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A GENETIC STUDY ON THE VIRULENCE MECHANISM OF *BURKHOLDERIA GLUMAE* AND, RICE RESISTANCE TO 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
Hari Sharan Karki
B.S. Institute of Agriculture and Animal Science, 2002
M.S. Louisiana State University, 2010
December 2013

I would like to dedicate this thesis to my parents: Shree Bahadur Karki and Bulu Maya for their constant support, love, efforts and encouragement brought me to the Louisiana State University, USA from Nepal.

ACKNOWLEDGMENTS

First, I would like to thank Dr. Jong Hyun Ham, for being a great advisor who gave me continuous supports for my creativity through-out the study period. He always gave me freedom of thought, good advice, made me work hard and think scientifically. I am thankful to my committee members Dr. Jeffrey W. Hoy, Dr. Lawrence Datnoff, Dr. Clayton Hollier and Dr. Prasanta Subudhi for their guidance and support, which enabled me to develop this dissertation. I would also like to thank present and past lab members, Bishnu Shrestha, Rebecca Melanson, Ruoxi Chen, Surendra Osti, Peng Jingyu, Felix Frances, Inderjit Kaur, Dr. Jung Nam Lee, Mr. Carl Dischler from LSU AgCenter, Rice Research Station (Crowley, LA) and student workers who worked in our laboratory. I am glad to thank Dr. Marc Cohn and late Dr. Milton C. Rush for their inspirational and supportive advices for me. It's my pleasure to thank all the faculty, staffs and students of the Department of Plant Pathology & Crop Physiology for making such a wonderful environment for working.

My most sincere appreciation goes to mom, dad, brothers and sisters. Their love, care and support are the main source of inspiration to achieve this. Needless to say, I give my greatest thanks to the most important three people, my wife Sarala, daughter Sonika and son Sohan, for their love, patience and their inspiration during the entire period. Without their support, I could not even imagine my success. Finally, I offer my admiration and blessing to all of people who directly or indirectly supported me in any respect during the completion of this research.

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ABSTRACT

Burkholderia glumae is a rice pathogenic bacterium that causes bacterial panicle blight. Some strains of this pathogen produce dark brown pigments when grown on the casamino-acid peptone glucose (CPG) agar medium. A pigment-positive and highly virulent strain of *B. glumae*, 411gr-6, was randomly mutagenized with mini-Tn5gus, and the resulting mini-Tn5gus derivatives showing altered pigmentation phenotypes were screened on CPG agar plates to identify the genetic elements governing the pigmentation of *B. glumae*. In this study, several positive and negative regulators, for the pigmentation of *B. glumae* were identified. During this study, a novel two-component regulatory system (TCRS) composed of the PidS sensor histidine kinase and the PidR response regulator was identified as an essential regulatory factor for pigmentation. Notably, the PidS/PidR TCRS was also required for the elicitation of the hypersensitive response in tobacco leaves, indicating the dependence of the hypersensitive response and pathogenicity (Hrp) type III secretion system of *B. glumae* on this regulatory factor. In addition, *B. glumae* mutants defective in the PidS/PidR TCRS showed less production of the phytotoxin, toxoflavin, and less virulence on rice panicles and onion bulbs relative to the parental strain, 411gr-6. In addition, the shikimate pathway genes *aroA* and *aroB* are required for the pigmentation of *B. glumae* in the CPG medium. This study revealed that *aroA* and *aroB* of *B. glumae* are also essential for virulence, growth in M9 minimal medium and tolerance to UV light but not required for the production of toxoflavin. Bacterial panicle blight and sheath blight of rice are the two most important diseases of Louisiana, and most of rice cultivars grown here are susceptible to these diseases. During this study, several rice lines showing superior phenotypes in disease

resistance and agronomic traits were developed through plant breeding techniques. LB-33, a recombinant inbred line derived from Bengal and LM-1, was superior to the parents in terms of the disease resistance to bacterial panicle blight and sheath blight as well as other important agronomic characters associated with high yield. In addition, pre-treatment with non-pathogenic strains of *B. glumae* suppressed the development of bacterial panicle blight and sheath blight disease.

