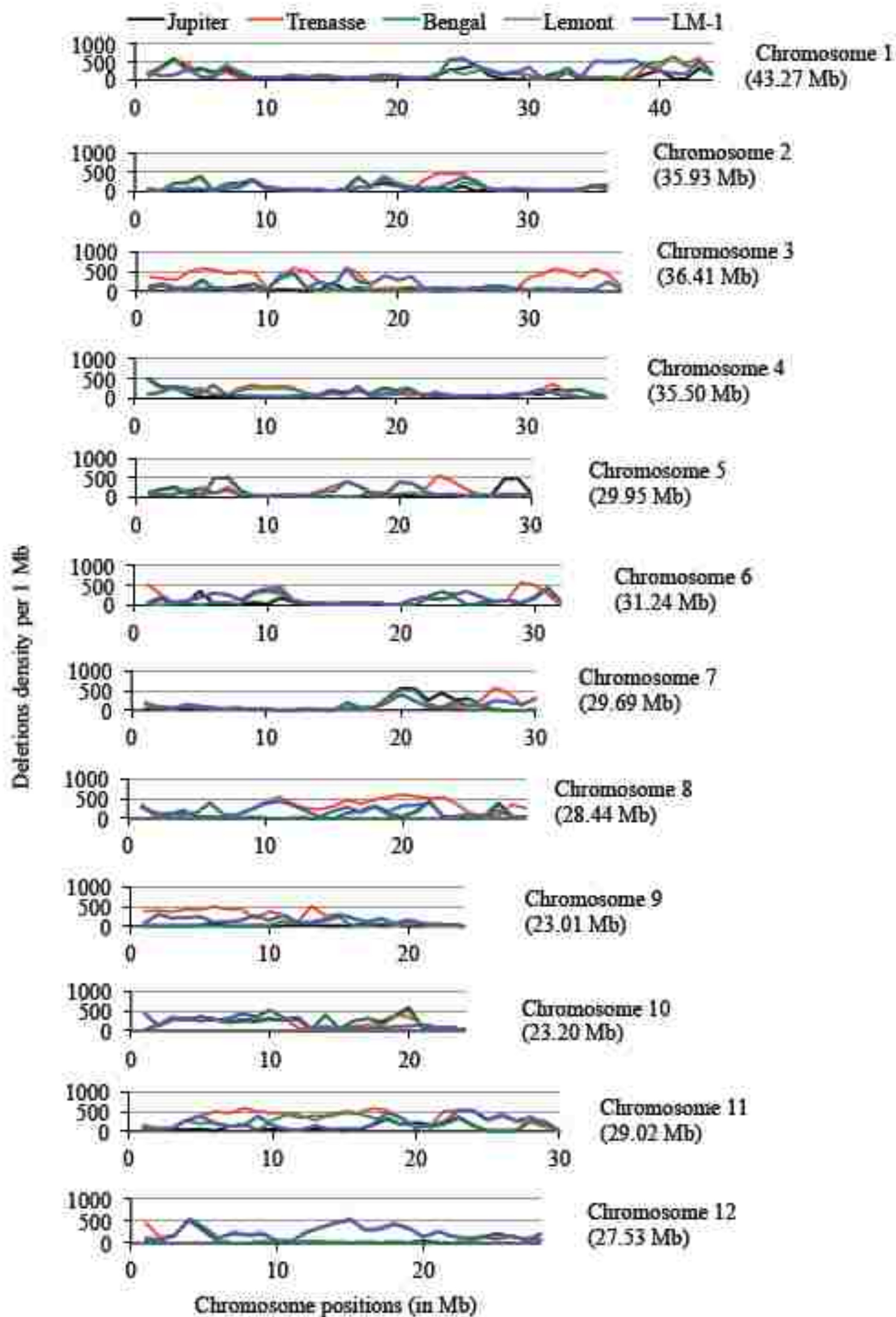


Figure 3.4 Distribution of deletions between the reference genome, Nipponbare and five-rice genotypes in 12 chromosomes. X-axis represents the physical distance of each chromosome in Mb, and Y-axis represents the number of deletions. Chromosome number and the size of each chromosome are given on the side of the graph.

program which was used for annotation of vcf files of the genome sequences, count variants that occur up to 5 Kb upstream and downstream of each gene. Similarly exon includes various types of variants including frameshift, exon, non-synonymous and synonymous coding, codon insertion and codon deletion, codon change plus codon insertion, codon change plus codon deletion, premature stop codon gained, and stop lost. Insertion and/or deletion generate frameshift variation. Variant that differs the amino acid product of codon is non-synonymous coding variant, and variant that changes the bases, but does not have any effect on the amino acid product, is synonymous coding variant. So, non-synonymous coding variants are more important to study than synonymous coding variants. Codon insertion and codon deletion type variants generated by the insertion and/or deletion of one or more codon which will generate inframe insertion and deletion, respectively, whereas codon change plus codon insertion and codon change plus codon deletion occur by the insertion or deletion within the codon there by generating disruptive inframe insertion and deletion, respectively.

Jupiter and Bengal had the lowest and Trenasse had the highest frequency of variants per 100 Kb each of the different regions among the five genomes. Lemont and LM-1 had similar frequency level of variants throughout all seven different regions, and had higher variant frequency than Jupiter and Bengal, and lower than Trenasse. The upstream, UTR5', intron and downstream regions had similar frequency of variants, whereas exon region had got higher variants frequency. On the other hand, the lowest frequency level of variants was observed in UTR3' and intergenic regions have the (Figure 3.5). SNPs that change codons of a genome resulting in altered amino acid are known as non-synonymous SNPs. A total number of SNPs in CDS regions ranged from 181, 000 to 469, 000. Among these, 96, 000 to 249, 000 of SNPs were identified as non-synonymous, and 85, 000 to 220, 000 were identified as synonymous SNPs in

all five rice genotypes. Similarly, 2000 to 5000 of insertions and deletions were identified in CDS regions, and similar number of insertions and deletions were found to cause frameshift in the five rice genomes (Appendix 14).

Table 3.3 Annotation of variants at various genomic regions identified in five rice genotypes compared to the reference genome

	Jupiter	Trenasse	Bengal	Lemont	LM-1
Upstream	815,677	2,104,611	980,273	1,689,888	1,556,965
UTR5'	13,429	30,666	14,811	24,820	23,476
Exon	189,479	489,598	228,698	406,990	378,445
Intron	165,791	434,003	207,332	347,026	325,436
UTR3'	19,321	46,448	22,728	35,407	34,030
Downstream	783,570	2,014,522	942,653	1,631,064	1,504,889
Intergenic	570,761	1,472,704	696,195	1,189,336	1,095,206

Nucleotide substitutions are the major reasons for the development of SNP variants. Based on the nucleotide substitutions, the SNPs identified between the reference genome and the genomes of five rice genotypes were classified into transitions (C/T and G/A) and transversions (C/G, T/A, A/C, and G/T). Nucleotide substitutions through transitions are higher than transversions in all five rice genotypes (Table 3.4). Within the transitions, the substitution of C/T is slightly higher than A/G substitutions in four rice genotypes including Jupiter, Trenasse, Lemont, and LM-1. However, A/G substitutions were higher in Bengal. Similarly, T/A substitutions were relatively higher in all five rice genotypes compared to other transversions including C/G, A/C, and G/T. The ratio of transitions to transversions was greater than 2.4 in all five rice genotypes.

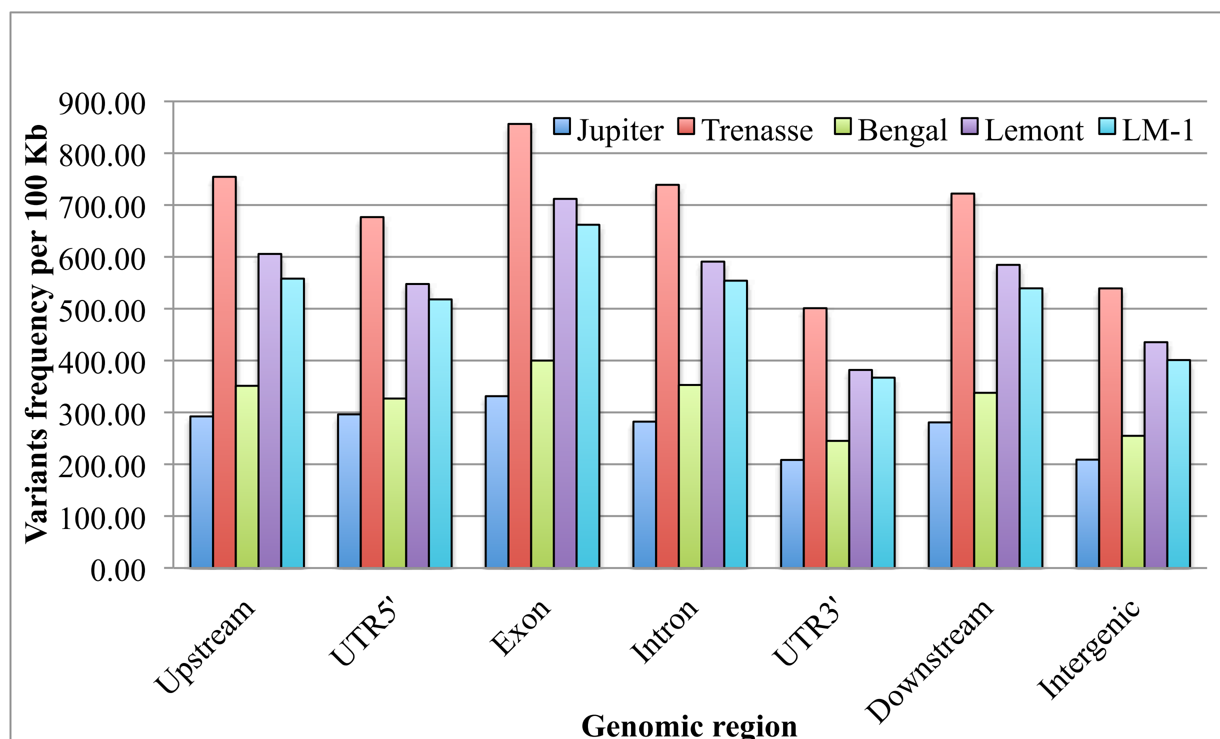


Figure 3.5 Frequency of variants at 100 Kb interval on various genomic regions identified between the reference genome and the five rice genotypes.

Table 3.4 Classification of nucleotide substitutions in SNPs: Nucleotide substitutions identified between the reference genome and five rice genotypes

	Reference (rice7) vs				
	Jupiter	Trenasse	Bengal	Lemont	LM-1
Transitions (Ts)					
C/T	290,775	767,627	358,738	616,433	571,423
A/G	290,473	767,190	358,746	615,814	570,833
Total	581,248	1,534,817	717,484	1,232,247	1,142,256
Transversions (Tv)					
C/G	44,696	112,560	54,631	92,490	84,881
T/A	69,829	179,926	85,768	146,855	136,043
A/C	61,043	156,584	75,121	126,458	117,100
G/T	61,068	156,004	74,290	126,005	116,754
Total	236,636	605,074	289,810	491,808	454,778
Ts/Tv ratio	2.46	2.54	2.48	2.51	2.51

3.4.4 Pairwise comparisons between the partially resistant medium-grain cultivar, Jupiter, and each of four rice genotypes

Based on the variants obtained from the comparison with the reference genome, comparison among the five rice genotypes were conducted. Jupiter, a medium-grain partially resistant cultivar for BPB, was use to perform pairwise comparisons with the other four genotypes. Trenasse had the highest and Bengal had the lowest number of number of SNPs, insertions and deletions among the four genotypes. Lemont and LM-1 had relatively similar number of variants and were in between Trenasse and Bengal (Table 3.5). At an individual level, chromosome 11 of Trenasse and Bengal and chromosome12 of Lemont and LM-1 had higher number of SNPs. However, chromosome 2 of Trenasse, Lemont and LM-1, and chromosome 12 of Bengal had lower number of SNPs. Similarly, chromosome 3 of Trenasse and chromosome 1 of Bengal, Lemont, and LM-1 had higher number of insertions and deletions. However, chromosome 11 had higher densities of insertion and deletion per 100 Kb in Trenasse (Figure 3.6b). Lowest number of insertions and deletions was identified within the genome of Trenasse in chromosome 2 and 7, respectively. In chromosome12 of Bengal and chromosome 7 of Lemont and LM-1, lower number of insertions and deletions were identified (Table 3.5). However, Bengal chromosome 8 and 12 had the lowest densities of insertions and deletions after normalization (Figure 3.6 [b, c]).

Within the individual chromosome, frequency of variants was differentially distributed. Several regions where the variant densities were higher for Trenasse had lower variants rate in the same regions for other genotypes including Bengal. Fifteen to twenty five Mb on chromosome 3, 1 to 5 Mb and 7 to 12 Mb on chromosome 4, 15 to 17 Mb on chromosome 5, 7 to 12 Mb and 15 to 17 Mb on chromosome 6, 9 to 24 Mb on chromosome 8, 1 to 11 Mb on chromosome 9 and 7 to 11 Mb on chromosome 10, 7 to 17 Mb on chromosome 11, and 11 to 20

Mb on chromosome 12 were the regions where higher SNPs frequencies were identified compared to Jupiter. The longest regions having higher SNPs frequencies for Lemont, LM-1 and Trenasse were in the region between 11 to 20 Mb on chromosome 12. For Trenasse, chromosome 8, 9, 10 and 12 had the longest regions of higher SNPs densities, whereas, for Bengal, only in the regions between 7 to 13 Mb on chromosome 4 and 10 to 17 Mb on chromosome 11 have higher SNP densities (Figure 3.11). Similar distribution patterns were also observed in insertions and deletions (Figures 3.12 and 3.13)

3.4.5 Annotations of the variants identified on individual genome of four rice genotypes when compared with Jupiter

Pairwise comparisons of the variants between Jupiter and each of four rice genotypes showed differential degree of variations. Trenasse showed higher number of variants in various regions of genomes, including upstream, downstream, exon, intron, UTR5', UTR3', and intergenic regions. Bengal had the lowest number of variants among the four rice genotypes. Exon tend to have the highest variants densities, and the UTR3' had the lowest variants densities in all four genomes when compared to Jupiter (Table 3.6 and Figure 3.7). Lemont and LM-1 had similar level of variants with Jupiter in all seven genomic regions.

Variants on each of the seven regions were important for further study, however, variants occurred in non-synonymously in CDS region were the most important due to change in amino acid product. SNPs in CDS regions in all four rice genotypes were ranged from 179, 000 to 440, 000 in which Trenasse had the highest and Bengal had the lowest number of SNPs. Within CDS, the number of non-synonymous SNPs were ranged from 92, 000 – 231, 000 (Appendix 15). Similarly, number of synonymous SNPs identified in four genotypes ranged from 87,000 to 208,000. In addition, number of variants due to insertions and deletions in CDS regions ranged

from 1800 to 4500. Frameshift variations due to insertions and deletions were found in the ranges between 2000 and 5200 in four rice genotypes (Appendix 15).

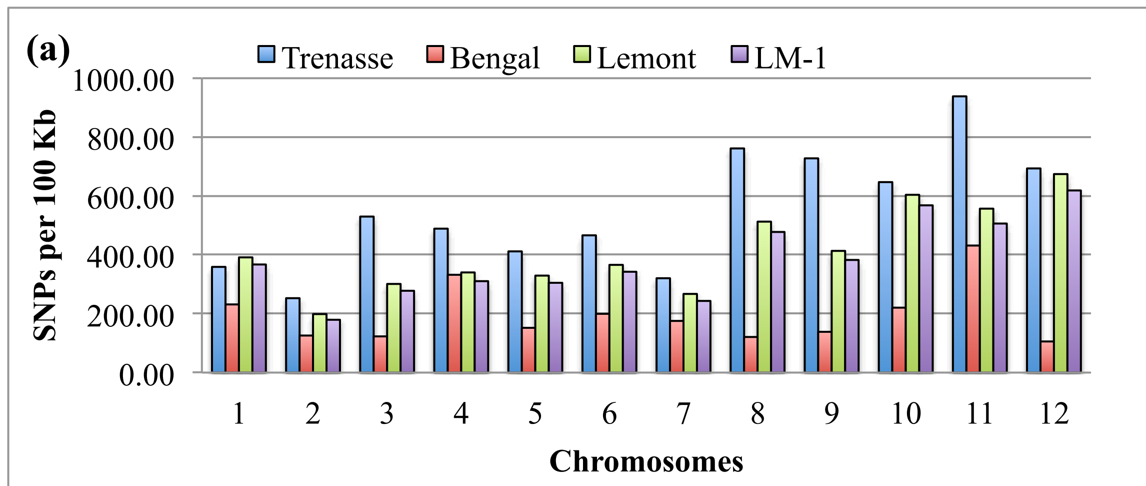
Total number of genes affected by several variants, such as non-synonymous SNPs, insertions and deletions, and frameshift was analyzed. Large number of genes was affected by non-synonymous SNPs followed by frameshift, insertions, and deletions. (Table 3.7 and Figure 3.8). Similarly, among the individual genotypes, the largest number of genes affected by nsSNPs was found in Trenasse and followed by Lemont, LM-1 and Bengal with 13, 696; 10, 460; 10, 153; and 6, 057 genes, respectively (Table 3.7).

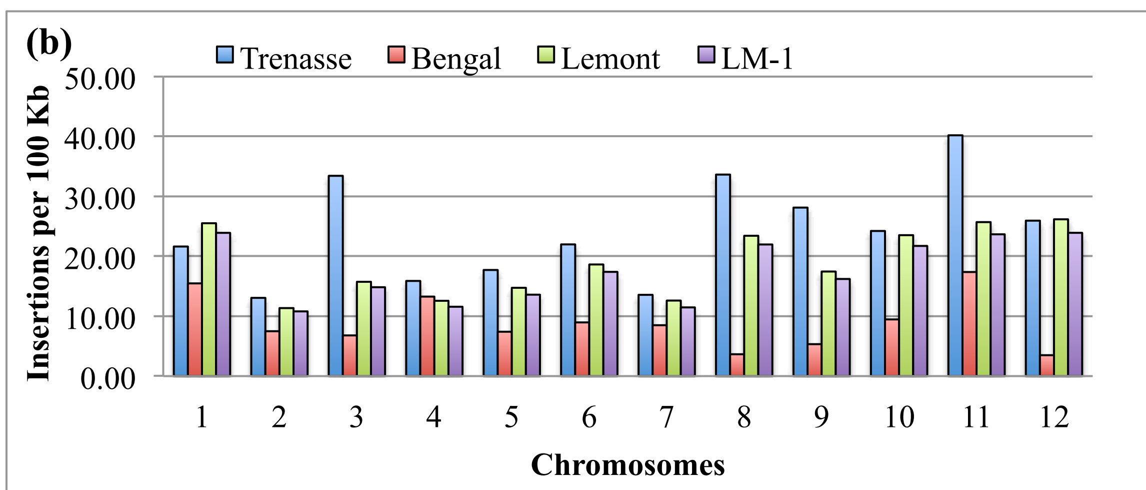
Since, Jupiter is partial resistant cultivar and Trenasse is susceptible cultivar for BPB, genes with non-synonymous SNPs found between these two genotypes were of great interest. So, gene ontology (GO) analysis of those genes affected by non-synonymous SNPs was performed to understand their functions using a web-based tool, agriGO (Du et al., 2010). A total of 21, 491 transcripts ID were assigned for GO analysis, however, only 8, 805 transcripts ID were found to be annotated in agriGO, and used as query list. Those transcripts ID were classified into 18 different GO terms. However, the results obtained did not have high level of gene enrichment in any GO terms.

GO terms, including signal transduction, response to stress, and protein modification, which were categorized in biological process have greater number of transcripts. Similarly, greater number of transcripts ID was grouped in nucleotide binding and kinase activity under molecular function (Figure 3.9).

Table 3.5 Number of SNPs, insertions, and deletions, on individual chromosome identified between the Jupiter and the four rice genotypes

	Trenasse	Bengal	Lemont	LM-1
SNPs				
Chromosome 1	155,199	100,099	169,186	158,744
Chromosome 2	90,748	45,183	71,352	64,418
Chromosome 3	192,799	44,731	109,543	101,111
Chromosome 4	173,417	117,846	120,662	110,157
Chromosome 5	123,230	45,510	98,604	91,281
Chromosome 6	145,550	62,332	114,225	106,938
Chromosome 7	95,086	52,068	79,282	72,226
Chromosome 8	216,367	34,359	145,715	135,740
Chromosome 9	167,313	31,846	95,043	87,963
Chromosome 10	150,008	51,131	140,010	131,721
Chromosome 11	272,063	125,128	161,460	146,798
Chromosome 12	190,753	29,083	185,469	170,175
Total	1,972,533	739,316	1,490,551	1,377,272
Insertions				
Chromosome 1	9,354	6,693	11,035	10,341
Chromosome 2	4,690	2,692	4,078	3,880
Chromosome 3	12,167	2,472	5,729	5,400
Chromosome 4	5,637	4,709	4,461	4,108
Chromosome 5	5,304	2,218	4,414	4,070
Chromosome 6	6,866	2,799	5,820	5,434
Chromosome 7	4,026	2,522	3,743	3,404
Chromosome 8	9,563	1,038	6,659	6,246
Chromosome 9	6,470	1,227	4,015	3,729
Chromosome 10	5,619	2,195	5,457	5,040
Chromosome 11	11,659	5,040	7,456	6,865
Chromosome 12	7,140	961	7,202	6,581
Total	88,495	34,566	70,069	65,098
Deletions				
Chromosome 1	8,593	6,225	9,772	9,351
Chromosome 2	4,217	2,392	3,702	3,481
Chromosome 3	11,195	2,296	5,431	5,197
Chromosome 4	5,254	4,441	4,047	3,804
Chromosome 5	4,846	2,053	3,880	3,684
Chromosome 6	6,662	2,695	5,512	5,283
Chromosome 7	3,761	2,452	3,528	3,286
Chromosome 8	9,128	920	6,015	5,622
Chromosome 9	6,054	1,288	3,859	3,548
Chromosome 10	5,364	1,669	5,097	4,834
Chromosome 11	10,423	4,821	6,520	6,078
Chromosome 12	6,670	790	6,512	6,028
Total	82,167	32,042	63,875	60,196





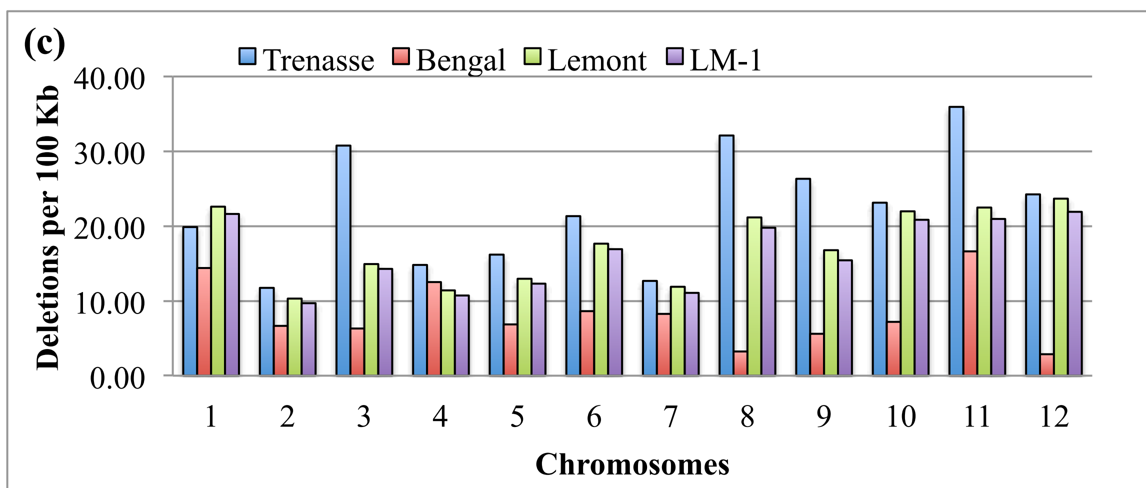


Figure 3.6 Frequency of variants on individual chromosome identified between the Jupiter and the four rice genotypes, (a) SNP densities, (b) insertion densities, and (c) deletion densities per 100 Kb.

Table 3.6 Annotation of variants at various genomic regions identified between the genome of Jupiter and other four rice genotypes

	Jupiter vs			
	Trenasse	Bengal	Lemont	LM-1
Upstream 5Kb	1,920,546	704,179	1,430,861	1,317,866
UTR5'	26,468	9,585	18,913	18,128
Exon	459,404	186,446	361,671	335,915
Intron	393,156	143,872	291,187	272,108
UTR3'	39,254	13,330	26,980	25,963
Downstream 5Kb	1,837,883	679,579	1,388,568	1,280,934
Intergenic	1,347,790	494,130	1,016,557	935,565

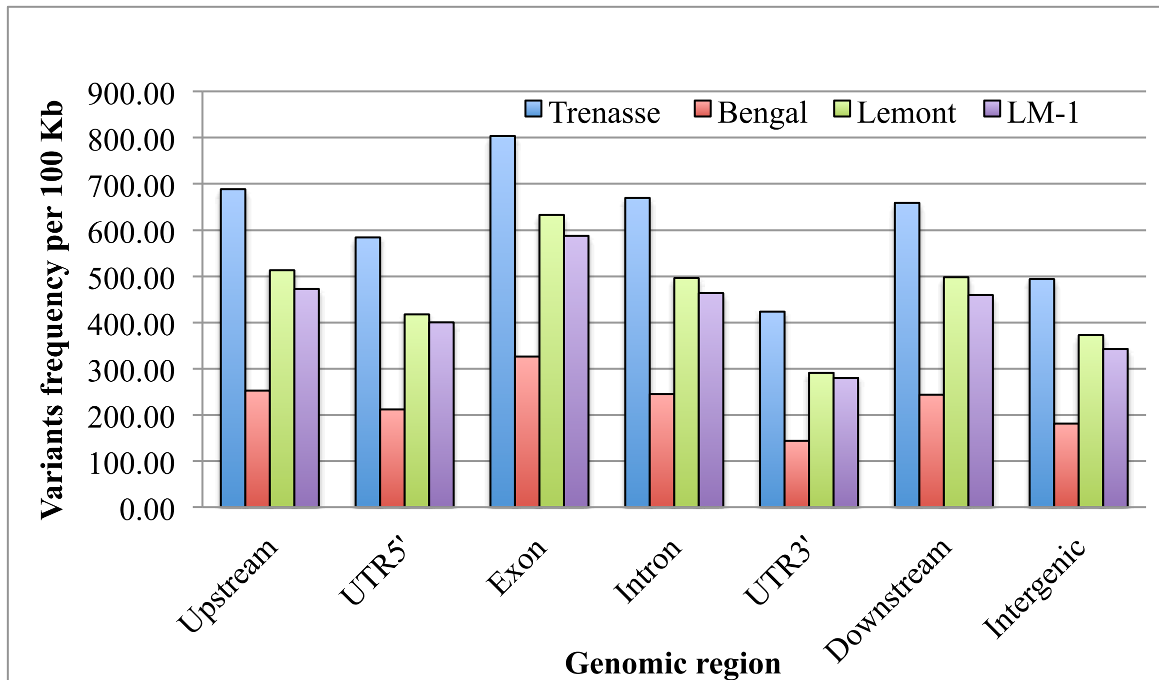


Figure 3.7 Frequency of variants at 100 Kb interval on various genomic regions identified between the genome of Jupiter and the genome of four rice genotypes.

Table 3.7 Total number of genes affected by non-synonymous SNPs, insertions, deletions and frameshift

	Non-synonymous SNPs	Insertions	Deletions	Frameshift
Jupiter vs Trenasse	13,696	1,432	1,267	2,007
Jupier vs Bengal	6,057	571	518	834
Jupiter vs Lemont	10,460	1,106	943	1,612
Jupiter vs LM-1	10,153	1,024	919	1,454

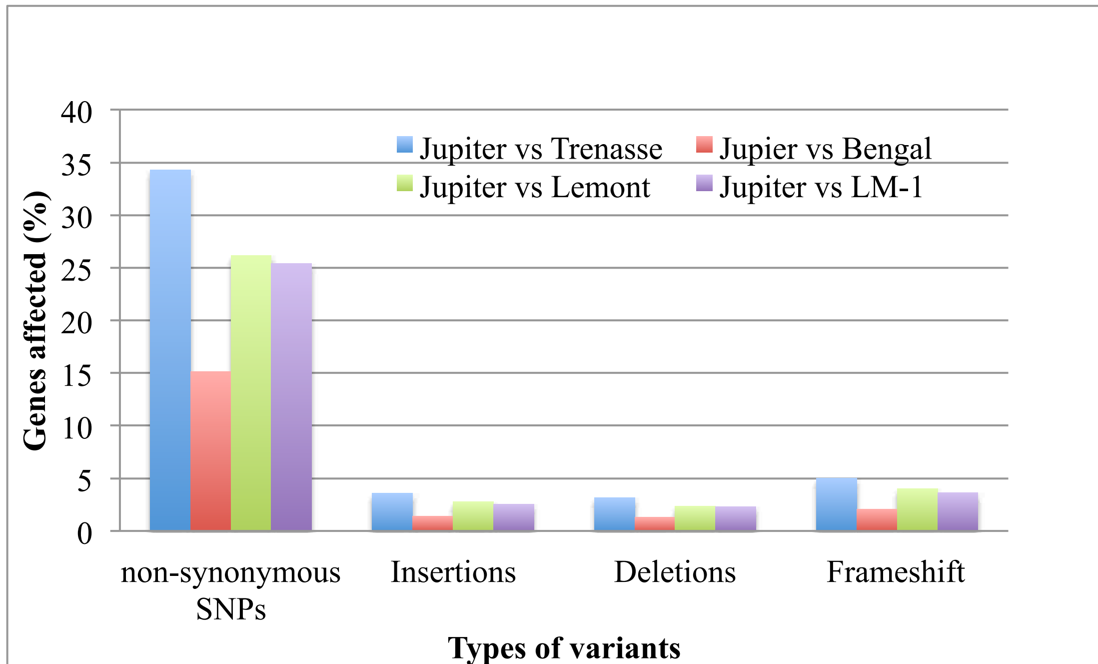


Figure 3.8 Percentage of genes affected by different variants including non-synonymous SNPs, insertions, deletions, and frameshift in Jupiter compared to four other rice genotypes

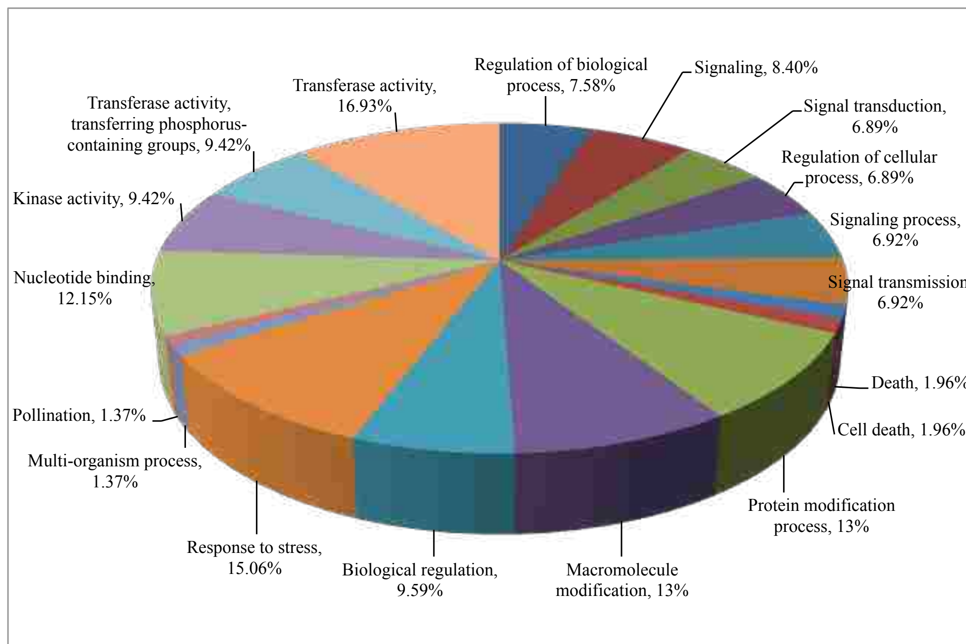


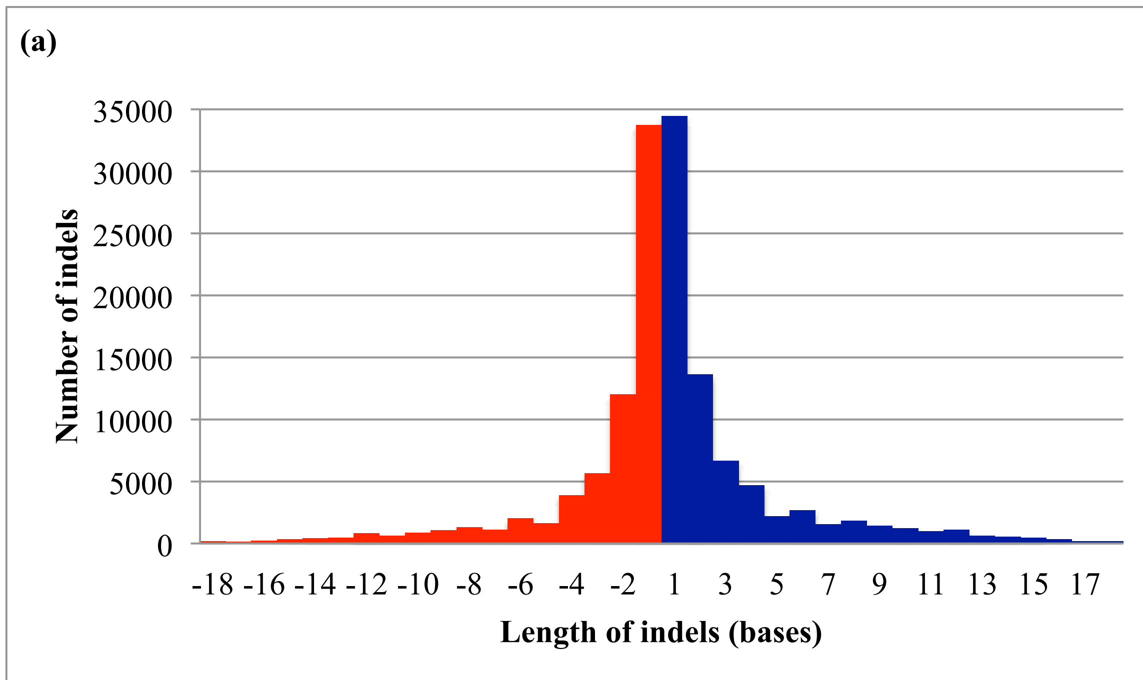
Figure 3.9 Grouping of genes in Trenasse with non-synonymous SNPs identified between Trenasse and Jupiter based on gene ontology using agriGO. agriGO divides the genes into 18 different groups.

Two types of nucleotide substitutions, including transitions (C/T and A/G), and transversions (C/G, T/A, A/C, and G/T), were observed in four rice genotypes. Transitions were occurred more than transversion in all four genotypes. Within transitions, number of C/T was higher than A/G in Trenasse, Bengal and Lemont; however, A/G was higher than C/T in LM-1. The highest and the lowest transitions occurred in Trenasse and Bengal, respectively. Similarly, in tranversions, T/A was the highest and C/G was the lowest in all four rice genotypes. Transitions to transversions ratios for all four genotypes were found to be 2.55, 2.50, 2.52 and 2.53 in Trenasse, Bengal, Lemont and LM-1, respectively (Table 3.8).

Table 3.8 Classification of nucleotide substitutions in SNPs: Nucleotide substitutions identified between the Jupiter genome and four rice genotypes

	Jupiter vs			
	Trenasse	Bengal	Lemont	LM-1
Transitions (Ts)				
C/T	708,909	264,152	534,104	493,517
A/G	708,530	264,109	533,468	493,669
Total	1,417,439	528,261	1,067,572	987,186
Transversions (Tv)				
C/G	102,800	38,989	78,629	72,144
T/A	167,197	64,324	129,017	119,032
A/C	142,652	54,174	107,867	99,721
G/T	142,445	53,568	107,466	99,189
Total	555,094	211,055	422,979	390,086
Ts/Tv ratio	2.55	2.50	2.52	2.53

There was a variation in length among the total identified insertions and deletions either in comparison with the reference genome, Nipponbare or with the Jupiter. The length ranged from 1 to 18 bases for both insertions and deletions (Figure 3.10 [a, b]). Majority of insertions and deletions were mononucleotide, and followed by 2 bases, and 3 bases (Figure 3.10 [a, b]).



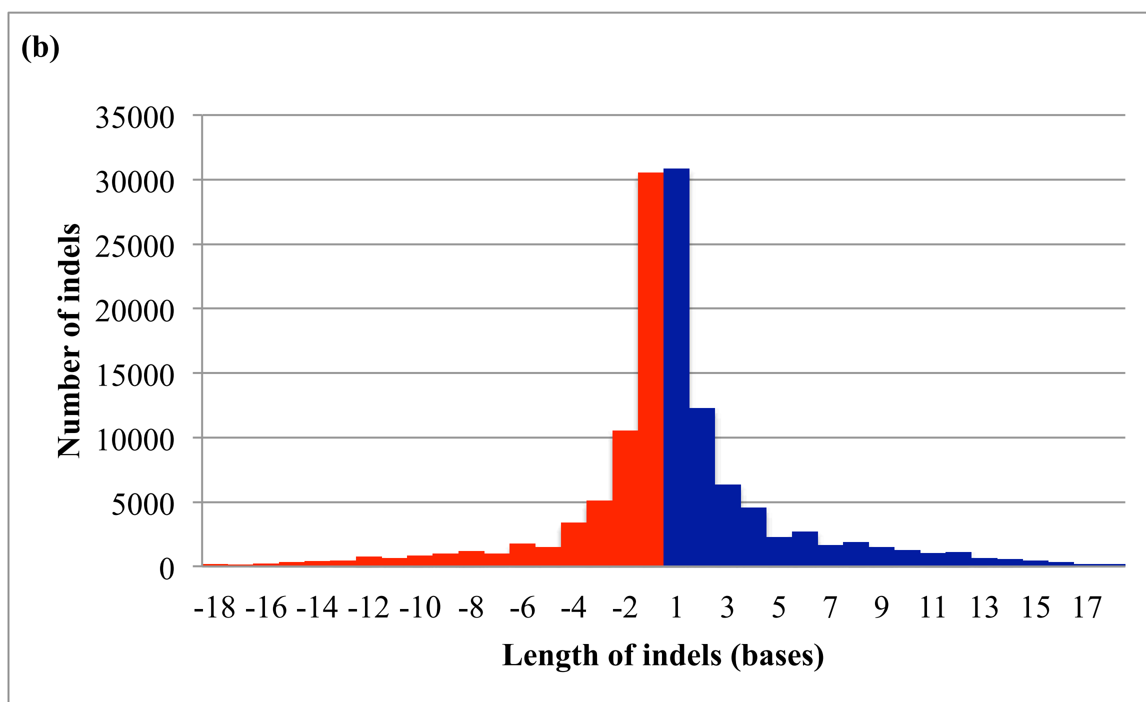


Figure 3.10 Distribution of insertions and deletions (indels) variants based on their length in, (a) five rice genotypes when compared with the reference genome, Nipponbare, and (b) four rice genotypes when compared with Jupiter. The x-axis shows the number deletions (red) and insertions (blue). The y-axis shows number of insertions and deletions.

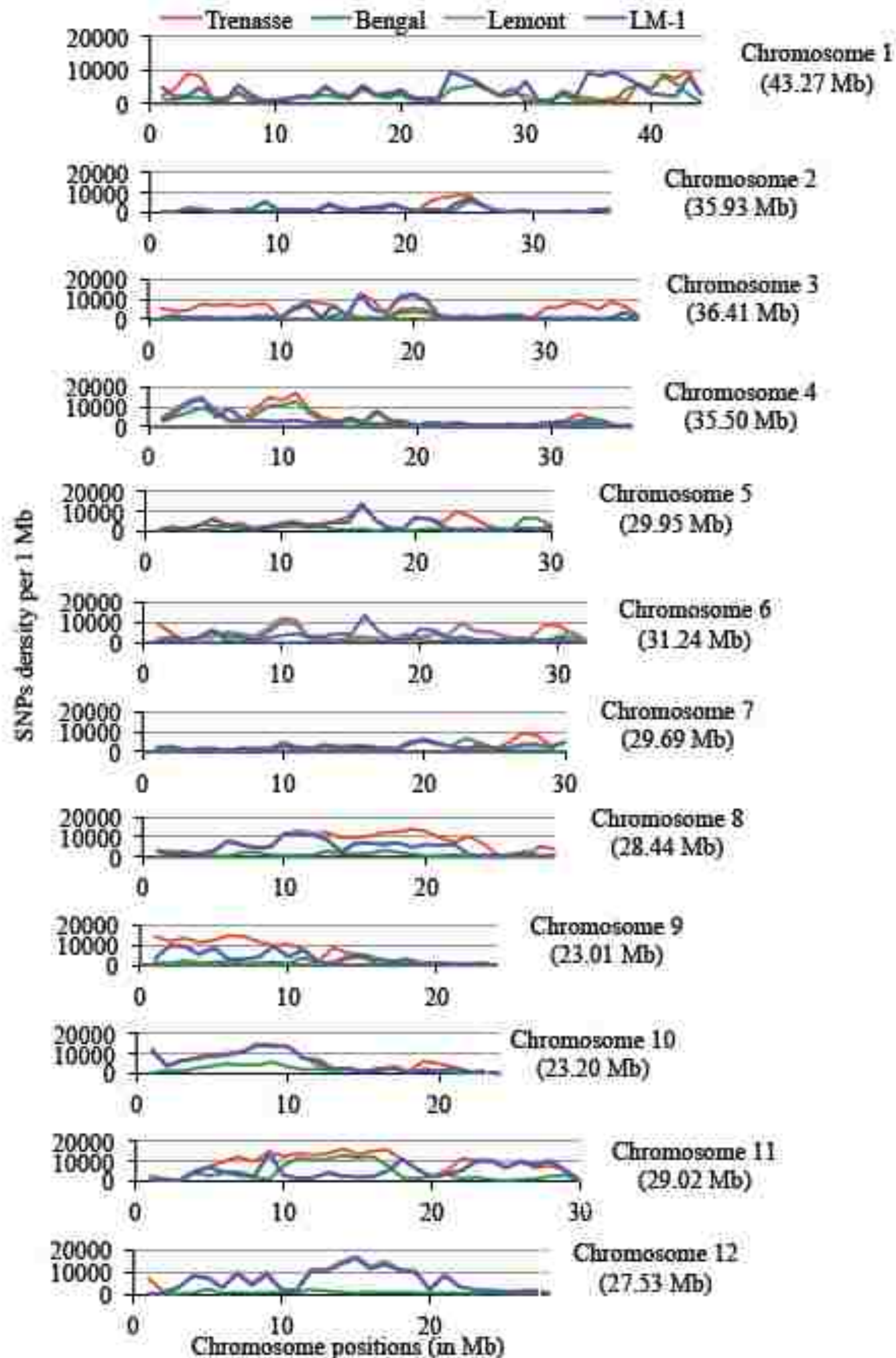


Figure 3.11 Distribution of single nucleotide polymorphisms (SNPs) between the Jupiter and four rice genotypes in 12 chromosomes. X-axis represents the physical distance of each chromosome in Mb, and Y-axis represents the number of SNPs. Chromosome number and the size of each chromosome are given on the side of the graph.

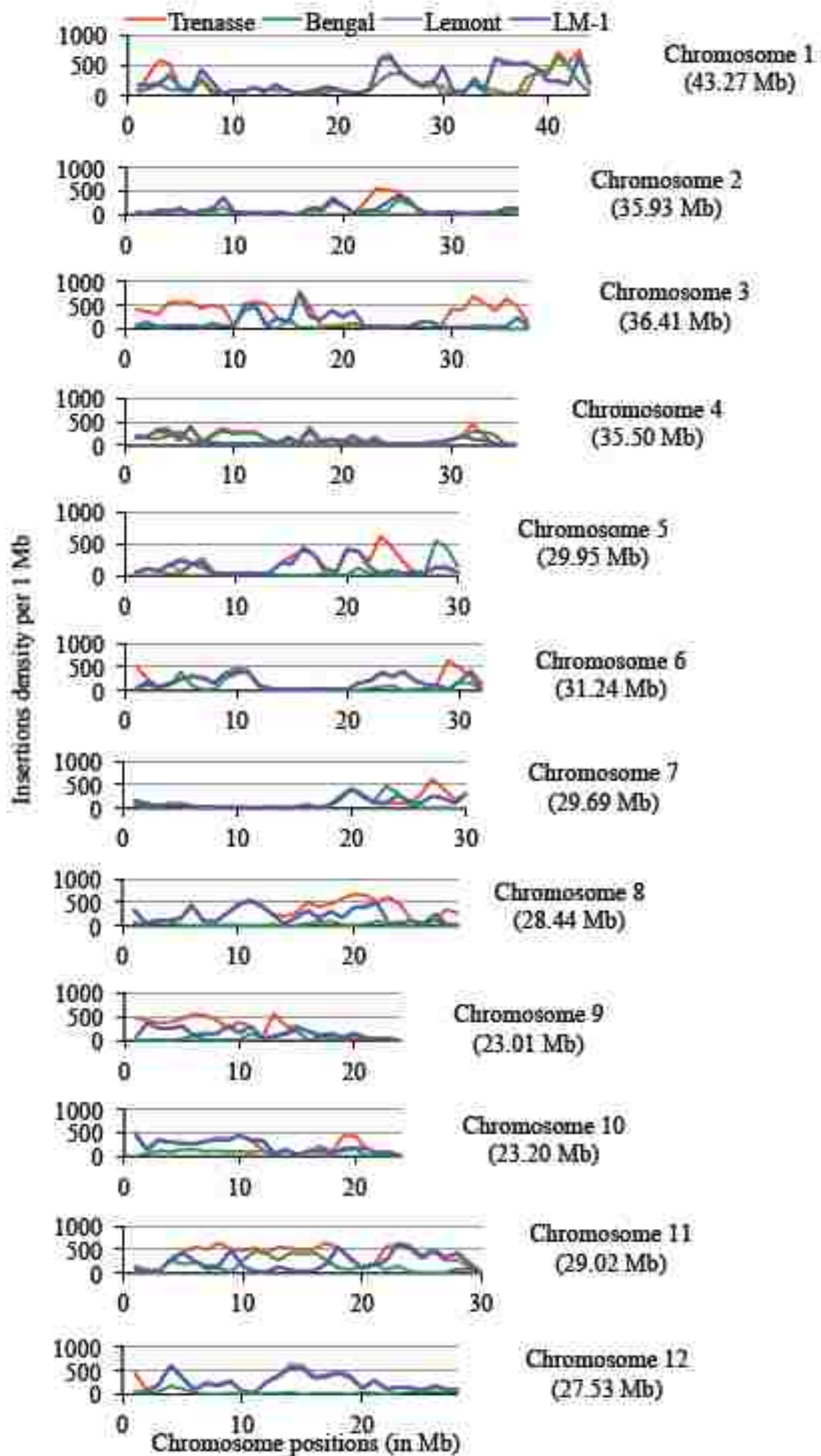


Figure 3.12 Distribution of insertions between the Jupiter and four rice genotypes in 12 chromosomes. X-axis represents the physical distance of each chromosome in Mb, and Y-axis represents the number of deletions. Chromosome number and the size of each chromosome are given on the side of the graph.

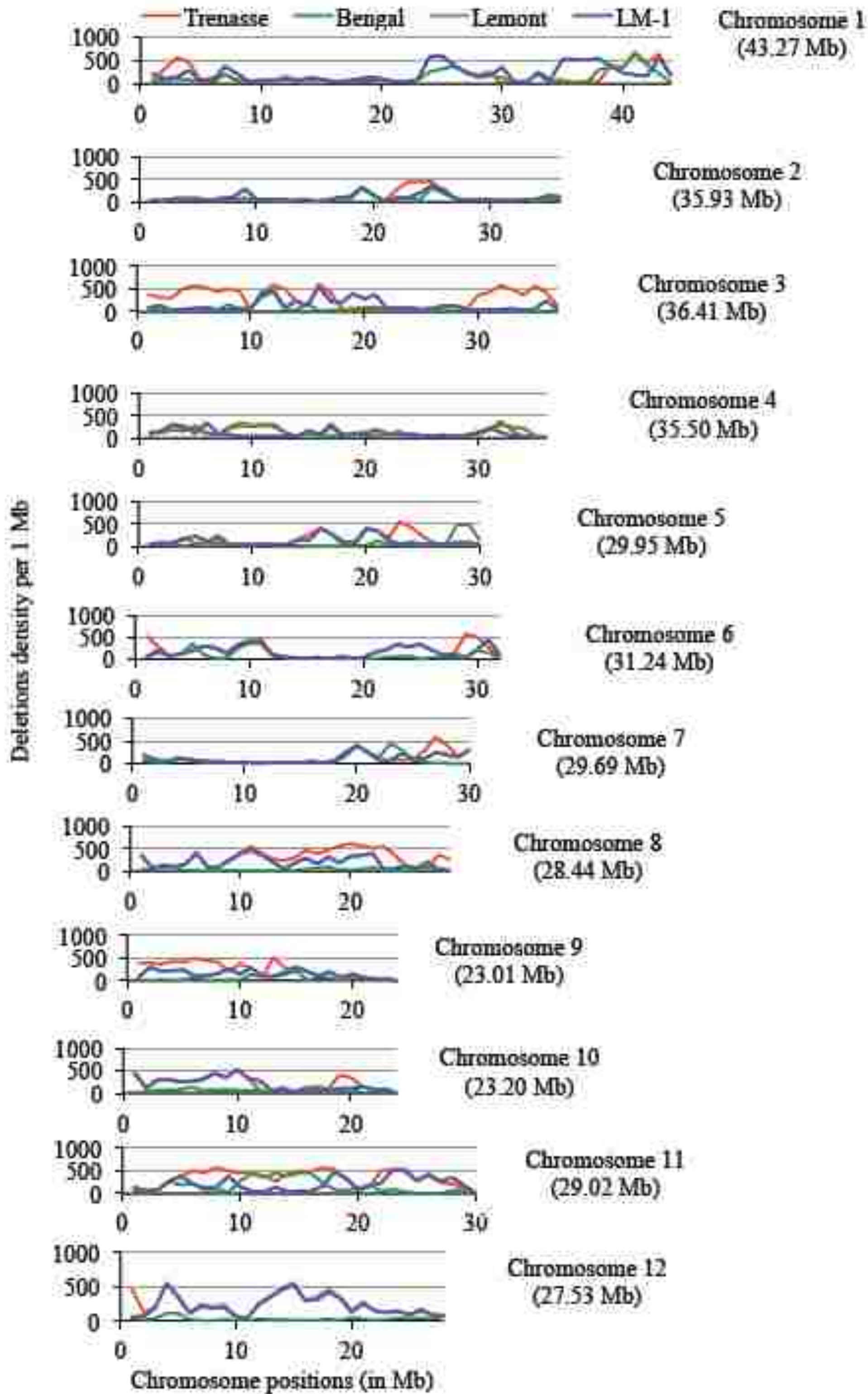


Figure 3.13 Distribution of deletions between the Jupiter and four rice genotypes in 12 chromosomes. X-axis represents the physical distance of each chromosome in Mb, and Y-axis represents the number of deletions. Chromosome number and the size of each chromosome are given on the side of the graph.

3.4.6 Population structure analysis

Population structure analysis using FRAPPE showed the five rice genotypes used in this study were located at the same place within tropical *japonica* group. Among five rice genotypes Trenasse and Lemont were located in the tropical *japonica* accession with some ancestry sharing of *indica* accessions in Trenasse. LM-1, a mutant derivative of Lemont is located close to Lemont. In contrast, Jupiter and Bengal have some admixture of tropical and temperate *japonica* ancestry (Figure 3.14).

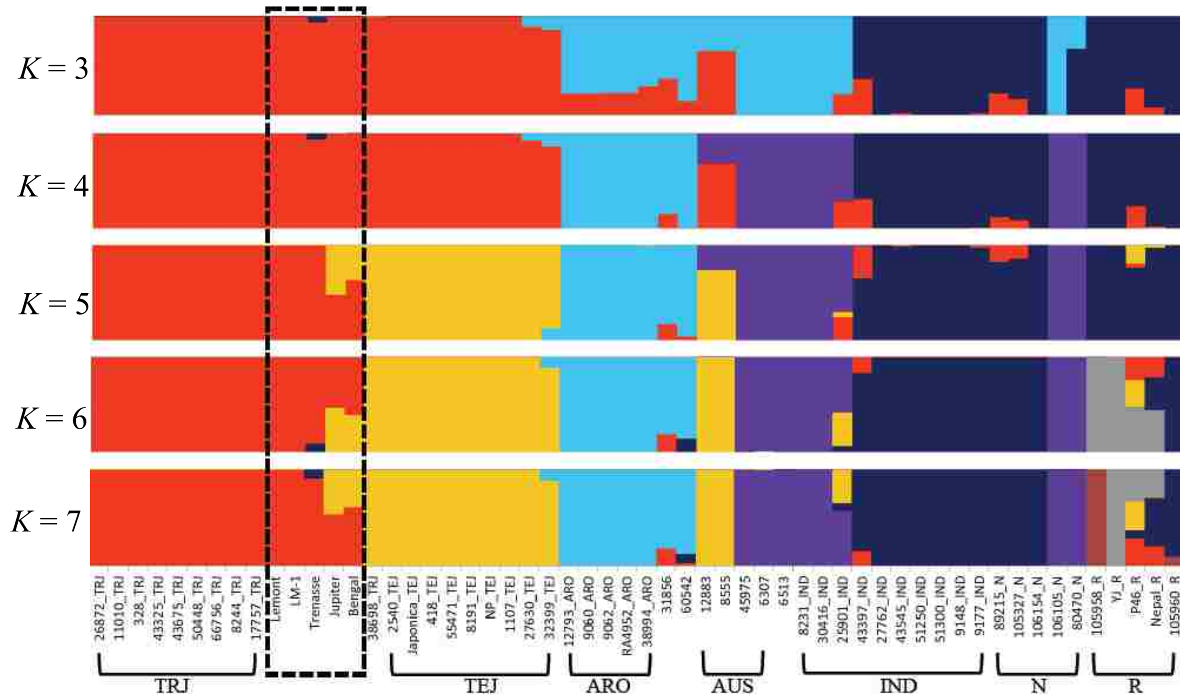


Figure 3.14 The five rice genotypes sequenced in this study (shown in the dashed box) in comparison with 50 rice accessions whose SNP data were publicly available (Xu et al., 2012). Total 1,188,460 non-ambiguous, biallelic SNPs were used for population structure analysis (*K* = 3 to 7, 10000 iterations). Five rice genotypes used in this study are clustered within tropical japonica. The 50 rice accessions were labeled as in Xu et al., 2012. TRJ, Tropical Japonica; TEJ, Temperate Japonica; ARO, Aromatic rice; AUS, *aus* rice; IND, Indica; N, *O. nivara*; R. *O.rufipogon*. Each accession is represented vertically and proportion of the ancestral populations contributions is represented by color segment in each vertical line.

3.5 DISCUSSION

Comprehensive identification of genome-wide DNA polymorphisms was performed from the whole genome sequence data of the commercially grown japonica rice genotypes at Louisiana. In-silico mapping of the paired-end sequences of each five genotypes covered about 92% of the reference genome, Nipponbare, unambiguously. We identified more than 400 SNPs per 100 Kb in Trenasse, Lemont and LM-1, but lower in Jupiter and Bengal which was only 200 SNPs per 100 Kb were detected (Figure 3.1a). The SNPs density found, in previous study, between Omachi and the reference genome, Nipponbare was higher than the SNP density between Nipponbare and two rice genotypes Jupiter and Bengal of this study, but lower than the SNP density found between the Nipponbare and remaining three rice genotypes Trenasse, Lemont and LM-1 used in this study (Arai-Kichise et al., 2011). However, SNP density found between six indica inbreds and the Nipponbare was higher than the SNP density found between Nipponbare and five rice genotypes used in this study (Subbaiyan et al., 2012). Moreover, pairwise comparison between a medium-grain cultivar, Jupiter and other four rice genotypes showed that higher SNP density was observed in Trenasse and the lowest in Bengal, suggesting that Bengal cultivar is closer to Jupiter (Figure 3.6a). Since Bengal is in pedigree of Jupiter, it is obvious that Jupiter to be closer with Bengal (Sha et al., 2006). These SNPs results was also supported by the population structure analysis of five rice genotypes in which Jupiter and Bengal are closer to temperate *japonica* where the reference genome Nipponbare is located (Figure 3.14). Distribution of insertions and deletions density per 100 Kb on five rice genotypes was observed like SNP density (Figures 3.1 [b, c], 3.3b and 3.6c). Higher variant density in three of the five rice genotypes used in this study suggested that higher genetic diversity might be present in those three rice genotypes compared to Jupiter and Bengal (Tenaillon et al., 2001).

Uneven distribution of SNP as well as insertions and deletions density within the chromosomes was observed. The same regions within the chromosome were found to have higher variants densities for some genotypes and have lower densities for other genotypes when compared with Nipponbare or with Jupiter. Higher densities of variants were detected in 11 Mb to 20 Mb of chromosome 12, 5 to 17 Mb of chromosome 11, 1 to 9 Mb of chromosome 9.Lower densities of variants also detected in several region within individual chromosome, including, 27 Mb to 33 Mb of chromosome 2, 22 Mb to 29 Mb of chromosome 3, 11 Mb to 22 Mb of chromosome 6, and 4 Mb to 14 Mb of chromosome 7 (Figures 3.2, 3.3, 3.4). These types of differences in the distribution of variants within the chromosomes have been described in previous studies of rice, wheat, and *Arabidopsis* (Arai-Kichise et al., 2011; Nordborg et al., 2005; Somers et al., 2006; Subbaiyan et al., 2012).