CHAPTER 1
GENERAL INTRODUCTION

1.1. *Burkholderia glumae*

Burkholderia glumae, formerly *Pseudomonas glumae*, was first described as a bacterial pathogen causing grain rot, seedling rot and seedling blight diseases of rice (*Oryza sativa*) in Japan (Goto and Ohata, 1956). Later, these disease symptoms were collectively described as a bacterial panicle blight of rice, and now are reported from major rice producing countries around the world (Nandakumar et al., 2009). *Burkholderia glumae* belongs to the genus *Burkholderia*. *Burkholderia* spp. were previously placed in the genus *Pseudomonas* based on phenotypic definitions. During the early 1970s, rRNA-DNA hybridization analyses showed a lot of genetic diversity among members of the genus *Pseudomonas* (Kersters et al., 1996). Five rRNA homology groups were created based on significant genetic diversities (Palleroni et al., 1973). The genus *Burkholderia* created in 1992, belonged to the rRNA homology group II of *Pseudomonas* and, containing seven different species (*B. caryophylli, B. cepacia, B. gladioli, B.mallei, B. pickettii, B. pseudomallei and B. solanacearum*) (Yabuuchi et al., 1992). Today, the genus *Burkholderia* contains about 60 valid species (Choudhary et al., 2013) which are adapted to a wide range of ecological niches ranging from contaminated soil to plant parts and; the respiratory tract of humans (Coenye and Vandamme, 2003). There are eight different species of *Burkholderia* reported as plant pathogenic species which include *B. ambifaria*, *B. andropogonis*, *B. caryophlli, B. cenocepaica, B. cepacia*, *B. glumae* and *B. plantarii* (Karki, 2010). The genome size of *Burkholderia* is about >8 Mbp, almost double the size of *Escherichia coli* and usually

consists of two to three chromosomes with frequently occurring plasmids (Holden et al., 2004b; Tumapa et al., 2008; Ussery et al., 2009). The important characteristic features of the *Burkholderia* genomes are flexibility and plasticity that enables them to colonize in diverse environmental conditions such as human, plant, soil, and other animal hosts (Chain et al., 2006; Holden et al., 2004a; Nierman et al., 2004). Recently several strains of *B. glumae* were sequenced; the whole genome of *B. glumae* BGR1 was first sequenced in Korea in 2009 and consists of two chromosomes and four plasmids (Lim et al., 2009). Chromosome 1 is 3,906,529 base pairs in size and has 3290 coding sequences, 144 pseudogenes, 3 rRNA operons and 56 tRNAs while chromosome 2 is 2,827,355 base pairs in size and has 2079 coding sequences, 192 pseudogenes, 2 rRNA operons and 8 tRNAs (Lim et al., 2009).

Different plant pathogenic bacteria produce different types of phytotoxins that are toxic to plant cells and support symptom development (Durbin, 1991). The bacterium *B. glumae* produces a phytotoxin known as toxoflavin which is the most important virulence factor for causing rice seedling rot, grain rot and wilting in many field crops (Iiyama et al., 1994; Jeong et al., 2003). It was reported that toxoflavin reduces the growth of leaves and roots of rice seedlings and induces chlorotic symptoms on the panicle (Iiyama et al., 1995). Toxoflavin shows antibacterial, antifungal, herbicidal activities and also toxic to mice (Nagamatsu, 2002). Toxoflavin UV spectrum has a maximum absorbance at 262 and 393 in methanol (=21, 5000) and minimum at 400nm (=5900) (Yoneyama et al., 1998). Toxoflavin is an active electron carrier between NADH, and oxygen and hydrogen peroxide can be produced that may by-pass the chytochrome system (Latuasan and Berends, 1961). As the level of hydrogen peroxide