About 59% of total SNPs detected were located in the intergenic region and only ~21% were located in coding region of the genome of each of the five genotypes. Within the coding regions, ~47% were synonymous and ~53% were non-synonymous SNPs. Similarly, in the case of pairwise comparisons with Jupiter, ~59% of total SNPs were located in intergenic region and ~22% of total SNPs were located in coding regions. Among the SNPs in coding region, ~48% were synonymous and ~52% were non-synonymous SNPs. On average, ratio of non-synonymous to synonymous SNPs ranged from 1.06 in Jupiter/Bengal comparison to 1.15 in Nipponbare/Bengal mapping (Appendices 14 and 15). Similar ratio was observed in other species of rice including *indica* and tropical and temperate *japonica* (Xu et al., 2012). Furthermore, about ~66% of indels were located in intergenic region, ~2.5% of were located in coding region, and ~2.6% of indels were found to cause frameshift substitution when five rice genomes were mapped with Nipponbare and similar trends were observed in pairwise

comparison between Jupiter and four rice genotypes (Appendices 14 and 15). It has been reported that gene families with essential functions have lower non-synonymous to synonymous SNP ratio, and gene families involved in signal transduction and regulatory process have higher ratio (McNally et al., 2009; Xu et al., 2012). Higher number of variants towards 5 Kb upstream and 5 Kb downstream region were detected (Tables 3.3 and 3.5). Such a large number of variants in the upstream and downstream region of genes have role in altering regulation of gene expression resulting in alter phenotypic traits (Thumma et al., 2009; Zhang et al., 2011).

Large number of genes was found to be affect with non-synonymous SNPs between Jupiter and Trennase. Gene ontology enrichment analysis of those affected genes revealed the greatest number of genes was found to be involve in signal transduction, response to stress, protein modification process, nucleotide binding, and kinase activity. The nucleotide binding and kinase activity related genes were reported to be involved in resistance to several rice diseases, including rice blast, bacterial blight of rice(Wang et al., 1999; Xiang et al., 2006).

Furthermore, the ratio of transitions to transversions in five rice genotypes was observed. This phenomenon is known as transition bias, and occurs in the nature during evolution. This transition bias helped us to understand the DNA-sequence evolution. The ratio of transistions to transversions in this study was about 2.5 in all five rice genotypes, which is higher than the ratio obtained in previous study of rice (Jain et al., 2014; Subbaiyan et al., 2012; Wakeley, 1996). Higher transitions to transversions ratio indicate higher frequency of transitions mutation occurred compared to transversions. Tranistions mutation were favored more, and had an important role in conserving the protein structure than transversions mutation in nature (Wakeley, 1996). Within transitions C/T substitution was higher in most of the genome except in Bengal and LM-1 when compared to Nipponbare and Jupiter respectively (Tables 3.4 and 3.7).

Similarly, among transversions, T/A substitution was found in large numbers (Tables 3.4 and 3.7).

The analysis of population structure of five rice genotypes showed that all of the five rice genotypes were clustered within tropical *japonica*. It was found that Bengal and Lemont were tropical *japonica*, and rice accessions that were used to develop Jupiter (Sha et al., 2006) and Trenasse (Linscombe et al., 2006) were also tropical *japonica* (Lu et al., 2005). Furthermore, Trenasse, a long-grain cultivar contained the admixture of *indica* accessions and tropical *japonica.* It was reported that one of the accessions in pedigree of Trenasse contains semidwarf gene from either IR-8 or Taichung Native 1 (Tseng et al., 1984). This might be the reason that Trensasse have large number of variants when mapped with the Nipponbare, which is a temperate *japonica.* Jupiter and Bengal were clustered in the border of tropical and temperate *japonica* showing some admixture of both groups. Since, Bengal which is a tropical *japonica* contains a temperate *japonica* accesseion, M-201 (Lu et al., 2005), in its pedigree, the portion of temperate *japonica*, in our study, in Jupiter and Bengal might have come from M-201 (Linscombe et al., 1993; Sha et al., 2006). LM-1, resulting from gamma radiation mutation was located closer to Lemont in tropical *japonica* group (Figure 3.14). The population structure analysis correlated with the known characteristics of the five rice genotypes and helped to understand the proportion of different ancestry accession in those rice genotypes.

In this study, genome-wide polymorphism in five rice genotypes commercially grown in southern region of the United States was identified. The identified SNPs and insertions and deletions will be potential assets for molecular marker development for genetic studies. In addition, the variants identified in this study will help to expand resources of DNA

polymorphisms that can be used along with available variants resources for high density QTL-mapping, association mapping, and comparative study of rice.

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CHAPTER IV: POLYMORPHIC MOLECULAR MARKERS SURVEY FOR GENOTYPING RECOMBINANT INBRED LINES DEVELOPED FROM THE CROSS BETWEEN JUPITER AND TRENASSE

4.1 INTRODUCTION

Bacterial panicle blight (BPB), caused by *Burkholderia glumae* and *B. gladioli*, is one of the major rice diseases in the rice-producing areas of the southern United States (Nandakumar, R et al., 2007; Nandakumar et al., 2005; Nandakumar et al., 2009). A typical BPB symptom in rice is shown in Figure 1.1. BPB reduces rice yield by as much as 40% in heavily infested fields (Nandakumar et al., 2009). Despite the importance of the disease, no effective control measure is available for BPB. In addition, no completely resistant rice cultivars were available. However, Jupiter, a medium-grain cultivar shows partial resistance to this disease (Nandakumar, R. et al., 2007; Sha et al., 2006). On the other hand, a commercially grown long-grain cultivar, Trenasse, is susceptible to BPB (Figure 1.1) (Linscombe et al., 2006).

Molecular basis for the quantitative traits, such as disease resistance, can be unravelled by genetic mapping. Genetic polymorphisms including, single nucleotide polymorphism (SNP), insertions and deletions are helpful to map genes responsible for quantitative trait variations. Those genetic variations are the basis for developing molecular markers for genotyping and genetic mapping study. Molecular markers, including amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and simple sequence repeats (SSRs) are powerful tools for association studies, quantitative trail loci (QTL) mapping, marker-assisted selection, and variety identification (Sharma et al., 2008). Among the available molecular markers, SSR were widely used due to better reproducibility and high polymorphism. Rice has a number of databases for SSR markers that has been used for QTL mapping and association studies for various traits (McCouch et al., 1988; McCouch et al., 2002; Price et al., 2000; Sato et al., 2006; Sharma et al., 2009; Wada et al., 2008). SNPs are becoming popular as molecular

markers for genotyping complex-traits and genome-wide association studies because of its abundance availability, and easier detection in the genome compared to other molecular markers (Huang et al., 2009; Lee et al., 2008; Rafalski, 2002). However, only few reports were available for QTL mapping for partial resistance to BPB and bacterial grain rot in rice caused by *B. glumae* (Mizobuchi, Ritsuko et al., 2013; Mizobuchi, R. et al., 2013; Pinson et al., 2010).

Development of high-throughput sequencing technologies in recent years provides useful genome sequence data from large number of samples in short period of time with low cost (Craig et al., 2008). Those data are being used for genetic studies of the individual organisms at genome level. High-throughput sequence data from rice enhance our understanding about genes and their roles on important agronomic traits, and genetic relatedness among the rice accessions. Comparative study of whole genome sequences of various rice cultivars will be helpful in identifying variations among them at genome level.

In our study, SSR markers available in the Gramene database, (www.gramene.org), were used for polymorphism survey. In addition, identification of SSR motifs and single nucleotide polymorphisms (SNPs) from high-throughput sequence data provided an alternative way to develop additional molecular markers (Kumar et al., 2012). In this study, identification and development of SSR and SNPs markers were done by comparative analysis of whole genome sequences between two cultivars. These molecular markers can be used for future QTL mapping studies for partial resistance to BPB.

4.2 OBJECTIVES

i. To identify polymorphic SSR markers between Trenasse and Jupiter from the available SSR markers databases

ii. To compare high-throughput sequence data of two rice cultivars, Jupiter and Trenasse, and identify SSR motifs and SNPs between them.

4.3 MATERIALS AND METHODS

4.3.1 Plant material and genomic DNA isolation

Genomic DNA from parent plants was isolated using CTAB method (Clarke, 2009). Briefly, 4 g of freshly emerging leaf tissues grown in the greenhouse were ground to fine powder in liquid nitrogen with mortar and pestle. The powder was mixed with 10 ml of CTAB buffer (2% CTAB) in a 50 ml of centrifuge tube. The tube with the mixture was incubated at 55°C for about 15 min in a recirculation water bath. The tube with plant tissue mixture was centrifuged at 12000g for 5 min to spin down cell debris after incubation, and the supernatant was transferred to a clean tube. Five ml of chloroform: isoamyl alcohol (24:1) was added to the supernatant and mixed properly. The mixture was centrifuged for 1 min at 13000 rpm. The upper aqueous phase after centrifugation was transferred carefully to a new clean tube. Transferred aqueous solution was mixed with 1 ml of 7.5M ammonium acetate followed by 10 ml of ice-cold absolute ethanol. The mixture was mixed properly and stored at -20°C for an hour to precipitate the genomic DNA. After an hour of incubation, the tube was centrifuged for 1 min at 13000 rpm, which helped to form a pellet at the bottom of the tube. Supernatant was removed from the tube and the DNA pellet was washed with 70% ice-cold ethanol for two times by centrifuging at 13000 rpm for 1 min. After washing, ethanol was discarded, the pellet was dried in the tube and resuspend in 1 ml of sterilized ddH_2O. The resuspended DNA was treated with 10 μg/ml of RNaseA. After resuspension, the DNA was incubated at 65°C for 20 min and stored at -20°C for further use.

4.3.2 Survey of molecular marker for polymorphism between Trenasse and Jupiter

In this experiment, SSR markers used were primarily selected from the Gramene database (www.gramene.org) (McCouch et al., 2002). About 1091 SSR markers representing all 12 chromosomes of rice were selected to detect polymorphism between Trenasse and Jupiter. Primers were obtained from Bioneer, Inc.(Alameda, CA). PCR amplification using selected SSR markers and genomic DNA of Trenasse and Jupiter was conducted in C1000^{TM} Thermal Cycler from BioRad (Hercules, CA).

Each PCR reaction contained 1 µl of genomic DNA (~ 50 ng/µl), 2.5 µl of 10X PCR buffer, 1.25 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTP mix, 1.0 µl of homemade *Taq* polymerase (~ 1.0U/µl), 1.25 µl of 10 µM forward and reverse primers, and 15.25 µl of sterilized ddH_2O in a total volume of 25 µl. The PCR program consisted of the initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 45 sec, 55°C for 45 sec and 72°C for 1 min, and the final extension at 72°C for 5 min. After amplification, PCR products were electrophoresed in 4.5% Agarose SFR^{TM} (Superfine Resolution Agarose) gel, (AMRESCO) at 180V for 4.5 hours, stained with (10 mg/µl) ethidium bromide, and visualized with a Kodak Gel Logic 1500 imaging system (Rochester, New York, USA).

4.3.3 Whole genome sequencing and reference-based assembly

One-week young leaves of Jupiter and Trenasse were used for genomic DNA extraction using DNeasy Plant Mini Kit, Qiagen (Valencia, CA). DNA was sent to the sequencing facility at Virginia Bioinformatics Institute, Virginia-Tech, for sequencing. 100 bp paired-end sequences reads obtained from the Illumina GAIIx platform was assembled using the reference sequence, Nipponbare, IRGSP pseudomolecules (Build 4.0), with reference-guided genome alignment option in SeqMan Ngen (DNASTAR).

4.3.4 SNPs identification, primer design and validation

Assembled sequences of Trenasse and Jupiter from the SeqMan Ngen were used in ArrayStar (DNASTAR) to identify variants, including SNPs, insertions and deletions between the two cultivars, and the reference. In the mean time, pairwise comparison between Trenasse and Jupiter was also performed. The identified SNPs between Trenasse and Jupiter, from pairwise comparison, were filtered based on their genotype (homozygous or heterozygous) and classification (synonymous or non-synonymos). To improve stringency of the identified SNPs, further filtering was performed based on the reads depth (≥ 8) and Q value (≥ 20).

Allele-specific primers were designed from identified SNPs and its flanking sequences. Primer length and PCR product length were optimized and designed with the help of a web-based primer designing tool, WebSNAPER (Drenkard et al., 2000). Those primers were tested for the validation of SNPs between Jupiter and Trenasse by PCR. Each PCR reaction contained 18 µl of sterilized ddH_2O, 2.5 µl of 10X PCR buffer, 0.75 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTP mix, 1 µl of home-made *Taq* polymerase (~1.0U/µl), 1.25 µl of 10 µM forward and reverse primers, and 1 µl of ~50 ng/µl of genomic DNA in a total volume of 25 µl. The PCR program consisted of the initial denaturation at 95°C for 3 min, and 33 cycles of 95°C for 20 sec, 70°C for 20 sec and 72°C for 20 sec; and the final extension at 72°C for 3 min. PCR products were later visualized in 2% agarose gel electrophoresis with 170V for 3 hours.

The flow chart for identification of SNPs, development and validation of allele-specific primers is shown in Figure 4.1.

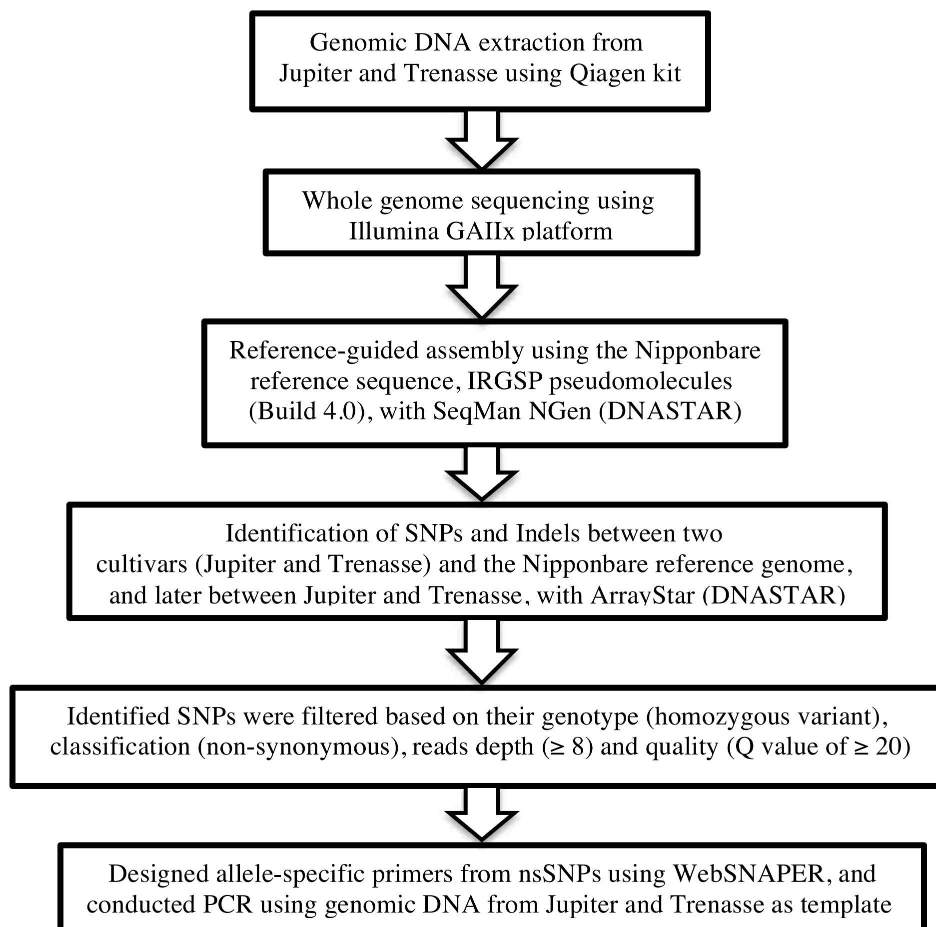

Figure 4.1: Flow chart of allele-specific, non-synonymous SNP marker development

4.3.5 SSR motifs identification, development of polymorphic markers between Trenasse and Jupiter and validation

The consensus sequences for Jupiter and Trenasse obtained from SeqMan Pro (DNASTAR) were used to identify SSR motifs using MIcroSAtellite (MISA) identification tool (Thiel et al., 2003). Search for perfect SSR repeats in both genomes were performed with 10, 7, 6, 5, 4, and 4 repeat units of mono-nucleotide repeats, di-nucleotide repeats, tri-nucleotide repeats, tetra-nucleotide repeats, penta nucleotide repeats and hexa-nucleotide repeats, respectively. Perfect SSR motifs obtained from consensus sequences of both Jupiter and Trenasse using MISA were compared based on the reference coordinates manually for the

difference in repeat number of motifs. Primer3 was used to design primers using flanking sequences of 300 bases including those motifs with differences in number of repeats (Rozen & Skaletsky, 1999). Those primers were tested for the validation of polymorphisms between Jupiter and Trenasse by PCR. Each PCR reaction contained 1 µl of genomic DNA (~ 50 ng/µl), 2.5 µl of 10X PCR buffer, 1.25 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTP mix, 1.0 µl of homemade *Taq* polymerase (~ 1.0U/µl), 1.25 µl of 10 µM forward and reverse primers, and 15.25 µl of sterilized ddH_2O in a total volume of 25 µl. The PCR program consisted of the initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 45 sec, 55°C for 45 sec and 72°C for 1 min; and the final extension at 72°C for 5 min. After amplification, PCR products were electrophoresed in 4.5% Agarose SFR™ (Superfine Resolution Agarose) gel, (AMRESCO) at 180V for 4.5 hours, stained with (10 mg/µl) ethidium bromide, and visualized with a Kodak Gel Logic 1500 imaging system (Rochester, New York, USA).

4.4 RESULTS

4.4.1 Survey of polymorphic markers from Gramene database

Out of 1091 SSR markers selected, only 28 usable polymorphic markers were identified between Trenasse and Jupiter (Appendix 16). These were not enough for representing all 12 chromosomes of rice, which will be required for further study of QTL mapping and association mapping.

4.4.2 Sequence alignment and SNP identification

About 40 millions of paired-end reads generated from each cultivars Jupiter and Trenasse were aligned with the help of reference genome, Nipponbare. The aligned reads of Jupiter and Trenasse covered an average of 94.72 and 91.21%, respectively, of the reference genome (Table 4.1). Comparative analysis between assembled sequences and the reference genome using

ArrayStar (DNASTAR) identified more than 1 million SNPs in each cultivar. Furthermore, pairwise comparison between Jupiter and Trenasse sequences detected more than 700,000 SNPs. Chromosome 8 had the highest SNP density of 28.77 SNPs per 10 Kb, and chromosome 7 had the lowest of 10.13 SNPs per 10 Kb (Figure 4.2) among two cultivars.

Table 4.1 Coverage of mapped reads with the reference genome Nipponbare

Accession	Chromosome	Reference genome coverage (%)	
		Jupiter	Trenasse
NC_008394	1	94.45	92.55
NC_008395	2	95.91	95.10
NC_008396	3	96.66	94.63
NC_008397	4	93.85	91.63
NC_008398	5	97.46	95.95
NC_008399	6	94.64	92.33
NC_008400	7	95.11	93.89
NC_008401	8	97.24	91.88
NC_008402	9	95.62	88.55
NC_008403	10	89.94	90.56
NC_008404	11	87.79	79.14
NC_008405	12	97.92	88.35
	Average	94.72	91.21

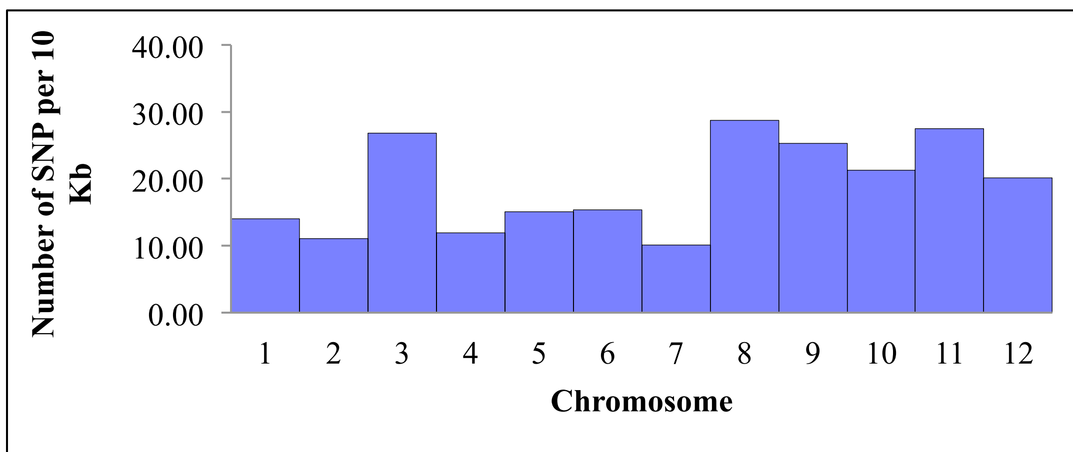

Figure 4.2 SNPs densities per 10 Kb on individual chromosome identified between Jupiter and Trenasse. The x-axis and y-axis represent the chromosomes and the number of SNPs per 10 Kb, respectively.

4.4.3 SNP primer design and validation by PCR

Out of 700, 000 SNPs detected between Jupiter and Trenasse, 685 homozygous, non-synonymous SNPs were selected for primer design. More than 250 allele-specific SNP primer sets were designed from selected nsSNPs along with their flanking sequence. Forward primers were allele-specific, but reverse primers are not allele-specific. In addition, forward primers have 2 to 3 bases mismatch at the 3' end of the primer sequence to improve the allele-specificity during PCR amplification (Hayashi et al., 2004).

Sixty-two of 250 primer sets were randomly selected for validation by PCR amplification from which 27 primer sets were found to be polymorphic, 14 primer sets did not show polymorphic, and 21 primer sets did not amplified on both DNA samples (Appendix 17). A representative picture of allele-specific amplification was shown in Figure 4.3.

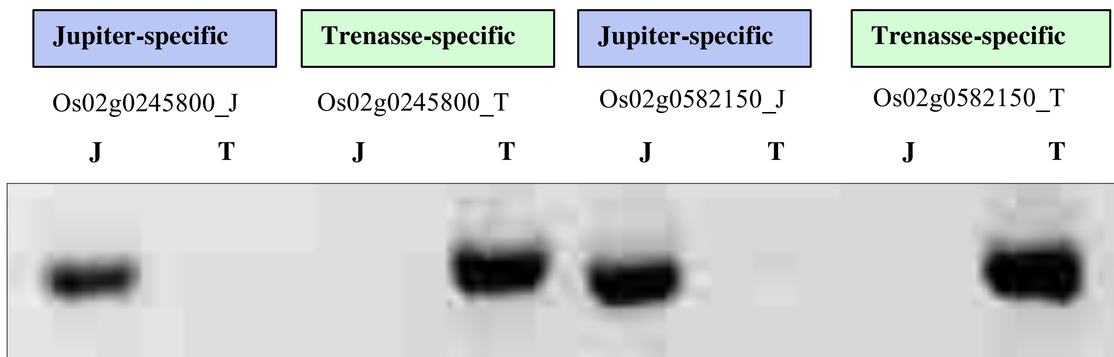

Figure 4.3 Analysis of PCR amplification of representative nsSNP primer pairs developed by using WebSNAPER. Jupiter-specific primer pairs Os02g0245800_J and Os02g0582150_J amplified genomic DNA from Jupiter only, and Trenasse-specific primer pairs Os02g0245800_T and Os02g0582150_T amplified genomic DNA from Trenasse only. PCR products were run in 2% agarose gel electrophoresis at 100 V for 2 hours. J indicates Jupiter and T indicates Trenasse.

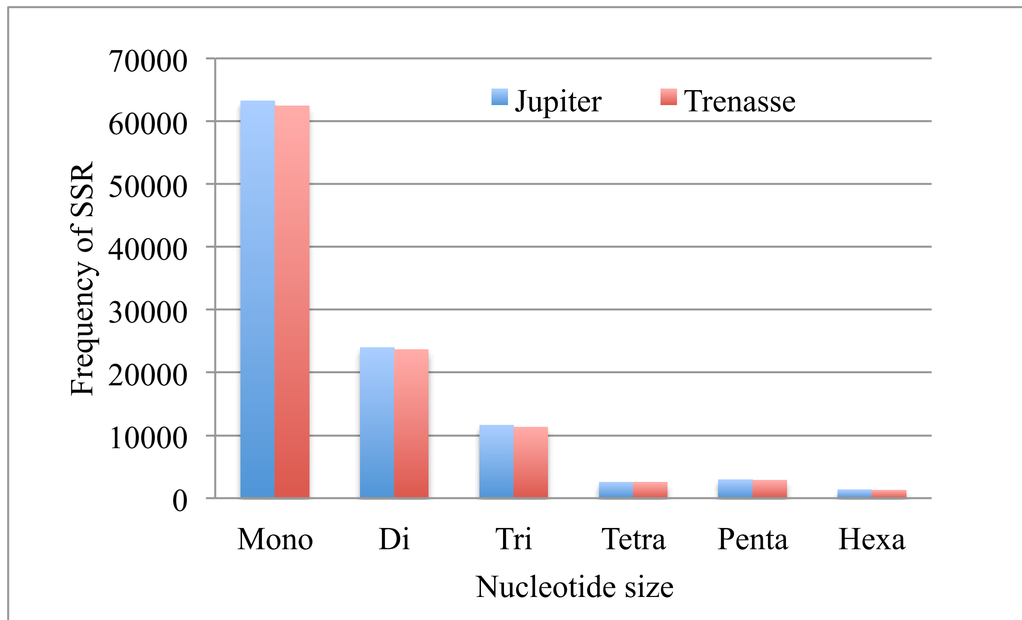

Figure 4.4 Frequency of different SSR motifs detected in the genome of Trenasse and Jupiter

4.4.4 SSR motifs detection, primer design and validation

Equal number of SSR motifs was observed between Trenasse and Jupiter. Mononucleotide type motifs were found in large number compared to other types (Figure 4.4). More than 50,000 perfect SSR motifs from each of the consensus sequences of both Trenasse and Jupiter were compared based on the reference coordinates manually for the difference in number of repeats in motifs. At least 12 base differences between motifs of two cultivars were filtered and selected for designing primers. Seven hundred and three SSR motifs that had with difference in repeat numbers between Trenasse and Jupiter from all 12 chromosomes were selected. A bioinformatics tool, SAMtools, was used to obtain about 300 bases of flanking sequence for all 703 SSR motifs. Those flanking sequences were used to design primers using primer design software, Primer3.

Thirty-seven of 703 primer pairs were randomly selected from chromosomes 1, 2 and 8. Out of 37, 14 primer pairs were found to be polymorphic between Trenasse and Jupiter (Appendix 18).

4.5 DISCUSSION

BPB in rice, caused by a bacterial pathogen *B. glumae*, is an economically important disease, but control measures for this disease have not been developed so far. Only a few partially resistant rice cultivars, such as Jupiter, are available. So, it is essential to identify the resistant genes to control bacterial disease. Only a few reports were available on genetic mapping for partial resistant traits in rice (Mizobuchi, Ritsuko et al., 2013; Pinson et al., 2010). Further study of QTL mapping for BPB resistance will be beneficial to increase the pool of loci that can be used in future breeding programs for developing BPB resistant cultivars. Molecular markers are now commonly used for genetic studies, study of genetic diversity, association and QTL mapping for an economically important traits.

So, while preparing database for polymorphic molecular makers between two rice cultivars, Jupiter (partially resistant cultivar for BPB) and Trenasse (very susceptible cultivar for BPB), survey of SSR markers available in Gramene database was the first choice because of its ready availability. Survey of SSR markers selected from Gramene database, however, showed very low percentage of usable polymorphic markers (28 of 1091 SSR markers) between two cultivars (Appendix 16). These 28 SSR markers were not enough to represent all 12 chromosomes of rice. Jupiter and Trenasse were developed by LSUAgCenter at Rice Research Station, Crowley, and rice accessions used to develop these two cultivars are in tropical *japonica* group (Linscombe et al., 2006; Lu et al., 2005; Sha et al., 2006). This might be one of the reasons of obtaining low level of polymorphism. In previous studies of genetic mapping for bacterial

panicle blight, rice blast and sheath blight diseases of rice, population generated from the crosses between *japonica* and *indica* with RFLP markers were used (Channamallikarjuna et al., 2010; Liu et al., 2009; Pinson et al., 2010; Tabien et al., 2000), however, only few studies were reported to identify QTLs for disease resistance with using population from the crosses between *japonica* and *japonica* group. SSR markers were used for genotyping the population where 18 to 44 percent of markers were polymorphic in $F_{2:3}$ and double haploid populations, respectively (Nelson et al., 2012; Sharma et al., 2009). Compared to these results of polymorphism, level of polymorphism between the parents, Trenasse and Jupiter, were lower.