increases in rice seedlings, different proteins involved in cellular and metabolic processes are down-regulated that play important roles in cell defense and other cellular activites related to photosynthesis and photorespiration, protein synthesis and degradation, signal transduction, and carbohydrate/energy metabolism (Wan and Liu, 2008). Toxoflavin can hinder several cellular and metabolic processes in the plant, and this could be the reason for toxoflavin being an important virulence factor of *B. glumae*. Toxoflavin production phenotype of *B. glumae* can be observed as bright yellow pigment in King's B, Luria Broth and Potato dextrose media. The production of toxoflavin is dependent upon the temperature in which maximum production is at 37°C, and no detectable amount is produced below 25 °C (Matsuda and Sato, 1988). The toxoflavin biosynthesis and transport depends on *tox* operons which is regulated by a transcriptional activator *toxJ* and a LysR type regulator *toxR* (Figure 1[B]) (Kim et al., 2004). ToxR requires toxoflavin as a co-inducer to activate toxoflavin biosynthesis and the transport operon (Kim et al., 2004). The toxoflavin biosynthesis operon consists of five genes (*toxA, toxB, toxC, toxD* and *toxE*) (Shingu and Yoneyama, 2004; Suzuki et al*.,* 2004) whereas the toxoflavin transport operon consists of four genes (*toxF toxG*, *toxH* and *toxI*) (Figure 1[B]) (Kim et al., 2004).

The plant cell wall contains cellulose and hemicelluloses which act as a barrier for the entrance of pathogens. Lipase is considered as a plant cell wall degrading enzyme secreted by many bacterial and fungal pathogens. For fungal pathogens lipase has been well reported as a virulence factor; however for bacterial plant pathogens it has been reported only in a few cases (Rajeshwari et al., 2005).