Another approach of obtaining molecular markers is by analysis of whole genome sequences of two cultivars. In this approach, we identified genome-wide DNA polymorphism between two rice cultivars, Trenasse and Jupiter, using high-throughput sequence data from next-generation sequencing technology. Only, homozygous, non-synonymous SNPs were selected for primer design, because non-synonymous SNP alters the amino acid sequence in protein resulting in alter phenotype. Allele-specific SNP primer sets were developed from the identified SNPs using WebSNAPER. In allele-specific SNP primers, forward primer sequences are allele-specific to Jupiter and Trenasse, whereas reverse primers are not allele-specific. It has been reported that transversions (T and G, and C and A) mismatch at the third base from 3’ end of forward primer sequence, and/or transversions (A and T) and transitions (A and G) mismatches will enhance the allele-specificity during PCR amplification (Hayashi et al., 2004; Hirotsu et al., 2010). SSR markers were also developed using the whole genome sequence, however, these markers resulted in few polymorphism. Close observations on the alignment sequence of two cultivars showed that there were several mismatches of bases in the SSR motifs during sequencing which restrict

precise estimation of repeats in the sequence. Validation of SNP markers suggested that more than 44% of SNP primers from this study could be used as molecular markers for genetic studies.

In conclusion, high-throughput sequence data is a good source to identify polymorphism between closely related rice cultivars like Jupiter and Trenasse where the molecular markers available in databases are not sufficient to get enough polymorphism for QTL mapping.

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CHAPTER V: AN NAC4-LIKE TRANSCRIPTION FACTOR IS RESPONSIVE TO EXOGENOUS APPLICATION OF *BURKHOLDERIA GLUMAE*, AND CHEMICAL ELICITORS, JASMONIC ACID AND ASCORBIC ACID

5.1 INTRODUCTION

Bacterial panicle blight (BPB) in rice, caused by *Burkholderia glume* and *B. gladioli*, is one of the major rice diseases in southern regions of the United States. BPB becomes prevalent when the flowering stage of rice coincides with hot and humid environment. No completely resistant rice cultivars have been found for BPB. It has been reported that most of the commercially grown rice cultivars are susceptible to this disease (Shahjahan et al., 2000). Jupiter, a medium-grain cultivar, and LM-1, a mutant-derivative of a long-grain cultivar, Lemont, show partial resistance to BPB (Groth et al., 2007; Nandakumar et al., 2007).

In an attempt to study the molecular mechanism of the partial disease resistance to BPB in Jupiter in comparison with Trenasse, gene expression analysis in response to *B. glumae* was previously conducted by Dr. Chuck Rush's group using a microarray technique. In that study, several genes related to defense, signal transduction, and seed development, were significantly upregulated in Jupiter compared to Trenasse in response to *B. glumae* (Nandakumar & Rush, 2008). For the further validation of the microarray results, reverse transcription PCR (RT-PCR) was conducted to investigate the expression of several promising genes encoding defensin, NAC4-like transcription factor, and prolamin. Among the selected genes, *BPR1* (**b**acterial **p**anicle **b**light **r**esponse **g**ene **1**) gene (Os01g0393100) encoding an NAC4-like transcription factor, was specifically expressed only in Jupiter but not in Trenasse upon inoculation with *B. glumae* 336gr-1, as well as its two mutant derivatives, one with deficient in toxoflavin production and the other one with deficient in toxoflavin production and functional type III secretion system (Shrestha, 2011). Transcription factors play a role in regulating expression of downstream genes, some of which are involved in plant defense system. NAC is derived from

three genes: NAM (no apical meristem), ATAF1/2 (*Arabidopsis* transcription activation factor) and CUC2 (cup-shaped cotyledon) (Aida et al., 1997; Souer et al., 1996). NAC is involved in various developmental processes, including embryo development, shoot apical meristem formation, and seed development (Kim et al., 2007; Sperotto et al., 2009). NAC transcription factors is also involved in regulating various biotic stress responses in potato (Collinge & Boller, 2001) and rice (Nakashima et al., 2007). In the rice genome, about 151 genes belong to the NAC family. These genes play vital roles in regulating physiology of plants (Nuruzzaman et al., 2010).

In this work, expression of Os01g0393100, encoding an NAC4-like transcription factor (will be used as *BPR1* onward) in different growth stages of Jupiter, and responsiveness of *BPR1* to *B. glumae* and some of the chemical elicitors, including salicylic acid, jasmonic acid, ascorbic acid and ethephon was studied.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials, bacterial inoculum and chemical elicitors

Rice varieties, Jupiter and Trenasse, were grown in pots in the greenhouse. A virulent pathogen of bacterial panicle blight (BPB) disease, *B. glumae* 336-gr1, and its two mutant derivatives deficient in toxoflavin production (*tox*$^-$), and deficient in both toxoflavin production and functional type III secretion system (*tox*$^-$*hrp*$^-$) were used in this experiment. Bacterial inoculum was prepared at the concentration of 1 X 10^8 CFU/ml (OD_{600} = 0.1) in deionized water. Chemical elicitors such as salicylic acid (SA), jasmonic acid (JA), and ethephon known to be involve in inducing systemic acquired resistance in several plants, These chemicals, were sprayed on the rice panicles. Concentration of each elicitor was 100 µM.

5.2.2 Bacterial inoculation at different rice growth stages

Jupiter was inoculated with freshly prepared bacterial inoculum of *B. glumae* 336 gr-1 and its two mutant derivatives in two different growth stages, including seedling and tillering stages, and samples were collected at different time points; 0, 48, and 96 h after spraying. Similarly, at 30% heading stage, several chemical elicitors, including SA, JA, ASA and ethephon, and the *B. glumae tox*$^{-}$ mutant deficient in toxoflavin production were sprayed on the panicles of both Trenasse and Jupiter. Water was used as control in both conditions. Each treatment was conducted with 3 replications. All applications of chemicals and bacteria were performed using hand sprayer.

5.2.3 Disease scoring and sample collection

Jupiter inoculated during seedling (15 days after planting) and tillering stages were scored 10 days after inoculation by using the standard scale of 0 to 9, where, 0 means no symptoms on the panicles, and 9 means more than 80 percentage of panicles showed BPB symptoms (Nandakumar et al., 2007).

Whole plants were collected for the samples in which treatments were done during seedling stages. Leaf samples were collected from plants in which treatments were done during tillering stage of rice. Samples were collected at different time point; 0 hours, 48 hours, and 4 days after the bacterial inoculation. Collected samples were flash-frozen in liquid nitrogen and stored at -70°C. Similarly, plants treated during 30% heading stages were collected at different time points, 0, 6, 12, 24, and 48 h after the treatment of different chemical elicitors and the toxoflavin deficient mutant. All samples were flash-frozen in liquid nitrogen and stored at -70°C.

5.2.4 Total RNA extraction and cDNA synthesis

Samples stored at -70°C were used for total RNA extraction using TRIzol^{R} Reagent (Invitrogen) (Moy, 2004). Briefly, 100 mg samples were ground in liquid nitrogen with autoclaved mortar and pestle. Each ground sample was transferred to a clean microcentrifuge tube and mixed with 700 µl of Trizol. Then the tube was gently shaked at least 5 min in order to mix the sample properly. Two hundred and ten µl of chloroform was added and mixed vigorously. The sample was incubated at room temperature for 2 to 3 minutes. Three distinct layers of RNA, DNA, carbohydrates, proteins and other cellular debris could be observed. After incubation, sample was centrifuged for 15 min at 12000 rpm at 4°C. Upper aqueous phase was transferred carefully to a new sterile tube to which 0.7X volume of isopropanol was added and mixed with transferred aqueous solution. The sample was incubated at -20°C for an hour followed by gentle agitation for about 10 min. and centrifugion at 12000 rpm for 10 min at 4°C. Supernatant was discarded and pellet formed at the bottom of micro-centrifuge tube was washed two times with 1 ml of icecold 75% ethanol. After washing, supernatant was removed carefully and pellet was dried for 15 min. Approximately, 100 µl of RNase-free water was used to resuspend the dried pellet. After resuspension, sample was incubated at 42°C for 15 min.

DNase treatment was performed using DNA-free^{TM} Kit (Applied Biosystems, Grand Island, NY) following the manufacture instructions. After DNase treatment, concentration of total RNA in samples was measured and adjusted to 300 ng/µl, using a Nano Drop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

5.2.5 Reverse transcription (RT) -PCR

cDNA synthesis from total RNA samples was performed using ProtoScript^R M-MuLV First Strand cDNA Synthesis Kit (NEB #E6300S, New England BioLabs Inc., Ipswich, MA) following manufacturer's protocol. After cDNA synthesis, PCR for the amplification of BPR1 was performed using the primers NAC-likeF (5' CCTGACCTGCCTCCGGGCTT 3') and NAC-likeR (5' TTGTCGCCCTTGGGAGCCCT 3') (Shrestha, 2011). Primers ActinF (5' TCCATCTTGGCATCTCTCAG 3') and ActinR (5' GTACCCGCATCAGGCATCTG 3') (Fukuoka et al., 2009) were used for the *actin* gene (X16280), which was used as an internal control. Each reaction of PCR contained 2-5 µl of cDNA (~ 50-100 ng/µl), 2.5 µl of 10X PCR buffer, 0.5 µl of 10mM dNTP mix, 0.2 µl (5.0U/µl), 1 µl of 10 µM forward and reverse primers, and 14.8 µl of sterilized ddH_2O in a total volume of 25 µl.

Two separate PCR programs were used for two different primers sets for *BPR1* and *Actin* gene amplification, due to different annealing temperature. The PCR program for the amplification of *BPR1* consisted of the initial denaturation at 95°C for 1 min; 35 cycles of 95°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec; and the final extension at 72°C for 10 min. For Actin, a PCR program of 95°C for 1 min; 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; and the final extension at 72°C for 10 min was used. The PCR products were electrophoresed in 1% agarose gel with 100V for 1 hour, and visualized with the help of KODAK Gel Logic 1500 Imaging System (Molecular Imaging Systems, Carestream Health, Inc., Rochester, NY).

5.3 RESULTS

5.3.1 Disease symptoms

BPB symptoms were evaluated in the seedling stage and tillering stage of Jupiter 10 days after bacterial inoculation, however, obvious BPB symptoms were not observed in any treatments.

5.3.2 Expression of *BPR1* gene in different growth stages of Jupiter

In Figure 5.1, expression of *BPR1* and *Actin* gene was shown in two different growth stages of Jupiter at 0 and 48 h time points. Lanes 1, 2, 3, and 4 in each figure represent four different treatments, including water control, *B. glumae* 336 gr-1, *B. glumae* 336gr-1 *tox*$^-$ and *B. glumae* 336gr-1 *tox*$^-$*hrp*$^-$, respectively. RT-PCR results showed that the *Actin* gene was expressed in all treatments in both growth stages at different time points; however, *BPR1* gene was not expressed in any treatments at both growth stages at any time point (Figure 5.1). Expression of *BPR1* was not observed 4 days after treatment (data not shown).

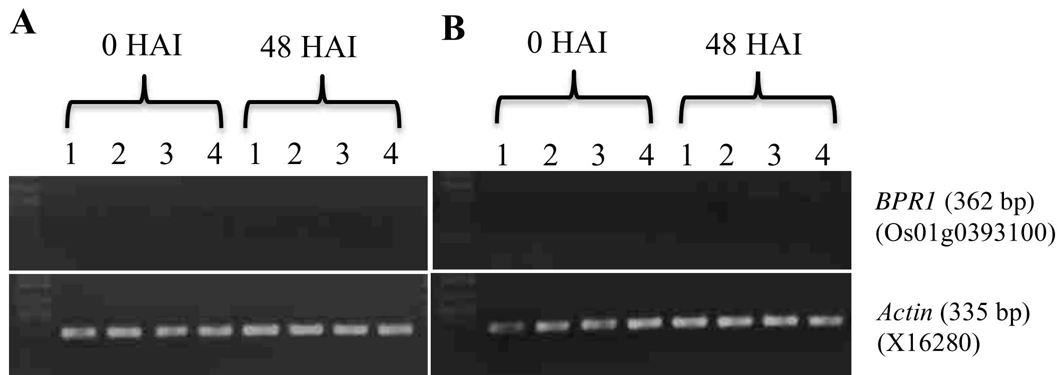

Figure 5.1 Expression patterns of *Actin* and *BPR1* genes in Jupiter after inoculation with *B. glumae* and its derivatives or water (control), analyzed by RT-PCR. Lane 1= cDNA samples from water treated rice samples, lane 2= cDNA from *B. glumae* 336gr-1 treated samples, lane 3= cDNA from *B. glumae tox*$^-$ treated samples, lane 4= cDNA from *B. glumae tox*$^-$*hrp*$^-$treated samples. (A) *Actin* and *BPR1* expression at seedling stage of Jupiter, (B) *Actin* and *BPR1* expression at tillering stage of Jupiter. HAI= hours after inoculation

5.3.3 Expression of *BPR1* gene during heading stage in response to various chemical elicitors

In the figure 5.2 expressions of *BPR1* and *Actin* genes in Trenasse and Jupiter after treatments of various elicitors, and toxoflavin deficient mutants were shown. RT-PCR results showed that *Actin* gene was expressed constitutively in all the treatments of both cultivars at any time points (Figure 5.2). Interestingly, *BPR1* gene was expressed in Jupiter treated with *B. glumae* 336gr-1 *tox*$^-$, jasmonic acid and ascorbic acid at 0 hour time point. But *BPR1* gene was not expressed in any treatments in Jupiter at any time points. In contrast, in Trenasse, *BPR1* gene was not expressed in any treatments (Figure 5.2). BPR1 was not expressed after 48 hours also (data not shown).

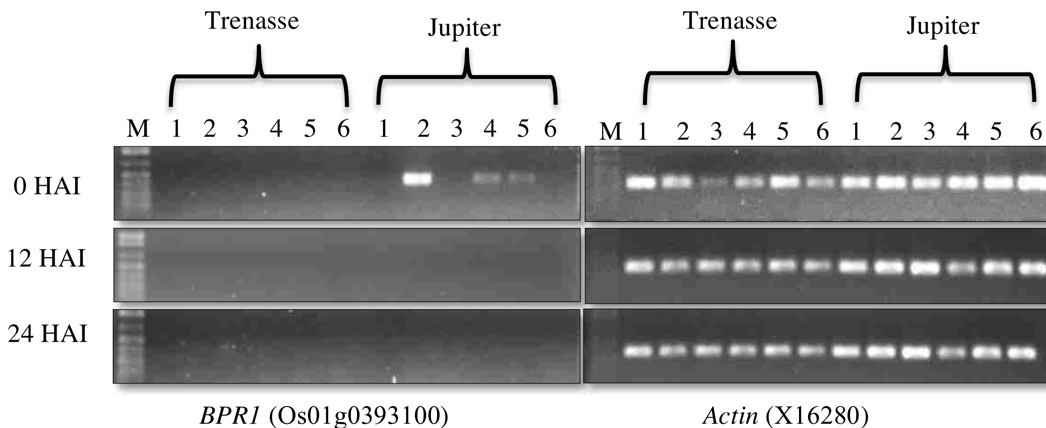

Figure 5.2 Expression patterns of *BPR1* and *Actin* genes in Trenasse and Jupiter at 0, 12 and 24 hours after inoculation with toxoflavin deficient *B. glumae* 336gr-1, salicylic acid, jasmonic acid, ascorbic acid, and ethephon, analyzed by RT-PCR. M= 1 Kb plus DNA ladder, lane 1= cDNA samples from water treated rice panicles, lane 2= cDNA from *B. glumae* 336gr-1 *tox*$^-$ treated panicles, lane 3= cDNA from salicylic acid treated panicles, lane 4= cDNA from jasmonic acid treated panicles, lane 5= cDNA from ascorbic acid treated panicles, lane 6= cDNA from ethephon treated panicles. First, second and third row of gel picture has expression of the genes *BPR1* and *Actin* at 0, 12, and 24 hours after the treatment, respectively. HAI indicates hours after inoculation.

5.4 DISCUSSION

NAC-like transcription factors are structurally distinct and functionally diverse transcription factors found in plants only which contain conserved N-terminal binding domain (Olsen et al., 2005). NAC proteins plays an important role in growth and development of plants, incuding leaf senescence, flowering, cell wall biosynthesis (Ricachenevsky et al., 2013), it is also involved in plant defense responses against various pathogens. *StNAC* gene was induced upon the pathogen attack in potato (Collinge & Boller, 2001). It was reported previously that various *NAC* genes, *OsNAC6*, *OsNAC4*, were involved in regulating hypersensitive responses, and disease resistance in rice against *Magnaporthe oryzae* and Rice dwarf virus (Kaneda et al., 2009; Nakashima et al., 2007; Yoshii et al., 2009). In this study pathogen-responsive *BPR1* (**b**acterial **p**anicle **b**light **r**esponse **g**ene **1**) gene encoding NAC4-like transcription factor was identified as biotic and abiotic stresses-responsive.

BPB symptom in the seedling and tillering stage of Jupiter were not observed. Expression patterns of NAC transcription factors were reported to be tissue-specific in previous studies (Lin et al., 2007; Meng et al., 2009; Xia et al., 2010). In this study *BPR1* gene was not expressed in seedling and tillering stages of rice. However, differential expression of *BPR1* gene was found in Jupiter during heading stage when treated with *B. glumae* 336 gr-1 and its mutant derivatives 48 h after inoculation (Shrestha, 2011) (Figure 5.3). Similarly, *BPR1* gene was expressed during heading stage in previous microarray study two days after bacterial inoculation (Nandakumar & Rush, 2008).

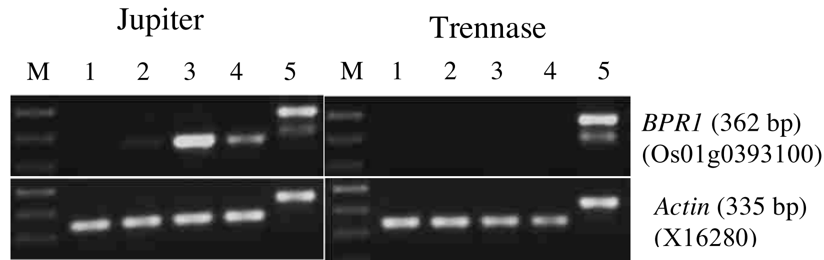

Figure 5.3 Expression patterns of *BPR1* and *Actin* genes in Jupiter and Trenasse 48 hours after inoculation with *B. glumae* and its mutant derivatives or water (control), shown by RT-PCR. Each lane represent cDNA sample from different treatments. M= 1 kb plus DNA ladder, lane 1= water treated, lane 2= *B. glumae* 336gr-1 treated, lane 3= *B. glumae tox*$^-$, lane 4= *B. glumae tox*$^-$ *hrp*$^-$, lane 5= genomic DNA from Jupiter and Trenasse used as a positive control.

In previous studies, early responses of *NAC* genes to biotic (Huang et al., 2012) and abiotic (Yun et al., 2010) stresses were reported. In our study also, treatment with toxoflavin deficient mutant of *B. glumae* enhanced the expression of *BPR1* gene in Jupiter at 0 hour (less than 15 mintues) after inoculation, but no expression of *BPR1* was observed in Trenasse. Various fungal elicitors and bacterial pathogen infection were reported to enhance the expression of *NAC* genes (Jensen et al., 2008; Wang et al., 2009). It was reported that several microbe-associated molecular pattern (MAMP) molecules that are found on the surface of bacterial cells, including lipopolysaccharides, peptidoglycan and flagellin are recognized by pattern-recognition receptors of the host innate immune system (Nürnberger & Kemmerling, 2006; Song et al., 1995). Perception of these MAMPs has shown to trigger signaling cascades, which activate the innate defense response in the host (Felix et al., 1999; Gomez-Gomez, 2004; Gómez-Gómez & Boller, 2000, 2002). So, recognition of MAMPs molecules in bacterial inoculum by the rice plants might have occurred resulting in rapid expression of *BPR1* gene.

Furthermore, *BPR1* gene was responsive to the treatment of jasmonic acid and ascorbic acid (Figure 5.2). A stress responsive *NAC* gene, *RD26*, in *Arabidopsis* was responsive to jasmonic acid and was induced by exogenous application of jasmonic acid, hydrogen peroxide, and pathongens (Fujita et al., 2006; Zimmermann et al., 2004). Many stress responsive *NAC*

genes in rice were also responsive to jasmonic acid (Nuruzzaman et al., 2013). In addition, these type of *NAC* genes were involved in regulating disease resistance pathways in rice (Nuruzzaman et al., 2013). Several *NAC* genes were reported to respond to exogenous application of chemical elicitors, including ethephon, salicylic acid, jasmonic acid in rice, and wheat (Nuruzzaman et al., 2012; Xia et al., 2010; Yoshii et al., 2010). Responsiveness of the *BPR1* gene to *B. glumae* infection and exogenous application of chemicals, including jasmonic acid and ascorbic acid, suggest there might be involvement of this gene in defense response against *B. glumae* infection. Therefore, further study is required for functional characterization of *BPR1* gene for disease resistance, and to understand molecular mechanisms underlying regulation of defense responses against the pathogen.

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CHAPTER VI: ISOLATION AND CHARACTERIZATION OF RICE-ASSOCIATED *BACILLUS* SPP. SHOWING ANTAGONISTIC ACTIVITIES AGAINST THE RICE PATHOGENS *BURKHOLDERIA GLUMAE* AND *RHIZOCTONIA SOLANI*

6.2 INTRODUCTION

A diverse range of microorganisms dwell in various parts of a plant, causing detrimental, neutral, or beneficial effects on plant health (Bashar et al., 2010; Bashi & Fokkema, 1977; Williamson & Fokkema, 1985). Some of these microorganisms can suppress plant diseases through competition, predation, antagonism against plant pathogens, or through induction of plant defense systems (Compant et al., 2005; Niranjan et al., 2006). Antagonistic bacteria isolated from plant surface, soil, and rhizosphere have been extensively used to control major crop diseases caused by various fungal and bacterial diseases (Kanjanamaneesathian et al., 1998; Kazempour, 2004). Those microorganisms can be used alone or in combination with other chemical or biological control agents for various crop diseases (Bashar et al., 2010; Bashi & Fokkema, 1977; Datnoff et al., 1995; Duffy & Weller, 1995; Kanjanamaneesathian et al., 1998; Paulitz et al., 1992; Shahjahan et al., 2001; Williamson & Fokkema, 1985).

Sheath blight (SB) is one of the most economically important rice diseases worldwide, which is caused by the fungal pathogen *Rhizoctonia solani*. This disease is characterized by oval to irregular lesions on rice sheath and leaf blades. *R. solani* is a soilborne pathogen having a broad host range including rice and soybean. Epidemics of sheath blight occur throughout temperate and tropical rice-growing regions. High nitrogen rates and plant density provide favorable microclimates for the development of sheath blight during early heading and grain-filling stages (Lee & Rush, 1983). Common practices for the management of sheath blight include crop rotation, fertilizer management, planting disease-tolerant varieties, and fungicide application. However, rice cultivars having vertical (or complete) resistance to the disease are not available, and crop rotation will not assure effective management of the disease because the

fungus can survive for a long period of time in the form of sclerotia, a primary source of inoculum that overwinters in soil and plant debris. Various fungicides are being used to control the disease (Araki & Yabutani, 1993; Groth, 2005; Miah et al., 1994). However, fungicide application increases the cost of cultivation and the risk of the emergence of fungicide-resistant pathogens (Bennett, 2012).

Bacterial panicle blight (BPB is caused by the Gram-negative bacterial pathogens *Burkholderia glumae* and *B. gladioli* is another important rice disease in many rice-growing regions around the world (Goto et al., 1987; Ham et al., 2011; Nandakumar et al., 2009; Shahjhan et al., 2000). The major symptoms of this disease include panicle discoloration, grain rot, and sterile florets. Prolonged high night-temperatures during the heading and flowering stages favor the outbreaks of BPB (Nandakumar et al., 2009; Trung et al., 1993; Tsushima, 1996). *B. glumae*, the chief causal organism of BPB (Ham et al., 2011), is a seed-borne bacterium and produces the yellow-colored phytotoxin, toxoflavin, as a major virulence factor (Sato et al., 1989). Despite the economic importance of BPB, there are few control measures for this disease. There is no known complete resistance for this disease and only a few partially resistant varieties are commercially available. Oxolinic acid is the only known commercial chemical for controlling this disease (Hikichi, 1993). However, this chemical is not registered for agricultural purpose in the United States (Nandakumar et al., 2009), and natural occurrence of oxolinic acid-resistant strains limits the use of this chemical (Hikichi et al., 2001).

Application of epiphytic and endophytic microbial antagonists as biological control agents will be an alternative control method for rice diseases (Mew et al., 2004). *Dermococcus nishinomiyaensis*, *Aspergillus niger, Trichoderma* strains and fluorescent pseudomondas including *Pseudomonas fluorescens* have been reported as biocontrol agents to suppress the

disease severity and lesion length caused by sheath blight in rice (De Costa et al., 2008; Devi et al., 1989; Gnanamanickam et al., 1992; Naeimi et al., 2010). Also, rhizosphere fungal strain *Chaetomium aureum* and its metabolites, and hyphae-colonizing *Burkholderia vietnamiensis* have been used to control sheath blight in rice (Cuong et al., 2011; Wang et al., 2013). Similarly, it has been reported that genetically engineered strain of *Burkholderia* sp. can be used as a bicontrol agent to reduce seedling rot in rice *in situ* caused by *B. glumae* (Cho et al., 2007).

In this study, rice-associated bacteria (RAB) were isolated from rice plants grown in the field and tested for their antagonistic activities against the SB pathogen, *R. solani*, and the BPB pathogen, *B. glumae*, as well as their efficacies for controlling SB and BPB. In addition, all the RABs tested were identified based on their 16S rDNA sequences and clustered based on other genotypes determined by 16S-23S intergenic transcribed spacer (ITS)-PCR, tDNA-intergenic spacer region (tDNA)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR and BOX-PCR analyses.

6.3 MATERIALS AND METHODS

6.3.1 Isolation of RABs

Leaves of rice plants at the 30% heading stage were collected from the rice field in the LSU AgCenter Rice Research Station at Crowley, Louisiana. The collected leaves were cut into ~4 cm-long pieces, and subsequently washed by stirring in 500 ml of sterilized ddH$_2$O for 10 min or in 500 ml of 10% bleach for 5 min. The bleach-sterilized leaf pieces were then stirred in sterilized ddH$_2$O for 10 min to remove the remaining bleach. The washed leaf pieces were placed on potato dextrose agar (PDA) plates, making the adaxial side contact to the medium, and incubated at room temperature for 72 h. Bacterial colonies grown out from the leaf samples were transferred to new PDA plates for isolation of RABs.

6.3.2 Measurement of RABs' antimicrobial activities against *R. solani* and *B. glumae*

A mycelial plug of *R. solani* was taken from one-week-old culture of *R. solani* on PDA using a cork borer (5 mm in diameter) and placed on the center of a fresh PDA plate. Each RAB was cultured overnight in Luria-Bertani (LB) broth (10 g tryptone, 10 g NaCl and 5 g yeast extract per L) in a shaking incubator at 37°C at 190 rpm. Fifteen hundred microliters of each culture was then washed twice with fresh LB and resuspended in 100 µl of LB. Ten microliters of the bacterial suspension was spotted on three locations around the mycelial plug on PDA. Observation of antifungal activities and measurement of inhibition zones were conducted 72 h after incubation at 25°C.

B. glumae strain 336gr-1 was cultured overnight in LB at 37°C and the overnight culture was washed twice with fresh LB. One hundred microliters of the bacterial suspension adjusted to $OD_{600} = 0.1$ (ca. 1×10^8 CFU/ml) was spread on a PDA plate. Bacterial suspensions of RABs were prepared as described above and 10 µl of each sample was spotted on three locations of a PDA plate previously spread with *B. glumae*. Observation of antibacterial activities and measurement of inhibition zones were conducted 72 h after incubation at 25°C.

6.3.3 Evaluation of RABs' inhibitory activities on sclerotial germination of *R. solani*

The effects of RABs on sclerotial germination of *R. solani* were observed following a previous method (Kazempour, 2004) with some modifications. Briefly, young and fresh sclerotia collected from mycelia of *R. solani* grown on PDA were surface-sterilized with 2% sodium hypochlorite solution for 2 min and washed with sterilized ddH_2O for 10 min. The surface-sterilized sclerotia were put in the overnight-grown cultures of RABs and further incubated in a shaking incubator for 24 h at 25°C at 200 rpm. The sclerotia incubated in a RAB culture were

gently taken out and placed on fresh PDA plates. Germination rate of sclerotia was determined 72 h after incubation at 25°C.