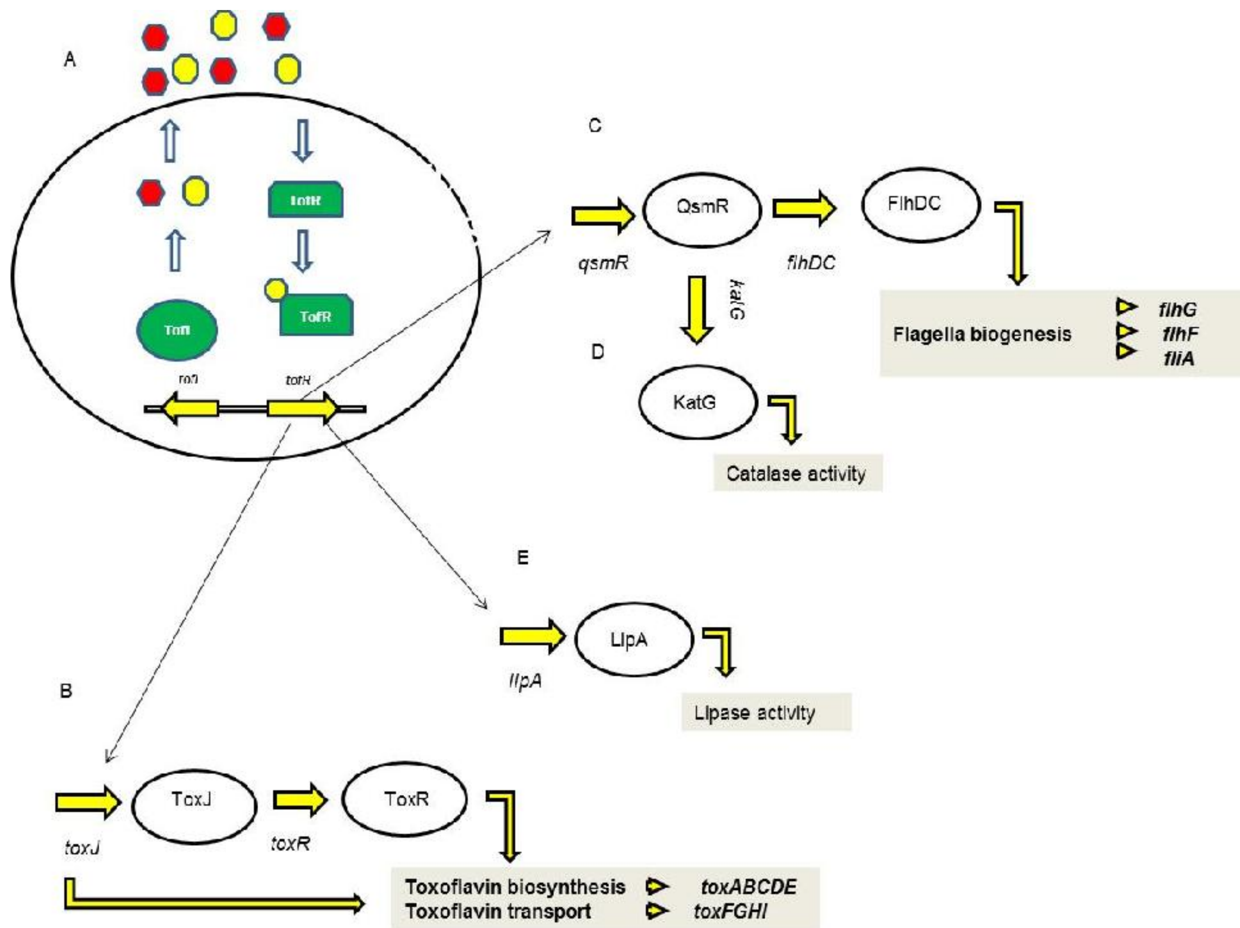

Figure 1.1. A schematic view of the global regulation of virulence by the quorum-sensing (QS) system in *B. glumae*. This figure is adopted from (Chun et al., 2009; Devescovi et al., 2007; Kim et al., 2007; Kim et al., 2004) A) QS in *Burkholderia glumae* consists of *N*-acyl homoserin lactone (AHL) synthase TofI, a LuxI homologue and AHL receptor TofR, a LuxR homologue. TofI synthesizes both N-hexanoyl homoserine lactone (C6-HSL) and N-octanoyl homoserine lactone (C8-HSL) and TofR is the cognate recepotor of C8-HSL. B) Toxoflavin biosynthesis circuit in *B. glumae* needs to bind to the TofR protein by C8-HSL and in turn activates the expression of *toxJ*. Then, ToxJ activates the expression of the LysR-type regulatory protein, ToxR as well as activates the transcription of the toxoflavin biosynthesis operon *toxABCDE* and toxoflavin transport operon *toxFGHI* in a cascade fashion. With a toxoflavin as a coinducer, ToxR can activate both toxoflavin biosynthesis and transport operons. C) QS system activates the expression of IclR-type transcriptional regulator, QsmR then it activates the expression of *flhDC* genes and FlhDC activates the expression of flagella biogenesis genes. D) QsmR activates the expression of the *katG* gene and QsmR is activated by QS. E) QS directly activate the expression of the *lipA* gene which is involved in lipase activity.

For instance, in *Fusarium graminearum*, a fungal pathogen of wheat and maize, secreted lipase is an important virulence factor for causing disease (Voigt et al., 2005). In the rice blight pathogen, *Xanthomonas oryzae* pv. *oryzae* xylanase or lipase plays an important role in its virulence (Rajeshwari et al., 2005). Similarly, secreted lipase is also an important virulence factor of *B. glumae* since lipase defective mutants are non-pathogenic to rice (Devescovi et al., 2007). Lipase is secreted from the outer membrane into the extracellular medium through the type II secretion pathway (Rosenau and Jaeger, 2000). In *B. glumae* the *lipA* mutant does not produce lipase (Devescovi et al., 2007) so the *lipA* gene is necessary for the production of lipase (Figure1[E]). Lipase production in *B. glumae* is enhanced by hexadecane and Tween 80 when supplemented in growth media (BoekeMa et al., 2007).

There are mainly two types of flagellum-mediated motility, direct movement through a liquid, called swimming (Moens and Vanderleyden, 1996), and over a surface as a biofilm, called swarming (Harshey, 1994). Another type of motility called twitching motility mediated by type IV pili, which is described as movement over a smooth surface is also reported from some other bacteria (Mattick, 2002). Bacteria flagella are important organelles for movement because it allows them to relocate at the infection sites of the host. Because of this, flagella are considered as an important organelle for virulence in plant pathogenic bacteria. The regulation system for flagellar biogenesis in bacteria can be broadly categorized in two groups based on their master regulators to activate the expression of flagellum genes. In general, bacteria belonging to the Enterobacteriaceae family have peritrichous flagellum systems and are often regulated by a transcriptional regulator FlhDC (Aldridge and Hughes, 2002; Soutourina and Bertin, 2003) while the

Pseudomonadaceae and Vibrionaceae have polar flagellum systems and are regulated by the sigma 54-dependent NtrC family of transcriptional activators (Arora et al., 1997). However, unlike many members of the Pseudomonadaceae family, *B. glumae*'s flagellum biogenesis genes are regulated by FlhDC (Kim et al., 2007). Briefly, in *B. glumae*, an IclR-type transcriptional regulator called QsmR directly activates the expression of *flhDC*, a regulator of flagella biogenesis genes and FlhDC activates the expression of genes related to flagellum biosynthesis, motor functions and chemotaxis (Figure 1[C]) (Kim et al., 2007). It was reported that *qsmR*, *fliA* and *flhDC* mutants of *B. glumae* are non-motile and toxoflavin sufficient but importantly lost its pathogenicity (Kim et al., 2007). So, toxoflavin production alone is not sufficient for *B. glumae* to cause bacterial panicle blight and flagella dependent motility and chemotaxis are required also for *B. glumae* to infect plant tissue efficiently (Kim et al., 2007).

It is believed that the toxic effect of toxoflavin is due to hydrogen peroxides (Chun et al., 2009). Plants respond to this toxic effect by producing reactive oxygen species (ROS), so it is important for the pathogen to survive under ROS (Levine et al., 1994). Bacteria protect themselves from ROS with the help of superoxide dismutase (SOD), catalases, and alkyl hydro peroxide reductase (Farr and Kogoma, 1991). These enzymes include SODs encoded by *sodA* and *sodB*, catalases encoded by *katE* and *katG*, glutathione synthetase encoded by *gshAB*, and glutathione reductase encoded by *gor* (Farr and Kogoma, 1991). In *B. glumae*, catalase activity is governed by the *katG* gene whose expression is up-regulated by QsmR, a transcriptional activator for flagella-gene expression (Figure1[D]) (Chun et al., 2009). The *katG* mutant of *B. glumae* exhibits less disease severity than wild type indicating that catalase activity plays an

important role in virulence of this pathogen by protecting the bacterial cells from visible lights (Chun et al., 2009).

In addition to these major virulence factors, the hypersensitive response and pathogenicity (Hrp) type III secretion system (Kang et al., 2008) and, PehA and PehB polygalacturonases (Giuliano et al., 2008) were already investigated in respect to their contribution to virulence of this pathogen. It was shown that Hrp type III secretion system of *B. glumae* is required for the full virulence in rice as well as in the colonization of host tissue (Kang et al., 2008). The *hrp* mutants of *B. glumae* still produced toxoflavin so reduced pathogenicity is due to their impaired growth in plant tissues (Kang et al., 2008). *Burkholderia glumae* secretes two very similar endo-polyygalacturonases, PehA and PehB, however neither PehA nor PehB is directly involved in the initial stage of pathogenicity (Giuliano et al., 2008). But the authors predicted that these enzymes could play an important role in providing nutrients to bacterial populations by breaking down the pectin layers of plant cell wall.