6.3.4 Evaluation of the RABs' inhibitory activities on the lesion development by *R. solani* on detached rice leaves

The detached leaf assay to examine the inhibition of SB lesion development by each RAB was performed following a previous method (Guleria et al., 2007) with minor modifications. Briefly, the second leaf from the base was taken from a two-month-old rice plant of the disease susceptible cultivar, Bengal, and cut into ~6 cm-long pieces. The leaf pieces were surface-sterilized with 1% sodium hypochlorite solution for 1 min and washed with sterilized ddH_2O. The sterilized leaf pieces were then placed on petri plates containing a wet filter paper, and were pressed with sterilized glass slides to keep the leaves flat during the experiment. Overnight grown RAB culture in LB broth in a shaking incubator at 25°C at 200 rpm was washed two times in a fresh LB broth, and resuspended in sterilized ddH_2O adjusting the RAB inoculum to ~6×10^8 CFU/ml. Each RAB inoculum was sprayed in each petri plates with leaf pieces until the leaf pieces got wet. A sclerotium collected from the one-week-old mycelia of *R. solani* was placed on the center of each leaf piece. Three leaf pieces were treated with each RAB for three replications. Leaf pieces without any RAB treatment and those treated with sterilized ddH_2O were also included as controls. The petri dishes containing rice leaf pieces placed with a screlotium were incubated at 25°C for 7 days with 12 h-light period per day. The relative lesion length on a detached leaf piece was calculated the following way (Kumar, K. V. K. et al., 2009):

$$\text{Relative lesion length} = [(\text{Lesion length}) / (\text{Leaf length})] \times 100$$

6.3.5 Evaluation of the RABs' biocontrol activities on SB and BPB

6.3.5.1 Treatment of rice plants with RABs

The medium-grain and disease susceptible cultivar, Bengal, was grown in the field at the Rice Research Station (Crowley, Louisiana) in 2012. Six rows each with ~ 1.2 meters for each of seven treatments including five RABs, one water-control and one non-inoculated treatment, were set up for each disease. Overnight cultures of RAB grown on LB agar were resuspended in deionized water and the bacterial suspension $OD_{600} = 0.1$ (~2×10^6 CFU/ml) of each RAB was sprayed to rice plants until it flows down.

6.3.5.2 Inoculation of rice plants with *R. solani*

The inoculum of *R. solani* was prepared in a mixture of rice husk and grain. Briefly, 600 gm of the mixture containing 2 parts of rice husk and 1 part of rice grain with 500 ml of water was sterilized at 121°C for 20 min. The sterilized mixture in a flask was inoculated with ~16 cm^2 of PDA plugs containing 7 days old *R. solani* mycelia, and incubated at 25°C for 10 days. After 10 days of incubation, the prepared inoculum was mixed with larger volume of the sterilized mixture of rice husk and grain at 1:2 ratio of prepared inoculum and sterilized mixture to increase the inoculum volume. After mixing properly the mixture was spread uniformly on a clean brown paper sheet and covered with a clean plastic sheet at room temperature. After 24 h of incubation at room temperature, the prepared *R. solani* inoculum was applied to each row of rice plants at the tillering stage at 24 h post treatment of RAB. The symptoms were observed during milk stage of rice. Disease severity was rated based on relative lesion height with the scale ranges from 0 to 9 (IRRI, 1996).

6.3.5.4 Inoculation of rice plants with *B. glumae*

B. glumae 336gr-1 was grown overnight on King's B agar at 37°C and the bacterial cells were resuspended in deionized water, with a concentration of ~1×10^{8}CFU/ml. The bacterial suspension was sprayed to rice plants at the 30% heading stage at 24 h post treatment of each RAB. The symptoms were observed 10 days after inoculation. Disease severity was rated based on discolored area and sterility of panicles with the scale ranges from 0 to 9 in which 0 indicated no BPB symptoms and 9 indicated more than 90 % of panicle discoloration and sterility. (Nandakumar et al., 2007) .

6.3.6 Identification of the antagonistic RABs

The 3% KOH test (Ryu, 1940; Suslow et al., 1982) was performed to initially categorize RABs into Gram-positive or Gram-negative bacteria. *B. glumae* and *Bacillus subtilis* were used as a control representing Gram-negative and Gram-positive bacteria, respectively. For identification by 16S rDNA sequencing, genomic DNA of each RAB was extracted using a previously described method (Pospiech & Neumann, 1995) and 16S rDNA sequence was amplified using the primers fD1 (5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3') and rD1 (5'CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3') (Weisburg et al., 1991). Each reaction of PCR contained 3 µl of genomic DNA (~ 100 ng/µl), 2.5 µl of 10X PCR buffer, 0.75 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTP mix, 1.0 µl of homemade *Taq* polymerase (~1.0U/µl), 1 µl of 10 µM forward (fD1) and reverse (rD1) primers, and 15.25 µl of sterilized ddH_2O in a total volume of 25 µl. The PCR program consisted of the initial denaturation at 95°C for 1 min; 35 cycles of 95°C for 2 min, 42°C for 30 sec and 72°C for 4 min; and the final extension at 72°C for 20 min. PCR products of 16S rDNA were purified using a QuickClean 5M

PCR Purification Kit (GenScript, Piscataway, NJ). Purified PCR products were sent to Macrogen Inc. (Seoul, Korea) for sequencing. The sequence data were searched against the National Center for Biotechnology Information (NCBI) database to identify the corresponding or homologous sequences, using BLAST.

6.3.7 16S-23S intergenic transcribed spacer (ITS)-PCR, tDNA-intergenic spacer region (tDNA)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR and BOX-PCR for DNA fingerprinting analyses

PCRs for ITS and tDNA regions were performed using the primer sets L1 (5'CAAGGCATCCACCGT3') and G1 (5'GAAGTCGTAACAAGG3') (Jensen et al., 1993), and T5A (5'AGTCCGGTGCTCTAACCAACTGAG3') and T3B (5'AGGTCGCGGGTTCGAATCC3') (Welsh & McClelland, 1991), respectively. Components of the 25 µl PCR reaction mixture for amplification of ITS and tDNA were: 1.0 µl of template DNA, 2.5 µl of 10X PCR buffer, 0.4 µl of 10 mg/ml bovine serum albumin (BSA), 0.5 µl of dimethyl sulfoxide (DMSO), 0.5 µl of 10 mM dNTPs, 1.0 µl each of the primers at 10 µM (L1 and G1 for ITS, and T5A and T3B for tDNA), 0.4 µl of Paq5000 (Agilent Technologies, California, USA), and 17.7 µl of sterile ddH$_2$O. The PCR condition for ITS- and tDNA-PCR was same as that used in a previous study (Freitas et al., 2008): the initial denaturation at 94°C for 10 min; 30 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 1 min; and the final extension at 72°C for 10 min.

Rep-PCR including enterobacterial repetitive intergenic consensus (ERIC)- and BOX-PCR was performed to study the phylogenetic relationships and genetic diversity of the RABs. The primer set of ERIC1R (5' ATGTAAGCTCCTGGGGATTCAC3') and ERIC2 (5' AAGTAAGTGATGGGGTGAGCG3') (Versalovic et al., 1991) was used for ERIC-PCR, while BOXA1R (5'CATACGGCAAGGCGACGCT 3') (Versalovic et al., 1994) was used for BOX-PCR. ERIC- and BOX-PCRs were performed following a previous study (Rademaker et al.,

2004) with some modifications. In brief, each reaction mixture (25 µl) for ERIC-PCR contained 1.0 µl of template DNA, 5.0 µl of 5X Gitschier-buffer (Rademaker et al., 2004), 0.4 µl of bovine serum albumin (BSA) at 10 mg/ml, 2.5 µl of DMSO, 1.25 µl of 100 mM dNTPs, 5.0 µl each of ERIC1R and ERIC2 at 10 µM, 0.4 µl of Paq5000^{TM} DNA polymerase (Agilent Technologies, California, USA), and 4.45 µl of sterile ddH_2O. Composition of a reaction mixture for BOX-PCR was similar to that of ERIC-PCR, except that 9.45 µl of sterile ddH_2O and 5.0 µl of only one primer (BOXA1R at 10 µM) were added to a 25 µl reaction mixture. The thermal cycle condition for both ERIC- and BOX-PCRs was same as that used in a previous study (Freitas et al., 2008): the initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 3 min; and the final extension of 72°C for 10 min. PCR products were separated by gel electrophoresis in 2% agarose at 50V for 14 h in 1X TBE buffer (10X TBE buffer contains:108 g of Tris base, 55 g of Boric acid, 9.3 g of (Ethylenediaminetetraacetic acid) EDTA in 1 L of ddH_2O) (Sambrook & Russell, 2001) and visualized with a Kodak Gel Logic 1500 imaging system (Rochester, New York, USA). The experiments were repeated three times and obtained similar fingerprints in all experiments.

6.3.8 Cluster analysis

DNA fingerprints generated by ITS-, tDNA-, ERIC-, and BOX-PCR were converted into binary matrix "1" or "0" which represent presence or absence, respectively, of a DNA band in the gel. Phylogenetic analyses were performed with the unweighted pair group mean averages (UPGMA) algorithm using MEGA5 (Tamura et al., 2011).

6.4 RESULTS

6.4.1 Isolation of RAB strains showing antimicrobial activities against *R. solani* and *B. glumae*

Twenty-nine RAB strains out of the total 127 RAB strains tested were initially screened based on their antagonistic activities against both *R. solani* and *B. glumae*. In the repeated experiments, 26 of the 29 RAB strains were confirmed to have antagonistic activities, but the rest three RAB strains (RAB1, RAB5 and RAB12) did not show any activity against *R. solani* or *B. glumae* (Figures 6.1, 6.2 and 6.3). According to the sizes of the inhibition zones generated by the RAB strains, the antibacterial activities of the 26 active RAB strains were overall less variable than their antifungal activities (Figures 6.2 and 6.3). RAB3 showed the lowest antibacterial activity, while RAB2S and RAB23S showed the highest antibacterial activities against *B. glumae* (Figure 6.2). In terms of the antifungal activities against *R. solani*, RAB2S, RAB3, RAB8, RAB13, RAB17R and RAB19 showed relatively lower antifungal activities, while RAB6, RAB9 and RAB17S showed higher antifungal activities against *R. solani* than other RAB strains (Figure 6.3).

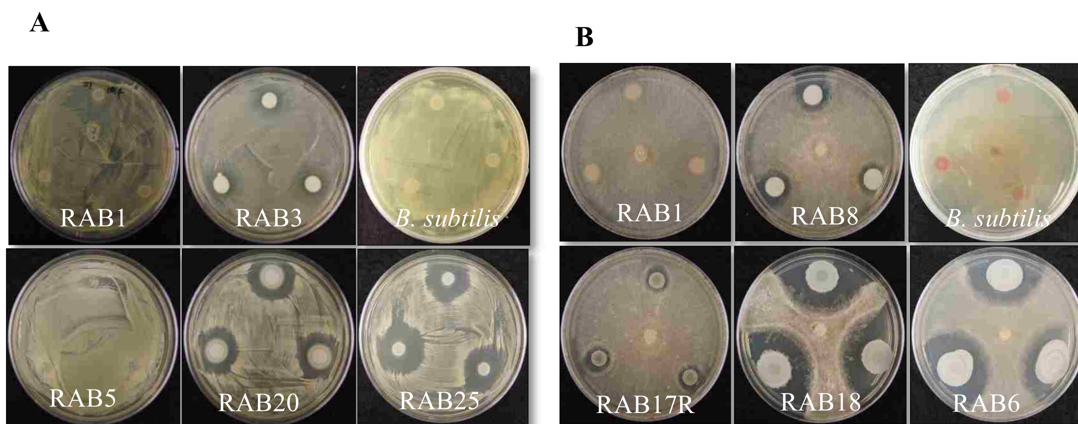

Figure 6.1 *Invitro* dual culture for antibacterial and antifungal activities of RABs. RABs inoculum was spotted after *B. glumae* inoculum was spreaded, and mycelial plug was placed in the center of the PDA media plate. Plates are incubated at 25°C for 72 hrs. (A) Antibacterial activities against *B. glumae* in PDA media. (B) Antifungal activities of RABs against *R. solani* in PDA media.

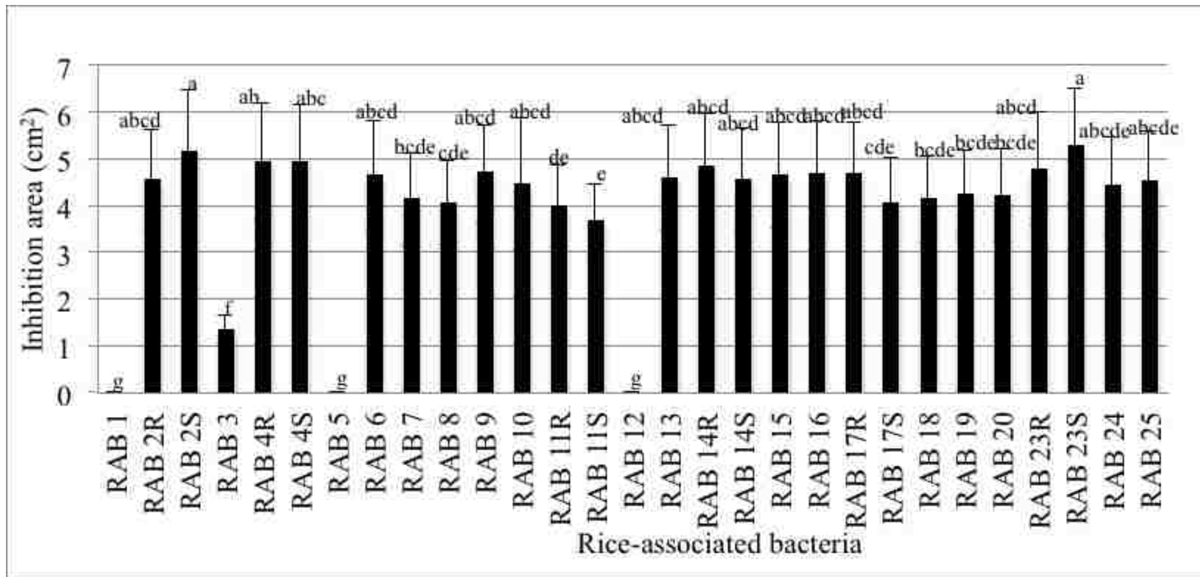

Figure 6.2 Differential antibacterial activities of RAB against *B. glumae*. RAB1, RAB5, and RAB12 did not show antibacterial activities, and RAB3 has the lowest activity and RAB23S has the highest inhibition activities. Antibacterial activities of RABs with different alphabets on the top are significantly different at α=0.05, p= <0.0001 from tukey's test. Each error bar indicates standard error from three replicates.

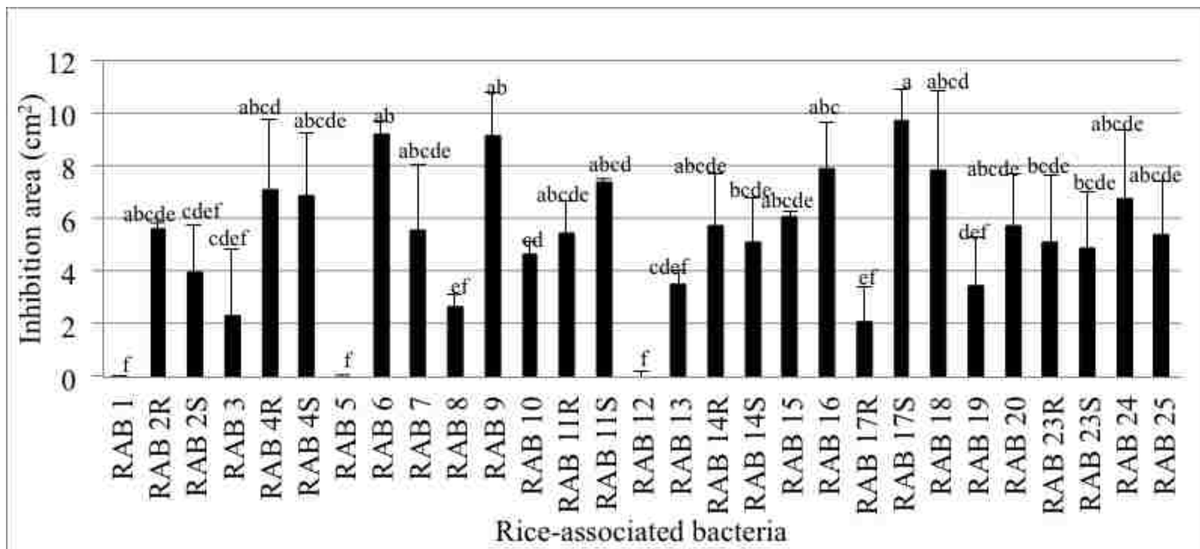

Figure 6.3 Differential antifungal activities of RAB against *R. solani*. RAB1, RAB5 and RAB12 did not show antifungal activities, and RAB3, RAB8 and RAB17R showed the lowest activities amonog the RABs. Antifungal activities of RABs with different alphabets on the top are significantly different at α=0.05, from tukey's test. Each error bar indicates standard error from three replicates.

6.4.2 *In vitro* inhibition of sclerotial germination, and suppression of sheath blight lesion development on detached-leaf assay

In vitro germination of sclerotia was completely inhibited by five selected RABs (RAB6, RAB9, RAB16, RAB17S, and RAB18) (Figure 6.4). These selected RABs showed prominent antifungal and antibacterial activities.

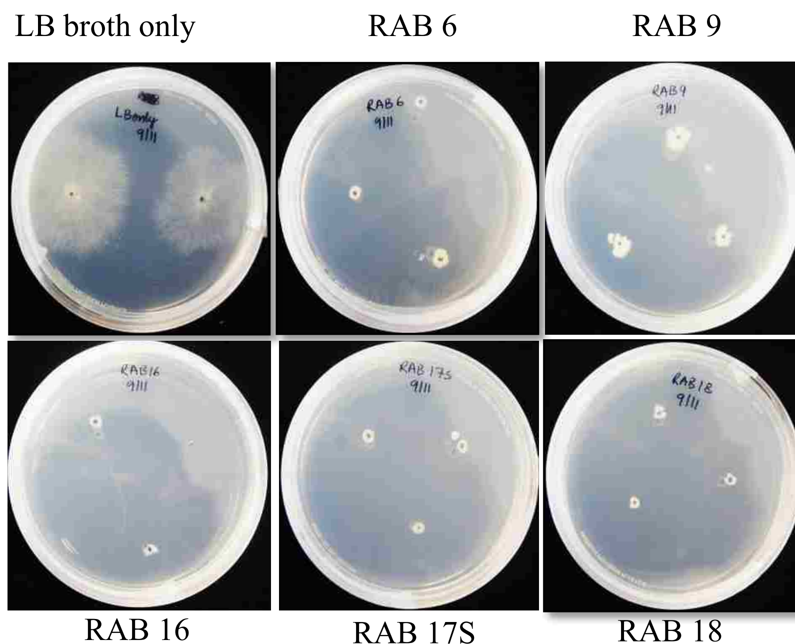

Figure 6.4 *Invitro* inhibition of sclerotial germination by RABs. Overnight cultured sclerotia with RAB inoculum in test tube were grown on PDA media and incubated at 25°C for 3 days. Sclerotia were placed at three spots on the PDA media.

On detached leaf assays, five selected RABs restrict the sheath blight lesion development. As compared to the RABs untreated leaves, RAB treated leaves showed lower disease severity (Figures 6.5 and 6.6). Disease score was ranged from 0-4 in which 0= no symptoms; 1= 1-10%; 2=11-25%; 3=26-50% and 4= >50% leaf area affected (Kumar, K Vijay Krishna et al., 2009). Among five RABs, RAB17S showed the maximum restriction of lesion development (Figure 6.6). Five-selected RAB were used in this experiment.

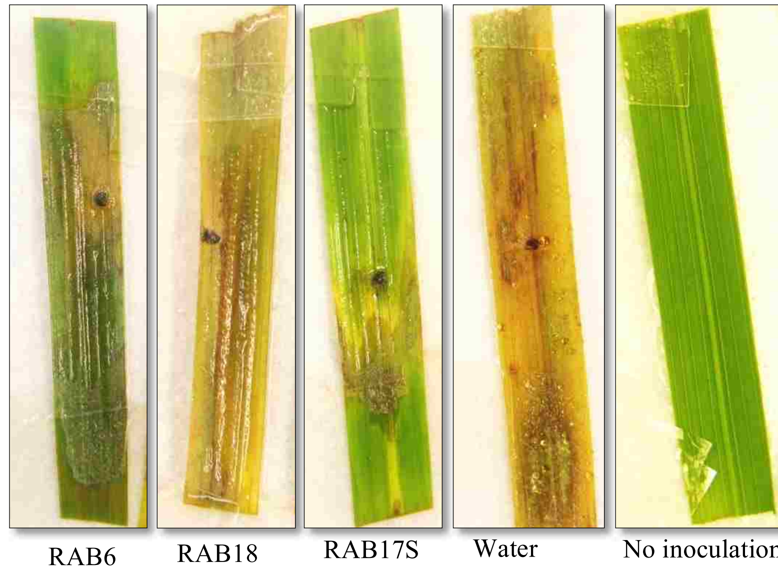

Figure 6.5 Reduction of sheath blight lesion was exhibited due to the pretreatment of RABs in detached-leaf assay. Detached-leaves in petri-dishes, lined with sterilized moist paper towel, were pretreated with RABs inoculum, sclerotia of *R. solani* was kept on the leaf at the center. Petri-dishes were incubated at 25°C with 12 h of light for 7 days. Leaf pieces were observed for sheath blight lesion after 7 days. Higher lesion was observed in the leaf pretreated with water only.

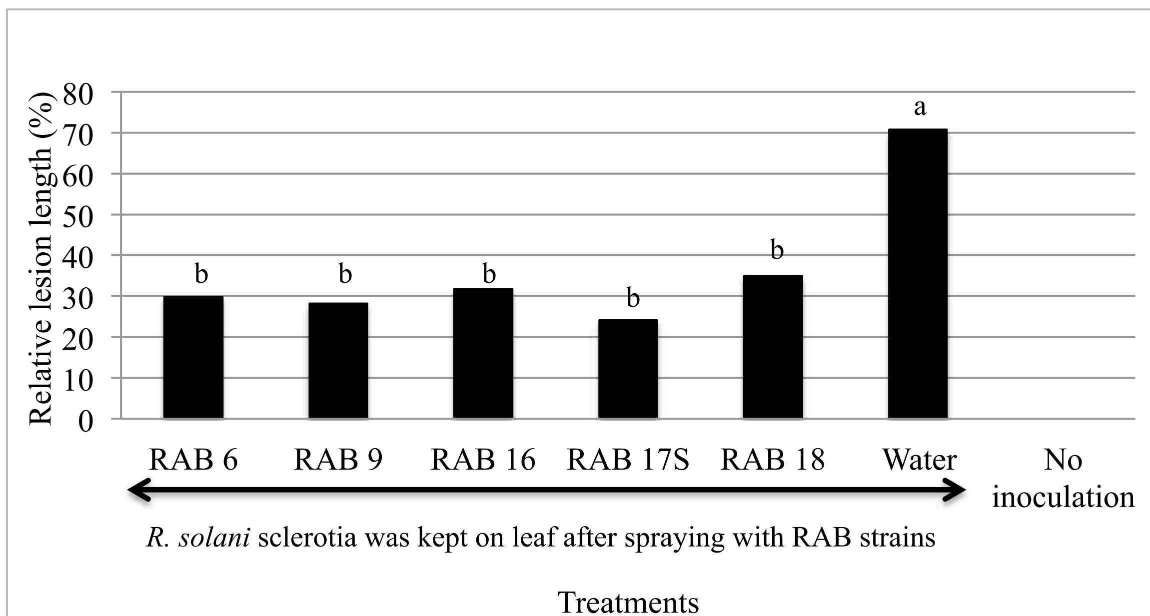

Figure 6.6 Suppression of sheath blight lesion in detached-leaf assay was observed by the pretreatment of RABs inoculum. All the selected RABs reduced equal amount of lesion, but more sheath blight lesion was observed on leaf in which only water was used. No lesion was observed on non-inoculated leaf. Each treatment was replicated for three times. Observations with different alphabets on the top are significantly different at α=0.05, from LSD.

6.4.3 Assessment of biological control activities of the antagonistic RABs

All of the five isolates that were used for pretreatment in the field in 2012 showed reduction in the symptoms of sheath blight in susceptible cultivar Bengal (Figure 6.7). Two of the isolates, RAB6 and RAB9 reduced the disease severity significantly with the score of 3.8 ± 0.33 and 3.0 ± 0.32, respectively (Figure 6.7). Other three isolates also inhibited the disease severity significantly, but had higher disease score than RAB6 and RAB9. Water-pretreated treatment has the highest disease severity of 6.95 ± 0.49 (Figure 6.7). Sheath blight symptoms were not developed in the treatment with no inoculation.

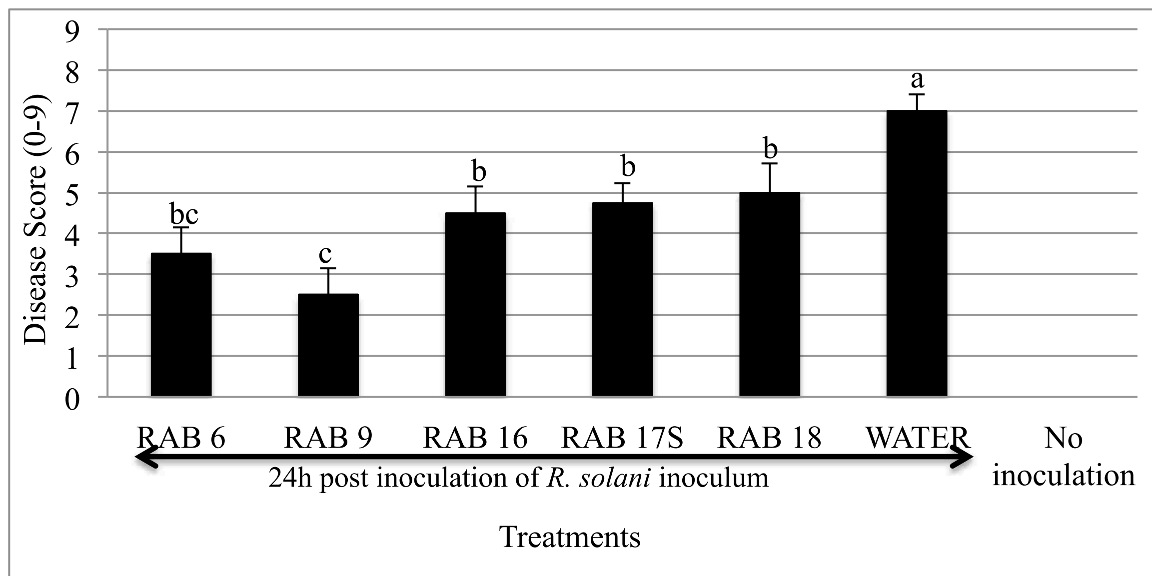

Figure 6.7 RABs suppressed sheath blight symptoms in rice in the field. Disease rating on the sheath of Bengal plant was done at milk stage of rice. *R. solani* inoculum was inoculated 24 after the inoculation of RABs inoculum at tillering stage of rice plants. Only water was used to spray rice plants as a control. No inoculation of *R. solani* was used as a negative control, which did not show any sheath blight lesions. Disease rating was done based on relative lesion height with the scale ranges from (0-9) (IRRI, 1996). Observations with different alphabets on the top are significantly different, between the treatments, at α=0.05, from LSD. Each error bar indicates standard error from three replications.

Similarly, pre-inoculation of the selected isolates 24 hours prior to the inoculation of *B. glumae* 336gr-1 suppressed the BPB symptoms on rice plants. RAB9 and RAB17S showed suppression of BPB symptoms significantly compared to water pretreated control. Other three isolates also suppressed the BPB symptoms, but did not suppress as much as RAB9 and RAB17S. RAB9 and RAB17S were the two of the selected five isolates with lower disease severity of 3.38 ± 0.61 and 3.33 ± 0.92, respectively. Water control has the highest disease severity of 6.38 ± 0.86 (Figure 6.8). RAB9 suppressed sheath blight and BPB symptoms with the lowest disease severity (Figures 6.7 and 6.8).

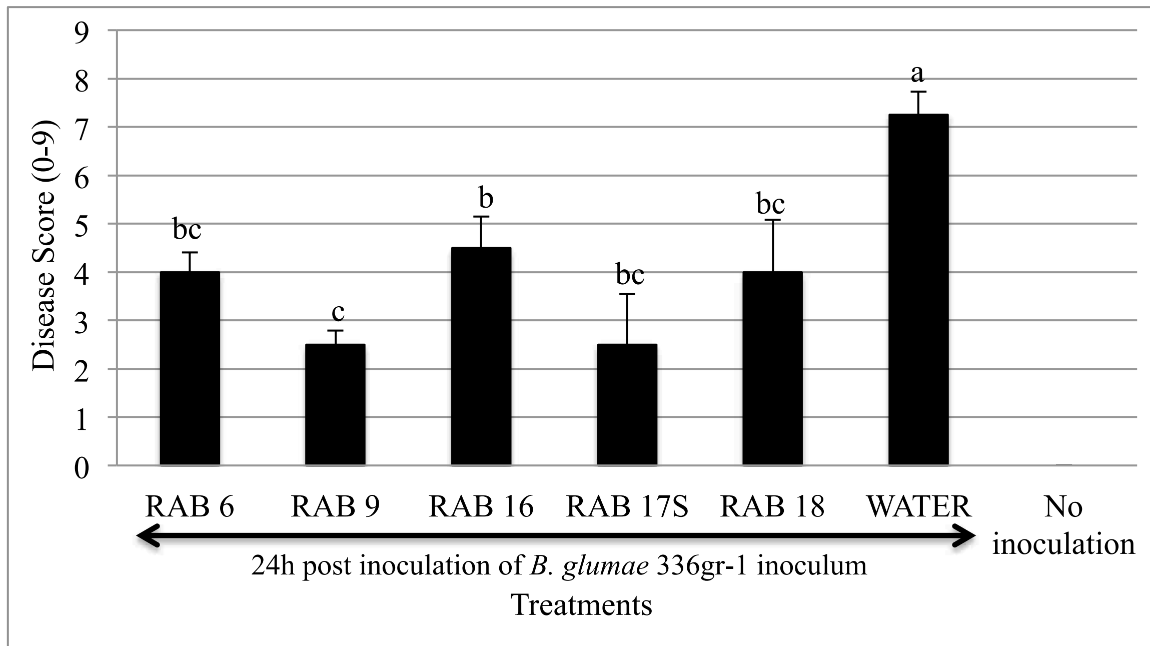

Figure 6.8 Suppression of BPB symptoms in rice was observed after pretreatment of RABs in the field. Disease rating on the panicles of Bengal plant was done at 10 days after inoculation of *B. glumae* 336gr-1. 1×10^8 CFU/ml of bacterial inoculum was inoculated 24 hrs. post-inoculation of various RABs inoculum at 30% heading stage. Only water was used to spray rice panicles as a control. No inoculation of *B. glumae* 336gr-1 inoculum was used as a negative control,which did not show any BPB symptoms. Disease rating was done using standard scale (0-9). Observations with different alphabets on the top are significantly different, between the treatments, at α=0.05, from LSD. Each error bar indicates standard error from three replications.

6.4.4 Identification of RAB isolates

During the 3% KOH test, *B.glumae* cell formed mucous thread of DNA, which indicated Gram-negative, however *B. subtilis* and 29 other RAB isolates did not form any thread-like structure of DNA which indicates that all the isolates belong to Gram-positive bacteria (Table 6.2). Moreover, 16S rDNA sequence analysis was performed from 29 RAB isolates. All 16S rDNA sequences were given in Appendix 19. A BLAST search of these sequences against NCBI database showed the highest sequence identity to *Bacillus* species (Table 6.2).

Table 6.2 3% KOH test and the closest species in Gene bank database with partial 16S rDNA sequence

Strains	Closest Gene Bank species with partial 16S rRNA sequence	Query coverage (%)	Identity (%)	E-value	Gram reaction based on 3% KOH test
RAB1	*Lysinibacillus sphaericus*	100	100	0.0	Gram +
RAB2R	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB2S	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB3	*Bacillus methylotrophicus*	100	99	0.0	Gram +
RAB4R	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB4S	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB5	*Bacillus* sp.	100	96	0.0	Gram +
RAB6	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB7	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB8	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB9	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB10	*Bacillus* sp.	100	99	0.0	Gram +
RAB11R	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB11S	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB12	*Lysinibacillus sphaericus*	99	99	0.0	Gram +
RAB13	*Bacillus subtilis*	100	100	0.0	Gram +
RAB14R	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB14S	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB15	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB16	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB17R	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB17S	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +

(Table 6.2 continued)

Strains	Closest Gene Bank species with partial 16S rRNA sequence	Query coverage (%)	Identity (%)	E-value	Gram reaction based on 3% KOH test
RAB18	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB19	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB20	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB23R	*Bacillus subtilis*	99	100	0.0	Gram +
RAB23S	*Bacillus amyloliquefaciens*	100	100	0.0	Gram +
RAB24	*Bacillus amyloliquefaciens*	100	99	0.0	Gram +
RAB25	*Bacillus amyloliquefaciens*	97	99	0.0	Gram +
Bacillus subtilis (as control)	*Bacillus subtilis*	98	99	0.0	Gram +
Burkholderia glumae 336gr-1 (as control)					Gram -

6.4.5 16S rDNA phylogenetic analysis

Phylogenetic analysis with the partial sequence information of 16S rDNA grouped the RABs into two major clusters (Figure 6.9). Twenty-six of 29 RABs were clustered in a one group and are close to *B. amyloliquefaciens* ATCC 23842 (EU689157) and *B. subtilis* subsp. *spizizenii* ATCC 6633 (AB018486) (Figure 6.9). These 26 RABs have shown various levels of antibacterial and antifungal activities, *invitro*. However, RAB1, RAB5 and RAB12, which did not show antibacterial and antifungal activities, *invitro*, to the cell growth of *B. glumae* 336gr-1, and mycelial growth of *R. solani* respectively, were clustered as a different group and kept separately from other RABs (Figures 6.2, 6.3 and 6.9). Phylogenetic tree was prepared by using neighbor-joining method and genetic distances based on 16S rDNA sequences of *Bacillus* species were calculated using Kimura 2-parameter method. *B. anthracis* ATCC 14578 (AB190217), which is the etiological agent of anthrax was separated from our RABs group suggesting that our RABs were not related to *B. anthracis*.

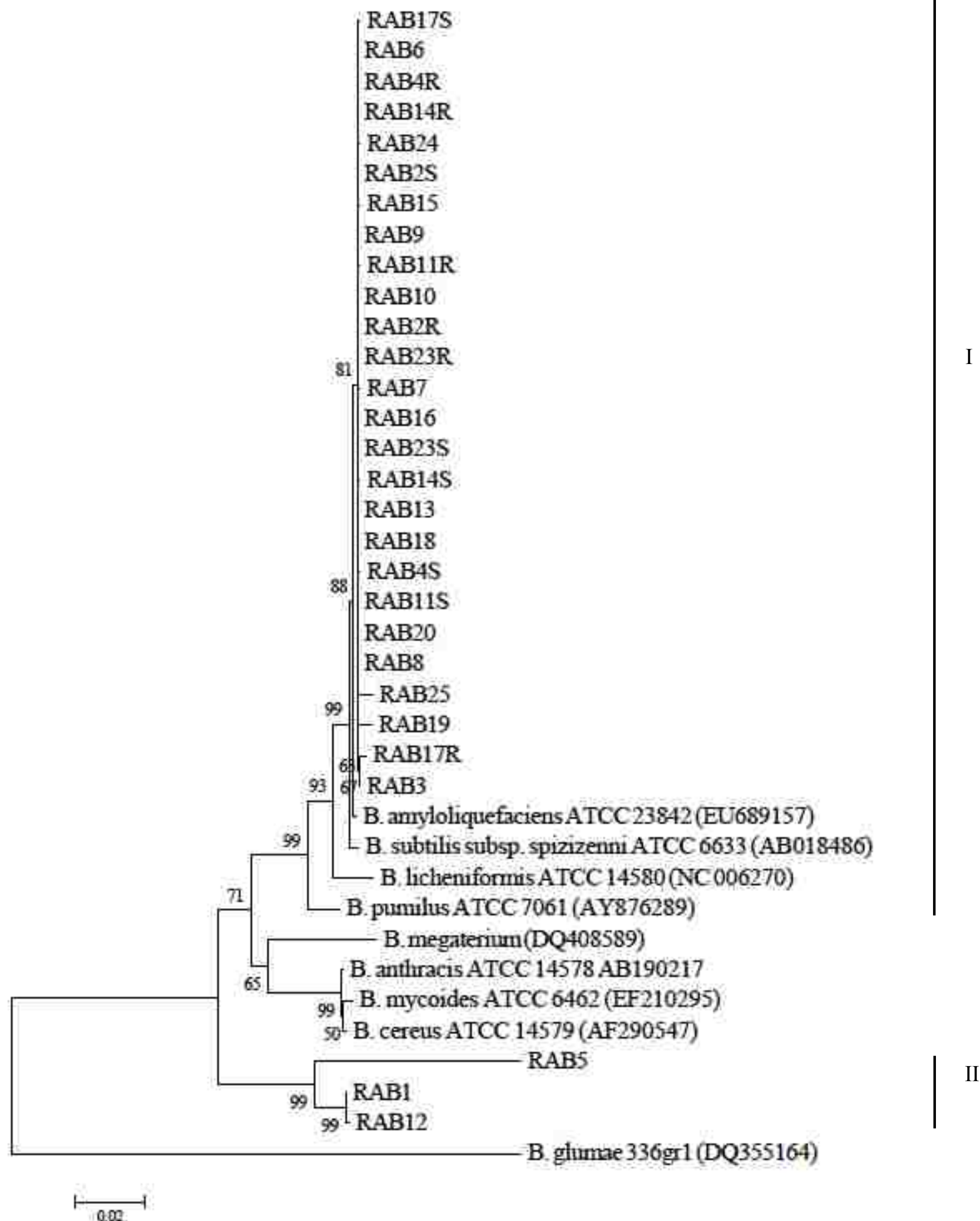

Figure 6.9 Phylogenetic tree, using neighbor-joining method, and genetic distances were calculated using Kimura 2-parameter method, based on 16S rDNA sequences of 29 RABs and randomly selected other species of *Bacillus* including, *Bacillus amyloliquefaciens* ATCC23842, *B. subtilis* subsp. *spizizenni* ATCC6633, *B. licheniformis* ATCC14580, *B. pumilus* ATCC7061, *B. megaterium*, *B. anthracis* ATCC14578, *B. mycoides* ATCC6462, and *B. cereus* ATCC14579. *Burkholderia glumae* 336gr-1 was used as an out-group. 16S rDNA sequences of randomly selected *Bacillus* and *B. glumae* 336gr-1 were obtained from NCBI database. Numbers at nodes indicate percentage of occurrence in 1000 bootstrap replicates. Gene bank accessions numbers were given in parenthesis.

6.4.6 ITS-, tDNA- and rep-PCR, and DNA fingerprinting

Eleven different banding classes were found in ITS-PCR, ranging from 200 bp to 1650 bp, and 15 band classes in tDNA-PCR, ranging from 100 bp to 650 bp (Figures 6.10 and 6.12, respectively), based on three independent PCRs. *B. subtilis*, which was used as a reference strain had unique profile compared to RABs in ITS-PCR, however it has similar profiles with most of the RABs profiles in tDNA-PCR (Figures 6.10 and 6.12). RABs were divided into two major groups and five sub-groups based on ITS- and tDNA-PCR fingerprinting and unweighted-pair group method with arithmetic mean (UPGMA) cluster analysis (Figures 6.11 and 6.13). RAB3, RAB1, RAB5 and RAB12 were separated into different group from rest of the other RABs in both ITS- and tDNA-PCR.

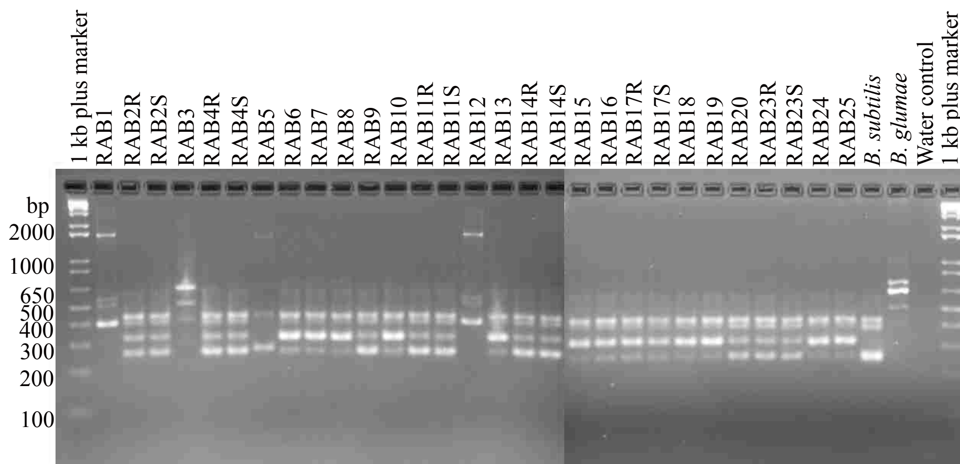

Figure 6.10 ITS-PCR fingerprinting patterns from 29 isolates of rice-associated bacteria isolated from healthy rice leaves in 2% agarose gel. *B. glumae*, and sterile ddH_2O were used as controls, and *B. subtilis* was used as a reference strain. 1 Kb plus DNA ladder was used as marker.

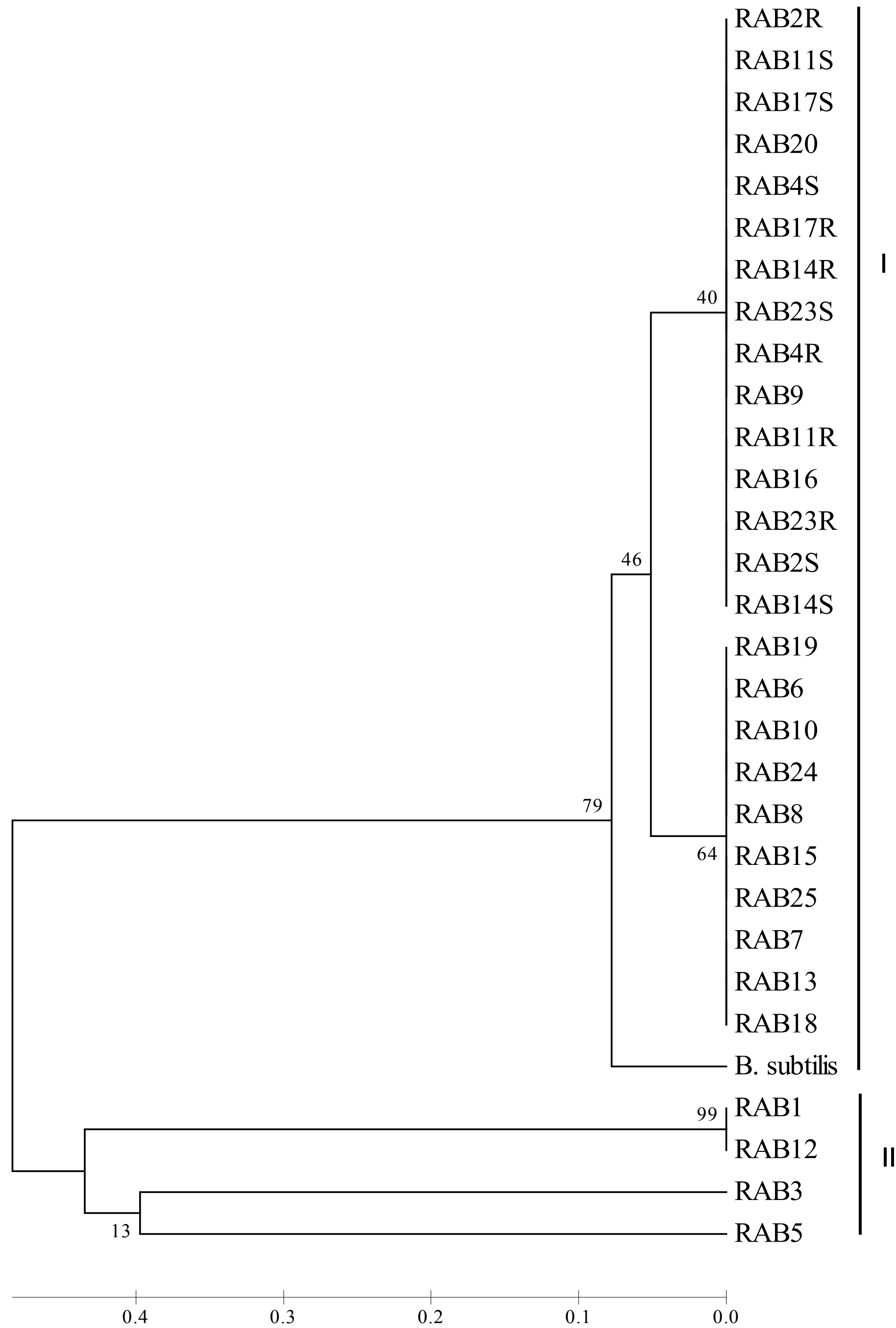

Figure 6.11 Phylogram constructed based on ITS-PCR fingerprinting shown in Figure 6.10. Phylogram tree was obtained from UPGMA analysis with 1000 bootstrap replications using MEGA5 tool. Numbers at nodes indicate percentage of occurrence in 1000 bootstrap replicates.

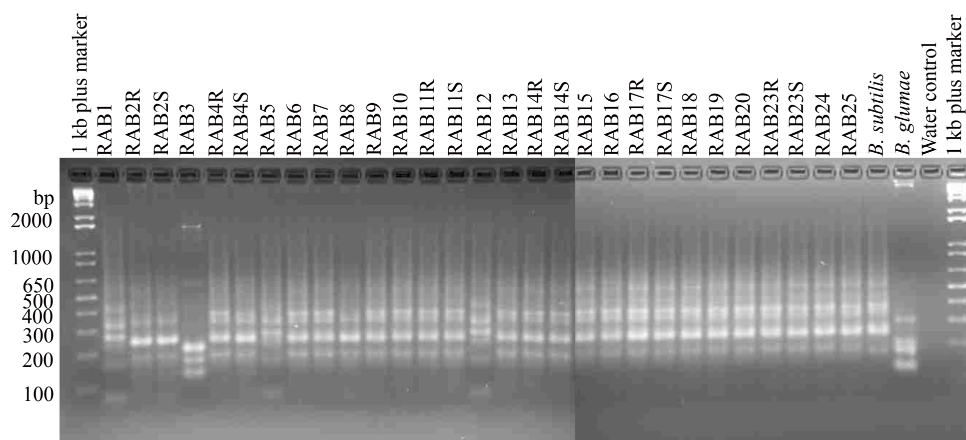

Figure 6.12 tDNA-PCR fingerprinting patterns from 29 isolates of rice-associated bacteria isolated from healthy rice leaves in 2% agarose gel. *B. glumae*, and sterile ddH_2O were used as controls, and *B. subtilis* was used as a reference strain. 1 Kb plus DNA ladder was used as marker.

Among these four RABs, RAB1, RAB5, and RAB12 did not show any antibacterial and antifungal activities against *invitro B. glumae* cell growth and *R. solani* mycelial growth, respectively, whereas RAB3 has the lowest antibacterial and antifungal activities against *B. glumae* and *R. solani* (Figures 6.2 and 6.3). *Bacillus subtilis*, which was used as a positive control, was grouped along with RABs that has shown both antibacterial and antifungal activities (Figures 6.11, and 6.13). However, in the phylogeny tree based on ITS-PCR fingerprinting *B. subtilis* was separated to a different sub-group (Figure 6.11).

Similarly, rep-PCR, including ERIC-, $(GTG)_{5}$-, and BOX-PCRs divided RABs into two major groups and several sub-groups. Twenty-eight and 30 classes of band were observed in ERIC- and BOX-PCR, ranging from 400 bp to 4000 bp and 200 bp to 1650 bp (Figures 6.14 and 6.15), respectively, based on three independent PCRs. Negative results were observed in RAB3 in both ERIC- and BOX-PCR whereas all of the tested RABs showed negative results for $(GTG)_5$ (data not shown).

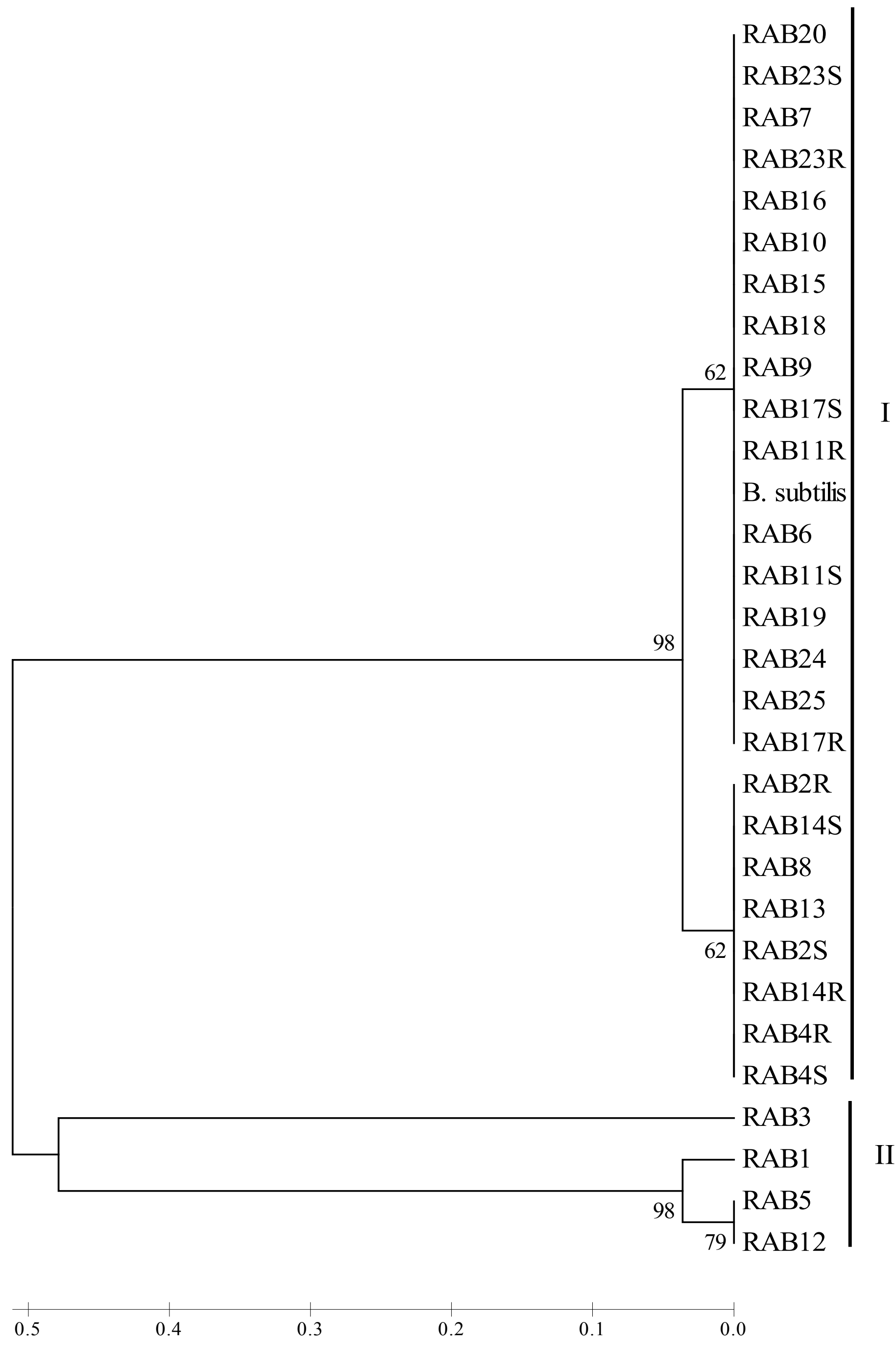

Figure 6.13 Phylogram constructed based on tDNA-PCR fingerprinting shown in Figure 6.12. Phylogram tree was obtained from UPGMA analysis with 1000 bootstrap replications using MEGA5 tool. Numbers at nodes indicate percentage of occurrence in 1000 bootstrap replicates.

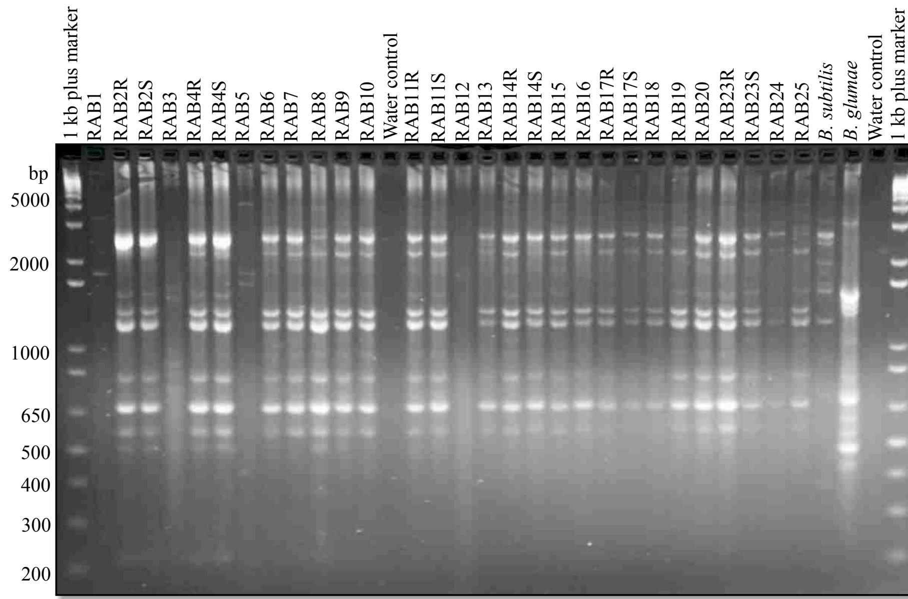

Figure 6.14 ERIC-PCR fingerprinting patterns from 29 isolates of rice-associated bacteria isolated from healthy rice leaves in 2% agarose gel. *B. glumae*, and sterile ddH_2O were used as controls, and *B. subtilis* was used as a reference strain. 1 Kb plus DNA ladder was used as marker.

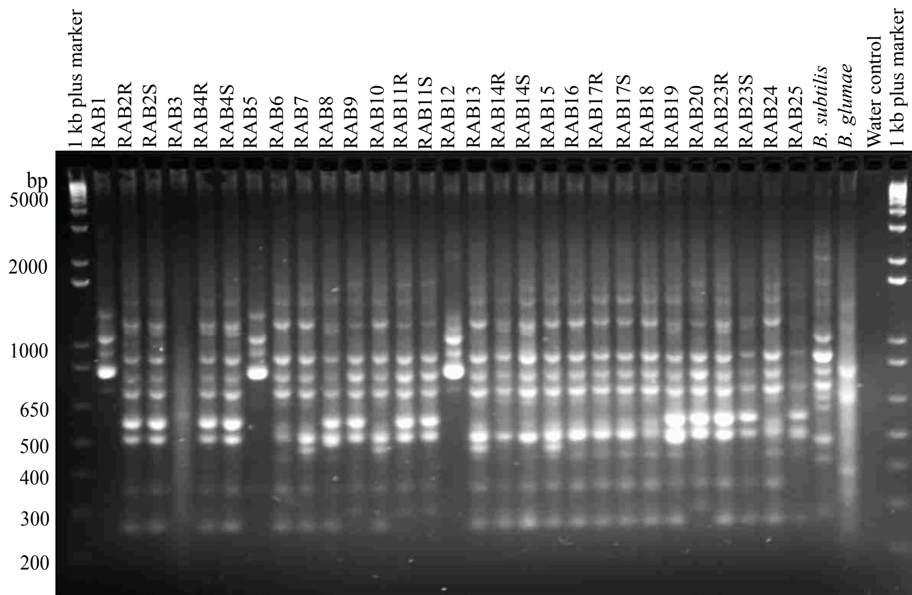

Figure 6.15 BOX-PCR fingerprinting patterns from 29 isolates of rice-associated bacteria isolated from healthy rice leaves in 2% agarose gel. *B. glumae*, and sterile ddH_2O were used as controls, and *B. subtilis* was used as a reference strain. 1 Kb plus DNA ladder was used as marker.

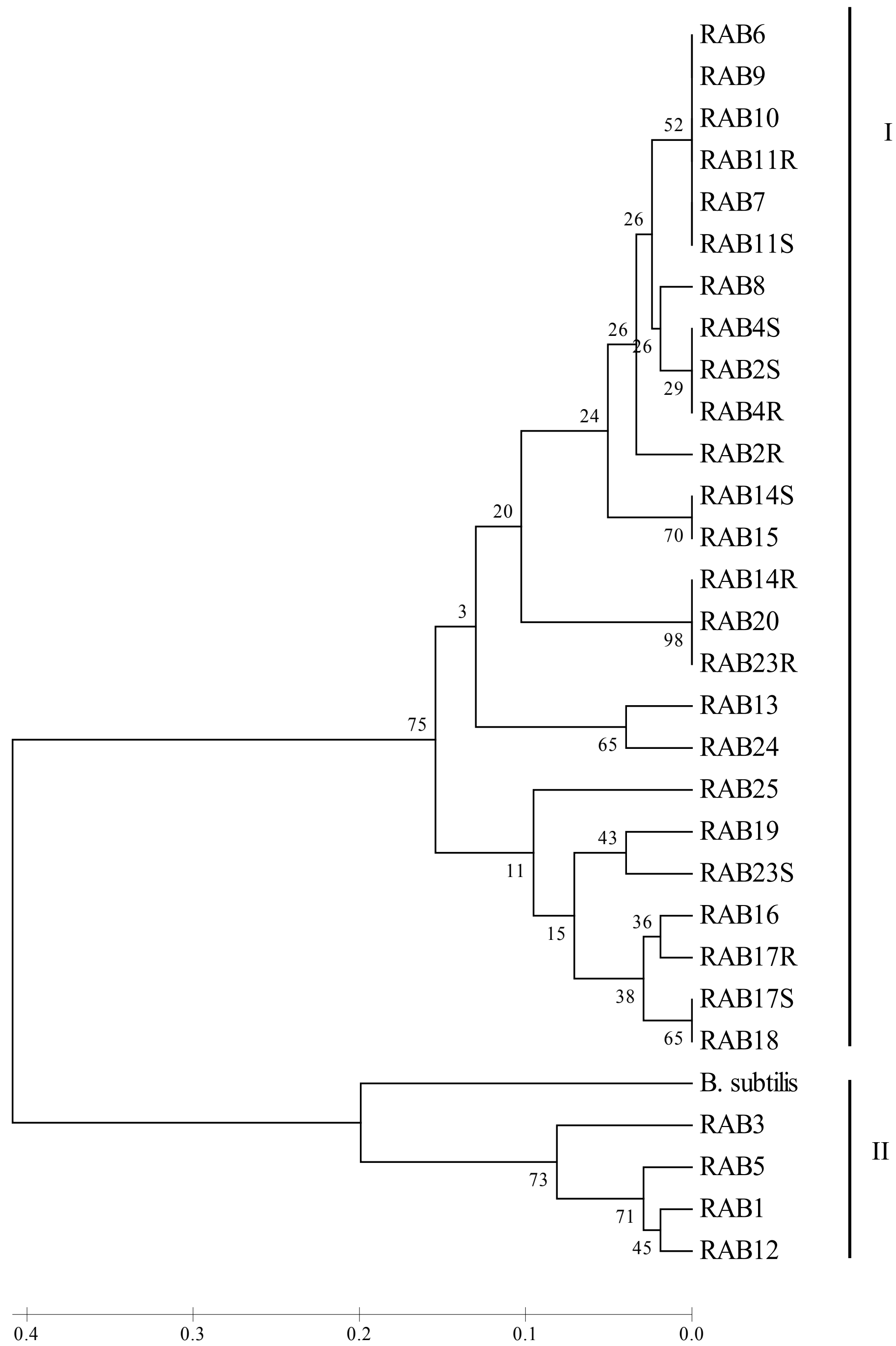

Figure 6.16 Phylogram constructed based on ERIC-PCR fingerprinting shown in Figure 6.14. Phylogram tree was obtained from UPGMA analysis with 1000 bootstrap replications using MEGA5 tool. Numbers at nodes indicate percentage of occurrence in 1000 bootstrap replicates.

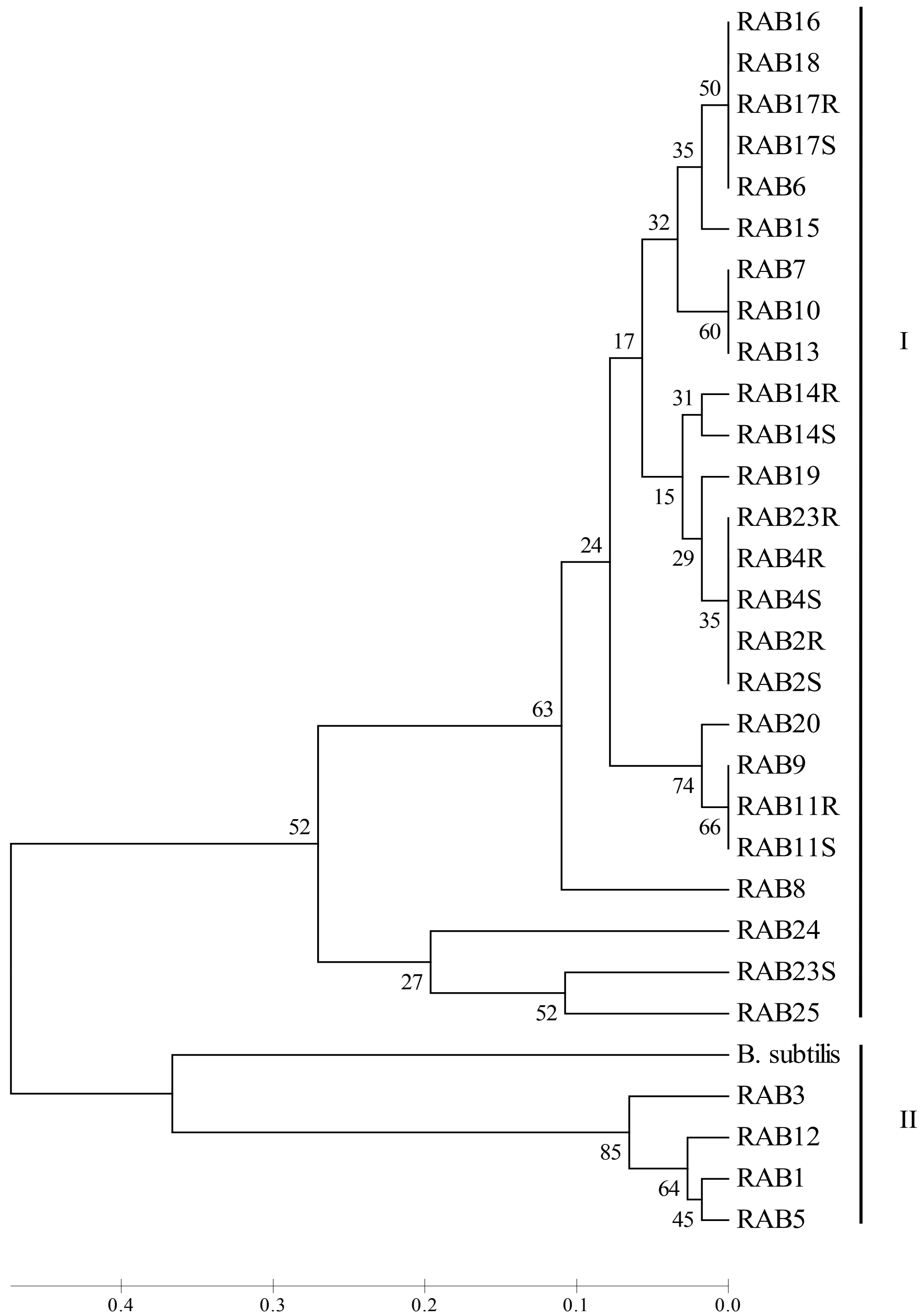

Figure 6.17 Phylogram constructed based on BOX-PCR fingerprinting shown in Figure 6.15. Phylogram tree was obtained from UPGMA analysis with 1000 bootstrap replications using MEGA5 tool. Numbers at nodes indicate percentage of occurrence in 1000 bootstrap replicates.

In UPGMA cluster analysis, RAB1, RAB5, and RAB12 were classified into same group, however, unlike ITS- and tDNA-PCR, *B. subtilis* classified into the same group with RAB1, RAB5 and RAB12 (Figures 6.16 and 6.17) that did not show any antibacterial and antifungal activities against *invitro B. glumae* cell growth and *R. solani* mycelial growth, respectively. All other remaining RABs were classified into separate group that have shown antibacterial and antifungal activities in dual culture assay (Figures 6.16 and 6.17).

6.5 DISCUSSION

In this study, 26 RABs having various antifungal and antibacterial activities against *R. solani* and *B. glumae*, respectively, were isolated from rice plants and all of them were identified as *Bacillus* spp. based on their 16S rDNA sequences. Regarding their antagonistic activities against the fungal and bacterial rice pathogens, these RABs can be potential biological agents for sheath blight and bacterial panicle blight and possibly other plant diseases caused by fungal and bacterial pathogens. Indeed, five RABs showing highest antimicrobial activities (RAB6, RAB9, RAB16, RAB17S and RAB18) were effective in suppressing the development of SB and BPB when sprayed to rice plants prior to pathogen inoculation. At this point, the mechanism of the biological control by the RABs remains unknown. Various mechanisms, including competition with pathogens for space, secretion of chemical compounds, which not only prevent pathogen growth and development on the plant surface, but also helps to induce systemic resistance in the plant systems, were involved in the suppression of disease symptoms (Niranjan et al., 2006). Several structural changes on plant system have been reported including callose deposition, and lignification at the pathogen infection sites. In addition, colonization of pathogens were reduced and/or restricted only on the outer side of epidermis in the plants treated with biocontrol agents (Kloepper, 1992; Mpiga et al., 1997).

Unlike the field and greenhouse experiments, detached leaf bioassay is a quicker method for pathogenicity tests because due to controlled-environments (temperature and moisture), and involvement of less space and time (Guleria et al., 2007; Kotamraju, 2010; Singh et al., 2002). *In vitro* experiments, including sclerotial germination and detached-leaf assay for *R. solani*, showed complete inhibition of sclerotial germination and mycelial growth of *R. solani* as well as reduction in lesion development on detached leaves (Figures 5.4 and 5.5). In earlier studies, various rhizobacteria including *Bacillus*, *Paenibacillus*, and *Arthorobacter* spp. also showed antagonistic activities against sheath blight lesion development in detached-leaf assays, sclerotia germination, and mycelial growth of *R. solani* in dual culture (Kotamraju, 2010; Kumar, K. V. K. et al., 2009). It has been reported that several secondary metabolites and enzymes including chitinase, β-1, 3-glucanase, siderophores, salicylic acid, and hydrogen cyanide were produced by several endophytic and epiphytic isolates, including *Pseudomonas florescens*, which inhibited mycelial growth in dual culture, sclerotial germination of *R. solani invitro* (Devi et al., 1989; Kazempour, 2004; Nagarajkumar et al., 2004).

All of the antagonistic RABs were identified to be Gram-positive bacteria by 3% KOH tests (Ryu, 1940) and *Bacillus* spp. by 16S rDNA sequence analyses (Janda & Abbott, 2007). However, 16S rDNA sequence analysis alone is not sufficient for the phylogenic or taxonomic studies of bacteria (Fox et al., 1992) because it has poor resolution for the identification at species level, and sometimes even at genus level (Janda & Abbott, 2007). DNA fingerprinting PCR methods, ITS-, tDNA-, ERIC, and BOX-PCR, were also used to characterize the variability among the isolated bacteria although 16S rDNA sequences were analyzed. ITS-, and tDNA-PCR were not sufficient to give better resolution to differentiate among the RABs. However, both methods divided RABs into two major groups (Figures 6.10, 6.11, 6.12, and 6.13). One of the

groups was antagonistic to *B. glumae* and *R. solani*. However, RAB3 which had small inhibition effect to both pathogens, but grouped along with RAB1, RAB5 and RAB12 that did not exhibit any antagonistic effect against *B. glumae* and *R. solani*. *B. subtilis*, which did not exhibit any activities, was grouped in the same clade with other RABs that showed antibacterial and antifungal activities, but has different branch (Figures 6.2, 6.3, 6.11, and 6.13). Rep-PCR, including ERIC- and BOX-PCR, on the other hand, has divided RABs into two major clades. Moreover, those two rep-PCRs have separated RABs into various sub-groups within the major clades. RAB16, RAB17S, and RAB18 that exhibited higher antagonistic activity against mycelial growth and growth of bacterial cell were grouped in one clade in both ERIC- and BOX-PCR (Figures 6.16 and 6.17). In these rep-PCRs also RAB3 was grouped along with the group that did not show any antagonistic effects on both pathogens. In contrast *B. subtilis* that did not exhibit any antagonistic effect to bacterial and mycelial growth was grouped with non-antagonistic RABs. It seems that DNA fingerprinting from ERIC- and BOX-PCR separated isolates according to their antagonistic characteristic against *B. glumae* and *R. solani* (Figures 6.2, 6.3, 6.16 and 6.17). These results from ERIC- and BOX- PCR showed variation among the RABs that may be helpful in commercializing the isolates.

In conclusion, RABs examined in this study were Gram-positive and showed differential levels of antagonistic activities to the growth and development of *B. glumae* and *R. solani*. Preinoculation of those selected RABs suppressed the disease symptoms caused by both pathogens in rice. So, these RABs can be used as a biocontrol agent for controlling bacterial panicle blight and sheath blight of rice. Various biological products from these beneficial microorganisms can be developed, commercially with minimal impact on enviroment. *Bacillus subtilis* was used in Germany in 1990s for seed dressing purpose in potatoes (Kilian et al., 2000).

Various strains of *Bacillus* species have been used alone or mixed with fungicides and chemical elicitors to suppress various crop diseases caused by several pathogens including *Aspergillus carbonarius* in table grapes (Jiang et al., 2014), *Streptomyces* sp. in potato and radish (Meng et al., 2013), *Colletotrichum orbiculare* in cucumber (Park et al., 2013a), *Ralstonia solanacearum* and *Phytophthora infestans* in tomato (Kabir et al., 2013; Tan et al., 2013), *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in common bean (Martins et al., 2013), *Pectobacterium carotovorum* SCC1 in tobacco (Park et al., 2013b), *Fusarium graminearum* in wheat (Moussa et al., 2013), *Pythium torulosum* (Shang et al., 1999), *Fusarium verticillioides* in maize root (Cavaglieri et al., 2005), *Xanthomonas oryzae* pv. *oryzae* in rice (Chithrashree et al., 2011), *Xanthomonas axonopodis* pv. *citri* (Huang et al., 2012) as well as soil-borne disease caused by *R. solani* and *Fusarium* spp. (Niranjan et al., 2006). Further research on the antagonistic activities of these RABs against other fungal and bacterial pathogens should be explored because these RABs have potential to develop commercial formulations for biological control of several bacterial and fungal diseases of several crops in an environmentally friendly manner.

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CHAPTER VII: CONCLUSIONS

Bacterial panicle blight (BPB) and sheath blight (SB) are two major rice diseases in the southern rice growing states of United States. These diseases reduce about 30-70% of total rice yield in severely infected field. Despite of its economic importance, no effective control measures are available for BPB and SB. In addition, no completely resistant rice cultivars for both diseases have been developed. However, a medium-grain cultivar and a long-grain line, Jupiter and LM-1, respectively, showed partial resistance to the diseases. In order to understand the mechanisms of rice resistance against BPB and SB, genetics and genomics studies of rice have been conducted. Recombinant inbred line (RIL) population was generated from a cross between a very susceptible cultivar, Trenasse and a partially resistant cultivar, Jupiter. Evaluation of phenotypic traits including days to 50% heading, BPB and SB disease ratings, and plant height of 300 RILs and their parents were performed in replicated trials for two years.

Trenasse showed earlier heading than Jupiter in 2012 and 2013. It took more days for heading in 2013 for both parents and RILs than in 2012 because of unfavorable environment for the growth and development of rice plant in 2013. The susceptible parent Trenasse had consistent BPB disease ratings of 8.75 and 8.70 in 2012 and 2013, and resistant parent Jupiter had 4.40 and 1.90 in 2012 and 2013. Mean BPB disease ratings for RILs were found in between the means of two parents in both years. Average BPB disease ratings of RILs were higher in 2012 than in 2013 suggesting that favorable environment was present for BPB development in 2012. Similarly, Trenasse had mean score of 9 and 8, and Jupiter had mean score of 2 and 3 for SB disease ratings in 2012 and 2014, respectively. Mean SB disease ratings for RILs were also found in between the mean scores of parents in both years. SB disease ratings for RILs were skewed towards higher disease score in 2012 and 2014. Furthermore, Trenasse grew taller than Jupiter in 2012, but shorter in 2013 and 2014.

Broad-sense heritability and correlations were calculated for the four traits in the RIL population. Traits including, days to 50% heading and plant height had high heritability indicating that these traits were not influenced by environmental variations. These traits were also negatively correlated with BPB and SB disease ratings. Heritability estimates for BPB disease ratings were 0.57, and 0.84 in 2012 and 2013, respectively, and for SB disease ratings were 0.91 and 0.63 in 2012 and 2014, respectively. These heritability results suggested that BPB and SB ratings were very much influenced by environmental variations. So, traits such as days to heading and plant height with higher heritability can be used for indirect selection method.

Comparative study of genomic characteristics of rice genotypes including Jupiter, Trenasse, Bengal, Lemont and LM-1 using whole genome sequence data was performed. Genome-wide DNA polymorphisms were identified among five rice genomes. Population structure analysis of these five rice genomes along with 50 rice accessions from the study by Xu et al., (2012) revealed that all the five rice genotypes were tropical *japonica*. Among five genotypes, Jupiter and Bengal had admixture of tropical and temperate *japonica*, whereas Trenasse showed some shared ancestry with *indica* rice accessions. The results from population structure analysis were congruent to the phenotypic characteristics, and the pedigree of individual rice genotypes. Pairwise comparisons between Jupiter and four other rice genotypes identified that Trenasse had higher number of variants, including SNPs and indels, whereas Bengal had lower number of variants. Lemont and LM-1 had similar degree of genome-wide DNA polymorphisms with Jupiter. The results from pair-wise comparisons were also supported the results obtained from population structure analysis.

Higher variants densities were found in exon and followed by intron, upstream and downstream regions of the genome of five rice cultivars. Variants in these regions of genomes

are very essential to study. For instance, variants detected in upstream regions of genes might have effect on the regulatory processes resulting in altering regulation of gene expressions. Those variations in regulatory and coding regions found in this study will be a base for the future study of the genomic basis of economically important traits such as disease resistance. Several high-impact variants, including, non-synonymous SNPs, insertions, deletions, and frameshift were also observed. Variations occurred due to non-synonymous SNPs cause missense mutation thereby resulting in different amino acid product. In this study, non-synonymous SNPs were found in greater number of genes in all genomes. Gene ontology analysis of the genes containing non-synonymous SNPs found between Trenasse and Jupiter showed that larger number of genes was found to be involved in signal transduction, response to stress, kinase activity and nucleotide binding activities. Further analysis of those genes involved in stress responses, kinase activities, and nucleotide binding is essential to understand genetic elements responsible for various phenotypic traits. This will help to enhance the genetic studies of US rice cultivars, and to develop elite lines.

Molecular markers are essential tools for the genetic mapping studies of quantitative traits like disease resistance. In an attempt to develop a polymorphic marker database for genetic mapping studies, simple sequence repeat (SSR) markers representing 12 chromosomes of rice from the database available in Gramene webpage were selected and screened for polymorphism between Trenasse and Jupiter. In this study very low percentage of usable polymorphic markers (28 of 1091 SSR) were identified between two parents. In order to increase the number of polymorphic markers between Trenasse and Jupiter, whole genome sequence data of the two cultivars were used. Allele-specific SNPs and microsatellite markers were developed and found that more than 44% and 38% of SNPs and microsatellites marker, respectively, developed in this

study can be used in genetic studies of mapping populations generated from the cross between Trenasse and Jupiter. These results suggested that whole genome sequencing is a useful source to identify polymorphism between the rice cultivars such as Trenasse and Jupiter where the molecular marker available in the databases are not sufficient to provide enough polymorphisms.

In the mean time, study of rice defense responses against *B. glumae* was conducted. Previous microarray studies, at Dr. Chuck Rush lab, showed that the gene encoding a NAC4-like transcription factor (Os01g0393100) named as **b**acterial **p**anicle **b**light **r**esponse gene **1** (*BPR1*) was highly up-regulated in Jupiter, but slightly up-regulated in Trenasse upon *B. glumae* infection. Expression of the *BPR1* gene at seedling and tillering stages of rice under different treatments of *B. glumae* and its mutant derivatives was not detected. However, *BPR1* gene showed rapid responses in Jupiter, but not in Trenasse when treated with *B. glumae* tox^- or chemicals, including jasmonic acid and ascorbic acid during heading stage. These results suggested that *BPR1* gene is tissue-specific and might be involved in defense response against *B. gluame* in rice. So, further study is required for functional characterization of *BPR1* gene to understand molecular mechanisms for defense responses against the pathogen.

Several rice-associated bacteria (RAB) isolated from healthy rice plants were tested for their ability to suppress BPB and SB in rice. All the RAB isolates were identified to be Gram-positive by 3% KOH test, and 16S rDNA sequence analysis identified RAB isolates as *Lysinibacillus* and *Bacillus* spp. Phylogenic tree using 16S rDNA sequences divided those isolates into two groups. One of the groups consists of RAB isolates, which showed antimicrobial activities against *B. glumae*, and *R. solani* in *in vitro* assays, and another group consists of RAB isolates, which did not show any activities against those rice pathogens. In addition, DNA fingerprintings generated by 16S-23S intergenic transcribed spacer (ITS)-PCR,

tDNA-intergenic spacer region (tDNA)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR and BOX-PCR were analyzed, which also divided RAB isolates into two major groups. The results from DNA fingerprinting and 16S rDNA sequence analyses showed similar grouping of RABs except for RAB3, which was grouped with the isolates that did not show any antimicrobial activities, in DNA fingerprinting analysis. These results showed that RABs, which had shown antimicrobial activities in *in vitro* assays, also suppressed BPB and SB symptoms in the field assays suggesting that these RABs are potential biocontrol agents for BPB and SB diseases.

APPENDICES

APPENDIX 1: ANALYSIS OF VARIANCE FOR DAYS TO 50% HEADING IN THE RILS OF F_5 GENERATION OF THE CROSS TRENASSE × JUPITER, 2012

Source of variation	df	Mean square	F value	Pr > F
RILs	299	41.69356	9.79	0.0001
Replications	1	6.82667	1.60	0.2064
Error	299	4.25810		
Corrected Total	599			

Coeff. of var. = 2.52

APPENDIX 2: ANALYSIS OF VARIANCE FOR BACTERIAL PANICLE BLIGHT RATING FOR THE RILS OF F_5 GENERATION OF THE CROSS TRENASSE × JUPITER, 2012

Source of variation	df	Mean square	F value	Pr > F
RILs	299	2.6330602	2.31	0.0001
Replication	1	6.2016667	5.43	0.0204
Error	299	1.141466		
Corrected Total	599			

Coeff. of var. = 15.93

APPENDIX 3: ANALYSIS OF VARIANCE FOR SHEATH BLIGHT RATING FOR THE RILS OF F_5 GENERATION OF THE CROSS TRENASSE × JUPITER, 2012

Source of variation	df	Mean square	F value	Pr > F
RILs	299	11.594760	11.30	0.0001
Replication	1	60.166667	58.63	0.0001
Error	299	1.026198		
Corrected Total	599			

Coeff. of var. = 16.93

APPENDIX 4: ANALYSIS OF VARIANCE FOR PLANT HEIGHT IN THE RILS OF F_5 GENERATION OF THE CROSS TRENASSE × JUPITER, 2012

Source of variation	df	Mean square	F value	Pr > F
RILs	299	135.22081	7.72	0.0001
Replications	2	72.56083	4.14	0.0164
Error	598	17.52349		
Corrected Total	899			

Coeff. of var. = 4.35

APPENDIX 5: ANALYSIS OF VARIANCE FOR DAYS TO 50% HEADING IN THE RILS OF F_6 GENERATION OF THE CROSS TRENASSE × JUPITER, 2013

Source of variation	df	Mean square	F value	Pr > F
RIL	299	19.272887	6.45	0.0001
Rep	1	15.360000	5.14	0.0241
Error	299	2.988763		
Corrected Total	599			

Coeff. of var. = 1.53

APPENDIX 6: ANALYSIS OF VARIANCE FOR BACTERIAL PANICLE BLIGHT RATING FOR THE RILS OF F_6 GENERATION OF THE CROSS TRENASSE × JUPITER, 2013

Source of variation	df	Mean square	F value	Pr > F
RIL	299	5.883361	6.29	0.0001
Replication	1	17.681667	18.89	0.0001
Error	299	0.935847		
Corrected Total	599			

Coeff. of var. = 17.83

APPENDIX 7: ANALYSIS OF VARIANCE FOR PLANT HEIGHT IN THE RILS OF F_6 GENERATION OF THE CROSS TRENASSE × JUPITER, 2013

Source of variation	df	Mean square	F value	Pr > F
RIL	299	132.16028	9.71	0.0001
Replication	2	46.58333	3.42	0.0332
Error	598	13.60452		
Corrected Total	899			

Coeff. of var. = 3.94

APPENDIX 8: ANALYSIS OF VARIANCE FOR DAYS TO 50% HEADING IN THE RILS OF THE CROSS TRENASSE × JUPITER, ACROSS YEARS (2012 AND 2013)

Source of variation	df	Mean square	F value	Pr > F
RIL	299	47.2162	13.03	0.0001
YEAR	1	281704.1633	77745.1	0.0001
Rep(YEAR)	2	11.0933	3.06	0.0475
RIL*YEAR	299	13.7503	3.79	0.0001
Error	598	3.6234		
Corrected Total	1199			

Coeff. of var. = 1.96

APPENDIX 9: ANALYSIS OF VARIANCE FOR BACTERIAL PANICLE BLIGHT RATING FOR THE RILS OF THE CROSS TRENASSE × JUPITER, ACROSS YEARS (2012 AND 2013)

Source of variation	df	Mean square	F value	Pr > F
RIL	299	4.864314	4.68	0.0001
YEAR	1	491.520000	473.23	0.0001
Rep(YEAR)	2	11.941667	11.50	0.0001
RIL*YEAR	299	3.652107	3.52	0.0001
Error	598	1.038657		
Corrected Total	1199			

Coeff. of var. = 16.80

APPENDIX 10: ANALYSIS OF VARIANCE FOR PLANT HEIGHT IN THE RILS OF F_7 GENERATION OF THE CROSS TRENASSE × JUPITER, 2014

Source of variation	df	Mean square	F value	Pr > F
RIL	299	168.38486	14.47	0.0001
Replication	2	26.54914	2.28	0.1031
Error	566	11.64058		
Corrected Total	867			

Coeff. of var. = 3.36

APPENDIX 11: ANALYSIS OF VARIANCE FOR SHEATH BLIGHT RATING FOR THE RILS OF F_7 GENERATION OF THE CROSS TRENASSE × JUPITER, 2014

Source of variation	df	Mean square	F value	Pr > F
RIL	299	4.505078	2.67	0.0001
Replication	1	3.375000	2.00	0.1582
Error	299	1.686037		
Corrected Total	599			

Coeff. of var. = 19.56

APPENDIX 12: ANALYSIS OF VARIANCE FOR PLANT HEIGHT IN THE RILS OF THE CROSS TRENASSE × JUPITER, ACROSS YEARS (2012, 2013, AND 2014)

Source of variation	df	Mean square	F value	Pr > F
RIL	299	289.45921	20.24	0.0001
YEAR	1	13653.65406	954.55	0.0001
Rep(YEAR)	4	48.56443	3.40	0.0025
RIL*YEAR	299	72.06776	5.04	0.0001
Error	1164	14.3037		
Corrected Total	1767			

Coeff. of var. = 3.89

APPENDIX 13: ANALYSIS OF VARIANCE FOR SHEATH BLIGHT RATING FOR THE RILS OF THE CROSS TRENASSE × JUPITER, ACROSS YEARS (2012 AND 2014)

Source of variation	df	Mean square	F value	Pr > F
RIL	299	10.584646	7.81	0.0001
YEAR	1	128.707500	94.91	0.0001
Rep(YEAR)	2	31.770833	23.43	0.0001
RIL*YEAR	299	5.515192	4.07	0.0001
Error	598	1.356118		
Corrected Total	1199			

Coeff. of var. = 18.45

APPENDIX 14: ANNOTATION OF SNPS, INSERTIONS, AND DELETIONS IN DIFFERENT GENOMIC REGIONS OF FIVE RICE CULTIVARS WHEN COMPARED WITH THE REFERENCE GENOME, NIPPONBARE

	Jupiter	Trenasse	Bengal	Lemont	LM-1
SNPs					
Intergenic	507,464	1,332,703	626,575	1,067,972	983,897
Genic	350,129	914,205	431,178	740,566	693,746
Intron	144,032	385,457	182,841	305,047	286,661
UTRs	24,950	59,639	29,236	45,958	44,224
CDS	181,147	469,109	219,101	389,561	362,861
Synonymous	85,078	220,282	101,782	182,892	171,881
Non-synonymous	96,069	248,827	117,319	206,669	190,980
Indels					
Intergenic	63,297	140,001	69,620	121,364	111,309
Genic	30,591	67,869	33,772	57,886	53,502
Intron	21,759	48,546	24,491	41,979	38,775
UTRs	6,465	14,344	6,829	11,677	10,855
CDS	2,367	4,979	2,452	4,230	3,872
Frame shift	2,426	5,509	2,685	4,907	4,389
Intragenic	39	68	34	66	52

APPENDIX 15: ANNOTATION OF SNPS, INSERTIONS, AND DELETIONS IN DIFFERENT GENOMIC REGIONS OF FOUR RICE CULTIVARS WHEN COMPARED WITH THE JUPITER

	Trenasse	Bengal	Lemont	LM-1
SNPs				
Intergenic	1,220,425	444,725	915,373	841,583
Genic	840,151	322,767	637,625	595,298
Intron	349,935	126,642	256,966	240,123
UTRs	50,647	17,144	34,754	33,523
CDS	439,569	178,981	345,905	321,652
Synonymous	208,179	86,709	164,986	154,415
Non synonymous	231,390	92,272	180,919	167,237
Indels				
Intergenic	127,365	49,405	101,184	93,982
Genic	59,990	23,795	46,911	43,932
Intron	43,221	17,230	34,221	31,985
UTRs	12,314	4,790	9,154	8,666
CDS	4,455	1,775	3,536	3,281
Frame shift	5,212	2,069	4,323	3,895
Intragenic	54	22	57	44

APPENDIX 16: USABLE POLYMORPHIC SSR MARKERS IN CHROMOSOMES 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, AND 12, BETWEEN TRENASSE AND JUPITER, FROM GRAMENE DATABASE (McCouch et al., 2002)

Primer name	Chr.	Motif	No. of repeats	Forward primer	Reverse primer	Position in chromosome (bp)	
						SSR start	SSR end
RM10198	1	AGG	8	CTCCACACAAGGACACACATGC	GCCTCAAAGGTAGGTTTGCTTCC	3,873,028	3,873,051
RM14133	2	AG	21	CGACAAATAATTGGAGCTGACAGTGG	CAGTTAGCAATGCCATGAGACAGG	34,306,280	34,306,321
RM14918	3	AAG	7	CTCGACATGCTGAGCTTTCTACC	GCTATTGCGCTTCACTGTCTCC	12,777,151	12,777,171
RM16459	4	AAT	16	TCCAGGAGTTTGCCTTGTAGTGC	TAGCGAAGTCAGGATGGCATAGG	5,178,652	5,178,699
RM16506	4	AC	10	GCAGTAGACCTCGTGCTGAATGC	CCACACCGCCGCAATATAAACC	6,888,967	6,888,986
RM16554	4	AG	11	GCAACCAAAGTTGGTAACGAGAGC	CCGGCGCAATCTATTAGACACC	8,639,749	8,639,770
RM18360	5	AG	19	TCGAGACTGATCGGAGTTTAGGC	CGCTCCTCCCTAACACCTCTACG	14,031,926	14,031,963
RM18398	5	AG	12	CCCTTTGCTCTGAATCTGATTACC	GGCTCAAAGTAGTGCTCCATCC	14,873,521	14,873,544
RM18751	5	AC	16	CCGTGTGTTGGCTTAGAATCAAGG	GCCACTTTCCAAACATCAGAAAGC	21,107,767	21,107,798
RM19235	6	AAG	9	CTCAATACGGTAGACTTGAGCAATCC	CCACGATCCATACGCCTTTACC	246,387	246,413
RM8121	6	AG	16	CATTGTCCCGCCGTATCTAGC	CCTTCTGGCTCATTTATGCTTGG	381,774	381,805
RM527	6	AG	17	CGGTTTGTACGTAAGTAGCATCAGG	TCCAATGCCAACAGCTATACTCG	9,874,150	9,874,183
RM20216	6	AATC	7	CGAGCTCATTTCACACAAACAGC	CGAAATGGAAAGGGTTTGACTCG	20,553,195	20,553,222
RM20535	6	AG	10	TGCAAGCTGTACAGTTCATGTGG	GGCCCATTACGGCTACAAAGG	26,781,109	26,781,128
RM20612	6	AG	13	TGTCTCTCGATACCTCCCATACC	GCCCACCTCTCTTGTCCTATCC	27,916,793	27,916,818
RM20852	7	AAG	11	GTAGCTCCATGCCAGTTTGTGG	AACCTTCTTGATTGGCCATCTCC	902,070	902,102
RM3710	8	AG	15	AGCAGCAGCCGCTTCTTGTCG	CGATTGTTTCCTCCGCCATTCC	370,263	370,292
RM152	8	CCG	10	AAGGAGAAGTTCTTCGCCCAGTGC	GCCCATTAGTGACTGCTCCTAGTCG	677,702	677,731
RM22899	8	AG	11	TTGCTGTAATGCTGTTCCATCC	CGAAGGCGACCTTTCTAGTCG	14,758,520	14,758,541
RM149	8	AT	10	GGAAGCCTTTCCTCGTAACACG	GAACCTAGGCCGTGTTCTTTGC	24,716,913	24,716,932
RM6760	8	ATC	8	AGTGATGGACCATGATGATGACG	CCTCCCTTTCTCCTTGTCTCTCG	27,495,915	27,495,938
RM3533	9	AG	12	CCTTCATTTCCCTTCCCTCTCC	CTTTCCAACCTGTCAGGGAATCG	17,833,841	17,833,864
RM23668	9	ACG	10	TGCATAGCATATCAACTAGCCCTACC	GCTGAAACAGAATGAAAGCACAGC	600,998	601,027
RM23959	9	AG	10	TGCCAAAGCTAGCTACACACTCC	TGCATCATCTTCATCTCTCCATCC	7,945,032	7,945,051
RM26646	11	ACC	7	GTTCATCGGATTCTCGGTTCAGG	GCGGAGGAACAAGACACGAAGC	14,832,719	14,832,739
RM27080	11	GGC	7	ACTCGGCCCAGTAAGCGTCAGC	GGGCCGCAATGTGTAAGAGAGG	23,246,740	23,246,760
RM27663	12	AC	12	TAGCTAGGATCGGATGAAAGATCTCC	GGAGAGAATGTGCGTGCTTGC	4,542,510	4,542,533
RM28261	12	AAC	7	GACATTTCGAATCCACATCTGACC	TTCAGAACAGGGAGGATGTCACC	19,188,093	19,188,113

APPENDIX 17: VALIDATING SOME RANDOMLY SELECTED NSSNP-MARKERS DEVELOPED WITH WEBSNAPPER USING WHOLE GENOME SEQUENCE DATA OF TRENASSE AND JUPITER. PRIMERS NAME ENDING WITH 'J' AND 'T' ARE SPECIFIC FORWARD PRIMES FOR JUPITER AND TRENASSE, RESPECTIVELY.

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
1	Os01g0118400	(A/G)	Os01g0118400_J	GCCACCACGACGCCACGTTTA	J[a]	NA[c]	65
			Os01g0118400_T	AGCCACCACGACGCCACGTTAG	T[b]		
			Os01g0118400_R1	GGCGGTGTCGCACAAGGTGC			
	Os01g0140400	(A/G)	Os01g0140400_J	GCCGGGGAACCACAGCTACAAGA	J	Y[d]	70
			Os01g0140400_T	GCCGGGGAACCACAGCTACAATG	T		
			Os01g0140400_R1	GTAGACGGAGAAGAGGCGGAGGTAGTAGC			
	Os01g0149350	(C/A)	Os01g0149350_J	TCTCTGAAAATGTCCTTATTAATTGTCTTAAAGCTC	J	N[e]	70
			Os01g0149350_T	TCTCTGAAAATGTCCTTATTAATTGTCTTAAAGGTA	T		
			Os01g0149350_R1	GAATGAATCTTCTGCAGCCAAAACCAGA			
	Os01g0167750	(A/G)	Os01g0167750_J	GAGAGGCGGCGGGGGCAATA	J	NA	65
			Os01g0167750_T	GAGAGGCGGCGGGGGCTAGG	T		
			Os01g0167750_R1	GCGTGGCCCTCTACCTCCGC			
	Os01g0168200	(T/C)	Os01g0168200_J	ATTCTACAAGTTACTTACAATTTTCCCATCCTTGAT	J	NA	65
			Os01g0168200_T	ATTCTACAAGTTACTTACAATTTTCCCATCCTTGAC	T		
			Os01g0168200_R1	ATGGGGTCCTGTGTGGGAAAGGTC			
	Os01g0174700	(G/A)	Os01g0174700_J	TCCTGCCGCTCCTTCTTGCTCATAG	J	NA	65
			Os01g0174700_T	CCTGCCGCTCCTTCTTGCTCATTA	T		
			Os01g0174700_R1	TCATCAACATCATCAAGCAGCCGG			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
2	Os02g0202300	(T/C)	Os02g0202300_J	CTTGTAGGCAGCTGTTTGCATAATACGTTCAT	J	Y	70
			Os02g0202300_T	TTGTAGGCAGCTGTTTGCATAATACGTTCAC	T		
			Os02g0202300_R1	AAACATGCTCATTGCATGCTTTCATTGTT			
	Os02g0218200	(T/G)	Os02g0218200_J	TGAAGCAGAGCATCGAGAGAGTGGGT	J	Y	70
			Os02g0218200_R1	GCATCTATCAAACTGACATAACCTGGAGAAACTG			
			Os02g0218200_T	GGTGAAGCAGAGCATCGAGAGAGTGAAG	T		
			Os02g0218200_R2	ACCTGGAGAAACTGGTCCATAGATGCAAG			
	Os02g0245800	(C/A)	Os02g0245800_J	CAAGTAAATGGCTGGGGTATGTTACATAATGC	J	Y	70
			Os02g0245800_T	GCAAGTAAATGGCTGGGGTATGTTACATAGAGA	T		
			Os02g0245800_R1	CCAATTTAGTTCAGACATGAAATTTCACTAAGGCTT			
	Os02g0582150	(A/T)	Os02g0582150_J	TTTTCGGATGGTTGGACTTCTGAAGGA	J	Y	70
			Os02g0582150_T	CTTTTCGGATGGTTGGACTTCTGAGGAT	T		
			Os02g0582150_R1	CAGGTTTCCACAAATGACTGCAATGATG			
	Os02g0601000	(G/A)	Os02g0601000_J	GGCAGGGGCCTGGTTCGCTG	J	Y	65
			Os02g0601000_T	GGCAGGGGCCTGGTTCGCTA	T		
			Os02g0601000_R1	GAACACCATGGTCGGCTGGAACA			
3	Os03g0103400	(T/G)	Os03g0103400_J	GCACGACCTCCTCCTCAAGCTCATCT	J	NA	65
			Os03g0103400_T	GACCTCCTCCTCAAGCTCGCCG	T		
			Os03g0103400_R1	GTGGAGAGGGTGTCGTGGATGAGC			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
3	Os03g0265700	(A/G)	Os03g0265700_J	CTGATAATGTCGAGTCTCTTGATTCGGA	J	Y	70
			Os03g0265700_T	CTGATAATGTCGAGTCTCTTGATTCGGG	T		
			Os03g0265700_R1	TTTCATGCTTAGAAAGATGCATTCCCCA			
	Os03g0320400	(C/T)	Os03g0320400_J	CAATGGAAAGTAACCAACACAAAGCAAC	J	Y	70
			Os03g0320400_T	CAATGGAAAGTAACCAACACAAAGCGTT	T		
			Os03g0320400_R1	TGTCACATGATTGGGTAGTCTTCCTCTC			
	Os03g0383800	(T/A)	Os03g0383800_J	GCCGCTGGCGGAGGATGT	J	NA	65
			Os03g0383800_T	CCGCTGGCGGAGGAGCA	T		
			Os03g0383800_R2	GTCCCAACGTCTGGAGGGAGTACTCTG			
	Os03g0758550	(C/G)	Os03g0758550_J	CAGGAAAACTGCACGGTTTTTTTCTCC	J	Y	65
			Os03g0758550_R1	CTGCCTCTTGAGGTCAACCTGGGAC			
			Os03g0758550_T	AGGAAAACTGCACGGTTTTTTTCGTG	T		
			Os03g0758550_R2	AAAAATCCTGCCTCTTGAGGTCAACCTG			
	Os03g0788300	(T/C)	Os03g0788300-b_J	GCCACAGTCAGAGGGCACAACGT	J	N	70
			Os03g0788300-b_T	GCCACAGTCAGAGGGCACAATCC	T		
			Os03g0788300-b_R1	TGAGGAAGCCCCCGCTCCA			
4	Os04g0578200	(T/C)	Os04g0578200_J	CCCTCCCCGCAGCGAATCAGT	J	Y	70
			Os04g0578200_T	CCTCCCCGCAGCGAATCTCC	T		
			Os04g0578200_R1	CTGAGGCTCCGACGCTGCTTCTT			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
4	Os04g0622600	(C/T)	Os04g0622600_J	CCTCTTCCTGTGACGAATTCACCTGAAC	J	N	65
			Os04g0622600_T	CCTCTTCCTGTGACGAATTCACCTGGTT	T		
			Os04g0622600_R1	CGCAACTGGTCTGCAAAGATCTGGTAA			
	Os04g0636500	(G/A)	Os04g0636500_J	GGAACGGCATCGCCACGG	J	NA	65
			Os04g0636500_T	GATGTGGAACGGCATCGCCACTA	T		
			Os04g0636500_R1	GATCTCCTCCAGGAGCGCCCTCT			
5	Os05g0473900	(A/G)	Os05g0473900_J	GGTGAGGCTACCGGCCGTCA	J	Y	70
			Os05g0473900_T	GGTGAGGCTACCGGCCAGCG	T		
			Os05g0473900_R1	CAGCTTCTTGCTAGGCCGCTTGG			
	Os05g0493500	(T/G)	Os05g0493500_J	ACGCCTACTCGCGGCAGCCT	J	Y	65
			Os05g0493500_T	GCCTACTCGCGGCAGCGG	T		
			Os05g0493500_R1	CTCCTTCAGCGCCATCTCAGAGAAGA			
6	Os06g0101000	(G/A)	Os06g0101000_J	GGGGTGGAAACCCAAATGGTACTGG	J	NA	65
			Os06g0101000_T	GGGGTGGAAACCCAAATGGTAGTGA	T		
			Os06g0101000_R1	GGCAAGCTCGCGCTTCATGG			
	Os06g0102100	(A/C)	Os06g0102100_J	CGCTGCTGGAGACCATCAACGA	J	NA	65
			Os06g0102100_T	CGCTGCTGGAGACCATCATGGC	T		
			Os06g0102100_R1	TCTTTTCTACTGGAGCACTGGAGCTGGA			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
6	Os06g0111600	(G/A)	Os06g0111600_J	AGGGCATACCTGGTGCTTAAGAGTAGAG	J	Y	70
			Os06g0111600_T	AGGGCATACCTGGTGCTTAAGAGTAGAA	T		
			Os06g0111600_R1	TTTCAGTTTCAGTTTCACAGGACTCCAA			
	Os06g0120200	(C/G)	Os06g0120200_J	TCGACCTCGGCCTCGCCAC	J	N	65
			Os06g0120200_T	TCGACCTCGGCCTCGCAGG	T		
			Os06g0120200_R1	TGGTAGTACTCGGCCAGGAAGTTCACC			
	Os06g0210400	(C/T)	Os06g0210400_J	AACGAGACGAGACGACGATCACCAC	J	Y	70
			Os06g0210400_T	ACGAGACGAGACGACGATCACGGT	T		
			Os06g0210400_R1	GGGCCTCCTATCCGGTCTCCACTAA			
	Os06g0612950	(G/A)	Os06g0612950_J	CGCCGTACGGCTTCCCGG	J	NA	65
			Os06g0612950_T	CATCCGCCGTACGGCTTCCCTA	T		
			Os06g0612950_R1	CGTCGGAGTTCGACTGGGCG			
7	Os07g0103500	(A/G)	Os07g0103500_J	CACCATCGTGCACCAGCAGCAGTA	J	N	70
			Os07g0103500_T	CCATCGTGCACCAGCAGCAGTG	T		
			Os07g0103500_R1	CCTGTACTCGCCGAAGGTGAAGCTC			
	Os07g0159700	(A/T)	Os07g0159700_J	GCAATTTTAACTGCTCCCTACACAGACA	J	NA	65
			Os07g0103500_T	CCATCGTGCACCAGCAGCAGTG	T		
			Os07g0103500_R1	CCTGTACTCGCCGAAGGTGAAGCTC			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
7	Os07g0184633	(A/G)	Os07g0184633_J	GCGATGCGGAGAGGGCTCGTTATA	J	N	70
			Os07g0184633_T	GATGCGGAGAGGGCTCGTTCAG	T		
			Os07g0184633_R1	CTTCGGCTGCCTCCACGGG			
	Os07g0204400	(C/T)	Os07g0204400_J	TCCCCTCTCAGGAGGCTCTGGGTAC	J	NA	65
			Os07g0204400_T	CCCCTCTCAGGAGGCTCTGGGATT	T		
			Os07g0204400_R1	GGACATGAACATCCTCATCCTGGCA			
	Os07g0623300	(G/A)	Os07g0623300_J	GTCCATATCTATCTCTACTCCGACCCCG	J	Y	70
			Os07g0623300_T	GTCCATATCTATCTCTACTCCGACCCCA	T		
			Os07g0623300_R1	AACTTTTATTGTTGGTGTTGTTGCCTCT			
8	Os08g0189700	(G/A)	Os08g0189700_J	AACAAGGGTGATGTATTCGTATTCCCGG	J	Y	65
			Os08g0189700_T	AACAAGGGTGATGTATTCGTATTCCACA	T		
			Os08g0189700_R1	GTCCGTAAGCGGACAATATTTAGGACAA			
	Os08g0233900	(T/C)	Os08g0233900_J	GGTTGAAGCAGCTATCGCCAAATCACT	J	N	65
			Os08g0233900_T	GGTTGAAGCAGCTATCGCCAAATCATC	T		
			Os08g0233900_R1	CATTGCCAGCTGCCGTTGGTTT			
	Os08g0305300	(A/G)	Os08g0305300_J	AAAATATCAGATGTTGACCTTGCACCAACATAAA	J	NA	65
			Os08g0305300_T	AAAATATCAGATGTTGACCTTGCACCAACATTAG	T		
			Os08g0305300_R1	ATGATGATGAAGAAATAGTTTTCAAGCCTCCAGT			
	Os08g0442000	(A/T)	Os08g0442000_J	GATGCCTGCAGTGGCAGTTTGTGA	J	Y	70
			Os08g0442000_T	AATAGATGCCTGCAGTGGCAGTTTGAAT	T		
			Os08g0442000_R1	GCAGCTGCACGATACTTTTCCACACA			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
8	Os08g0551500	(T/C)	Os08g0551500_J	CACAGCAACTAGTGGTTCCCAAGATGAT	J	Y	65
			Os08g0551500_T	CACAGCAACTAGTGGTTCCCAAGATGAC	T		
			Os08g0551500_R1	CAAAGATTGGCAGATCCTTTAAAAGGCG			
	Os09g0101800	(C/T)	Os09g0101800_J	TCCTGCAAACTACACGATACTACTGGGC	J	NA	65
			Os09g0101800_T	TCCTGCAAACTACACGATACTACTGGGT	T		
			Os09g0101800_R1	TGAATGTGGGAAATGCTAGAATAACATG			
9	Os09g0110200	(A/G)	Os09g0110200_J	CCAGTTTACGGAGGCTCTCACTGTCATA	J	Y	70
			Os09g0110200_T	CAGTTTACGGAGGCTCTCACTGTCAGG	T		
			Os09g0110200_R1	TCATGATATGTGGACTGTGCAAGCGAG			
	Os09g0127800	(T/G)	Os09g0127800_J	CATCAGAGCAAATCAGGACGGCATCT	J	N	65
			Os09g0127800_T	CAGAGCAAATCAGGACGGCATCG	T		
			Os09g0127800_R1	TCTTGCTGCCCACCCTGAGATGA			
	Os09g0131150	(T/G)	Os09g0131150_J	CAAGGGTCCCTCTCACTCTGGCTCAT	J	NA	65
			Os09g0131150_R1	TTTCCTGCGCTCCAATCAAACGAC			
			Os09g0131150_T	AAGGGTCCCTCTCACTCTGGCTCAG	T		
			Os09g0131150_R2	ATCCGATTCCTATGTTTTTCCTGCGCT			
	Os09g0332360	(A/G)	Os09g0332360_J	CGATAATTGCAATGAGTGTCTTTAGCCA	J	Y	70
			Os09g0332360_T	CGATAATTGCAATGAGTGTCTTTACCCG	T		
			Os09g0332360_R1	GGAGTCTTAATGATCCATGATGGCAAGG			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
10	Os10g0104500	(C/A)	Os10g0104500_J	GTGGTCTCCTTCGGGACTGACAAGC	J	Y	70
			Os10g0104500_T	TGGTCTCCTTCGGGACTGACGAGA	T		
			Os10g0104500_R1	GTAGCAGTTGCGTTTGTTATGGGCTTTG			
	Os10g0110600	(G/C)	Os10g0110600_J	CGACGCCGATGCGCTGAAG	J	Y	70
			Os10g0110600_T	GACGCCGATGCGCTGCTC	T		
			Os10g0110600_R1	CTCAAGATGCTGCTCCGGGTGTTC			
	Os10g0112700	(G/A)	Os10g0112700_J	CGCCTCTTCGAAACCCTCAAGCG	J	N	70
			Os10g0112700_T	AAACGCCTCTTCGAAACCCTCAAGAA	T		
			Os10g0112700_R1	CATCACACCGCTCATGGCGAAA			
	Os10g0141900	(T/C)	Os10g0141900_J	CCCGTGATCTCCTTGCATCCCTGT	J	NA	65
			Os10g0141900_T	TCCCGTGATCTCCTTGCATCCCTAC	T		
			Os10g0141900_R1	ACCAGCTGCGGCTATGAGGATATGATC			
	Os10g0334500	(C/T)	Os10g0334500_J	AGGGCAACCACACTCCACAGCAC	J	NA	65
			Os10g0334500_T	AGGGCAACCACACTCCACAGGGT	T		
			Os10g0334500_R1	CGATGGGAAACAGTCATAGCACCCT			
	Os10g0500500	(G/T)	Os10g0500500_J	AACTCTAGCAGCATCTTGGCTGAATTAG	J	N	65
			Os10g0500500_T	AACTCTAGCAGCATCTTGGCTGAAATGT	T		
			Os10g0500500_R1	CTCACAAATTTACCGCTGAGTTCCAGAA			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
11	Os11g0105600	(G/A)	Os11g0105600_J	CGGCTTCAGGCACGCGAAGTAG	J	Y	70
			Os11g0105600_T	CGGCTTCAGGCACGCGAAGTTA	T		
			Os11g0105600_R1	CTGATATGTCATATGCCCAGGCCCAG			
	Os11g0182100	(A/G)	Os11g0182100_J	TCCTCCGGAGCAACGGGAGAA	J	NA	65
			Os11g0182100_T	TCCTCCGGAGCAACGGGAGAG	T		
			Os11g0182100_R1	CTGCCTCCCTGCATGACGTCG			
	Os11g0225300	(A/G)	Os11g0225300_J	CCTGACCTCATTGTTCTCTCAATGCAAA	J	Y	70
			Os11g0225300_T	CTGACCTCATTGTTCTCTCAATGCACG	T		
			Os11g0225300_R1	TCCAATTCGAACAGCAATCTTCTTGAGG			
	Os11g0242700	(G/T)	Os11g0242700_J	GAAGACTTGGAGCTTCTTGGAACGTCG	J	Y	70
			Os11g0242700_T	GGAAGACTTGGAGCTTCTTGGAACGTCT	T		
			Os11g0242700_R1	TCACATTATGTCCATGTAACCATGACAA			
	Os11g0305400	(A/G)	Os11g0305400_J	ATGCCTCGAATTCCTAGCTTCTTCACTA	J	NA	65
			Os11g0305400_T	GCCTCGAATTCCTAGCTTCTTCACCG	T		
			Os11g0305400_R1	TCACCATTGGAGTGACCAGGATTGC			
	Os11g0485900	(C/T)	Os11g0485900_J	AAGTAACCGGCTGTATGGTGCTAGGAAC	J	N	70
			Os11g0485900_T	AAGTAACCGGCTGTATGGTGCTAGGAAT	T		
			Os11g0485900_R1	TTTCCATTTGTCTCGAATTGTCCTCCAA			
	Os11g0606600	(G/A)	Os11g0606600_J	GGAAGTTGCAGAAGACAACTTCAATGCG	J	N	65
			Os11g0606600_T	GGAAGTTGCAGAAGACAACTTCAATGGA	T		
			Os11g0606600_R1	TCTCCAACACAGTCAAAGATCGGACATG			

(APPENDIX 17: continued)

Chromosome	Gene	(Jupiter/Trenasse) SNPs	Primers name	Primer sequence	Allele	Polymorphism	Annealing temperature (°C)
12	Os12g0102400	(G/T)	Os12g0102400_J	TCACCATATGTCCATTTCCCTAACACCG	J	N	70
			Os12g0102400_T	TCACCATATGTCCATTTCCCTAACATTT	T		
			Os12g0102400_R1	CAATTCTCACTGCACCAACTGCTCCA			
	Os12g0119000	(G/T)	Os12g0119000_J	CAATTGCTGGATTCTCCTCATCTCCG	J	N	70
			Os12g0119000_R1	GTAGTCTCCCTCTTCTCTCACACACACA			
			Os12g0119000_T	GCCAATTGCTGGATTCTCCTCATCACTT	T		
			Os12g0119000_R2	AGTCTCCCTCTTCTCTCACACACACACC			
	Os12g0151000	(G/T)	Os12g0151000_J	CCAACCCGGCTTGCAACAAGAAAG	J	Y	70
			Os12g0151000_T	CAACCCGGCTTGCAACAAGAGGT	T		
			Os12g0151000_R1	TGAGGTGTGTGTTTCTTTGTACGAATGC			
	Os12g0225300	(C/T)	Os12g0225300_J	GAAGGGGAACCCGAGCCAGC	J	NA	65
			Os12g0225300_T	GAAGGGGAACCCGAGCCGCT	T		
			Os12g0225300_R1	ACCAACTCCCGCTCTTGCCAGAA			
	Os12g0613200	(A/G)	Os12g0613200_J	TTAACAGGGGCCATTTTCTTATTCTACA	J	Y	70
			Os12g0613200_T	TTAACAGGGGCCATTTTCTTATTCTCGG	T		
			Os12g0613200_R1	GGCGGGTCTATGCTGCATCAGAATT			

[a] = Jupiter specific allele, [b] = Trenasse specific allele, [c] = Not amplified, [d] = Polymorphic, [e] = Non-polymorphic

APPENDIX 18: POLYMORPHIC SSR MARKERS IDENTIFIED BETWEEN TRENASSE AND JUPITER, IN CHROMOSOMES 1, 2, AND 8, DEVELOPED BY USING WHOLE GENOME SEQUENCE DATA

Primer name	Chr.	Motif	No. of repeats	Forward primer	Reverse primer	Position in chromosome (bp)	
						SSR Start	SSR End
Chr1TJ001	1	AT	11	GATGTTTGATCCAGCAGCCT	ATCCAGCGAACTTGAGCAAT	82,261	82,282
Chr1TJ013	1	GA	16	AACAGCGAAAACGCAAACTT	GAGACAGCAAGAAATCCCGA	4,851,731	4,851,762
Chr1TJ024	1	CTT	19	TCGAATTTGCATCCATTTGA	ACGAACTAGAGCATGGGCAC	9,611,474	9,611,530
Chr1TJ030	1	TC	20	AAGAAACTCTCCGCTCCTCC	CCTGAGAGACAAACGCATCA	11,906,704	11,906,743
Chr1TJ032	1	CT	12	GATGGCTGGTGTTGTTGTTG	CAACAGCCCTTGAAGTGTCA	12,451,430	12,451,453
Chr1TJ039	1	TA	15	CGGAAGAGAAGCTCAACACC	GAACTTTGCGGAATAGCGAG	20,319,310	20,319,339
Chr1TJ049	1	AG	12	CCCATCGGATTTATTCTCCA	GGCCATTTTAAAACAAGCACA	24,347,616	24,347,639
Chr1TJ053	1	CGG	8	AGGGTGAGGAGAAAACCCAT	AAAGCAACGAGAGATCCGAA	25,823,698	25,823,721
Chr1TJ088	1	TA	20	CACTGTCACCACAAAGCTGA	CACGAAGCCAAACGTAGTTG	43,133,416	43,133,455
Chr2TJ001	2	TAA	16	GCATGCACTGCAGATACCAA	TTTGCAGCAGAGCAGAAAAA	427,164	427,211
Chr2TJ032	2	TG	17	GACAAGGTGGATACCGGAGA	GACACAGTTGTTCGACCCCT	19,770,679	19,770,712
Chr2TJ045	2	GCT	11	ATTCCACCTCAACTTGCACC	GAGGAGGAGGAGGAGCACTT	26,157,146	26,157,178
Chr8TJ033	8	GAA	27	CAAATGCACAGTTGCGAATC	ACGGAGTACATACCAAGGCG	19,845,666	19,845,746
Chr8TJ045	8	AG	13	TGACCTCACTTCACTTCCCC	CGATGAGCTCTCCACATCAA	22,481,799	22,481,824

APPENDIX 19: PARTIAL SEQUENCE OF 16S RDNA OF RICE-ASSOCIATED BACTERIA

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VITA

Bishnu Kumar Shrestha was born in Shivanagar-4, Chitwan, Nepal. He received his Bachelor's degree in Agriculture Science from the Institute of Agriculture and Animal Sciences, Tribhuwan University, Nepal, in 2005. Then he worked as a research associate in REGARD-Nepal until 2008. He joined Ham's lab at the Department of Plant Pathology and Crop Physiology at Louisiana State University in the summer of 2008 where he received his MS degree in Plant Health in 2011, and continued for his Ph. D. at the same lab.