Bacteria have a signaling-molecule-mediated cell-cell communication system for sensing and response pathways to control the expression of genes in a population, growth stage dependent manner described as quorum sensing (QS). Many bacteria coordinate their activities through this intercellular communication system in a population dependent manner and the term intercellular communication system is given based on signal molecules (auto-inducers) (Atkinson and Williams, 2009). In bacteria, QS regulates many behaviors including, virulence, symbiosis, biofilm formation, antibiotic production, and (Schauder and Bassler, 2001). Different bacteria utilize different signal molecules for their respective QS system. However, *N*-acyl homoserine

lactones (AHLs), oligopeptides and autoinducer-2 (AI-2) are the major group of signal molecules utilized by bacteria. Mostly AHLs are utilized by Gram negative bacteria (*Agrobacterium*, *Burkholderia*, *Pseudomonas, Vibrio*) (Aguilar et al., 2003; Fuqua et al., 1994; Miller and Bassler, 2001; Quinones et al., 2004) and oligopeptides by Gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*) (Grossman, 1995; Ji et al., 1995) while AI-2 is utilized by both Gram negative and Gram positive bacteria (Surette and Bassler, 1999). The principle behind quorum sensing or cell-cell communication is, when a single bacterium releases signal molecules, its concentration is too low for detection by other bacteria. As sufficient bacterial population are present, the signal molecules concentration reaches a certain threshold level which allows bacteria to sense a critical cell mass and thus bind and activate transcriptional activators that induce the expression of certain genes. In general, the QS system relies on two major components, diffusible signal molecule and a transcriptional activator protein which accumulates in a population level dependent manner and activates the expression of targeted genes. In the genus *Burkholderia*, QS system is well conserved and is homologous to *luxI* and *luxR* QS of *Vibrio fischer* which is a major global regulator system for the production of virulence factors. Particularly in *B. glumae*, QS is mediated by AHL signal molecules and typically consists of *tofI* and *tofR* genetic elements that are homologous to *luxI* and *luxR* that controls the production of most of the known virulence factors such as toxoflavin (Kim et al., 2004), lipase (Devescovi et al., 2007), flagella dependent motility (Kim et al., 2007) and catalase activity (Chun et al., 2009). The gene *tofI* encodes AHL synthases for the production of *N*-hexanoyl-L-homoserine lactone (C6-HSL) *N*-octanoyl-L-Homoserine lactone (C8-HSL) and *tofR* encodes an

AHL synthase receptor where C8-HSL binds and forms a TofR-C8-HSL complex (Figure 1[A]) (Kim et al., 2004). In the known toxoflavin production and transport cascade, TofR-C8-HSL complex activates the expression of *toxJ* and ToxJ activates the expression of *toxR* which in turns activates the expression of both toxoflavin biosynthetic and transport operons (Figure 1[A and B]) (Kim et al., 2004)). Similarly, the TofI/TofR QS system is required for the flagella dependent motility in *B. glumae* which is crucial for its virulence. TofR-C8-HSL complex activates the expression of *qsmR*; QsmR activates the expression of *flhDC* genes and FlhDC activates the expression of flagella biogenesis genes (Figure 1[C]) (Kim et al., 2007). TofI/TofR QS is also involved in the catalase activity of *B. glumae* by activating the expression of *qsmR*; QsmR up-regulates the expression of a catalase gene, *katG* (Figure 1[D]) (Chun et al., 2009). Not much is currently know about how QS regulates lipase activity in *B. glumae*; however it was shown that TofI/TofR QS is involved in lipase activity through the expression of *lipA* gene (Figure 1[E]) (Devescovi et al., 2007). Recently, it was reported that another gene in the inter-genic region of *tofI* and *tofR*, named as *tofM*, positively regulates toxoflavin production and is dependent upon the growth conditions of bacterium of the bacterium (Chen et al., 2012). *tofM* is homologous to *rasM*, a negative regulator of AHL synthase in *Pseudomonas fuscovaginae* (Mattiuzzo et al., 2011) and conserved among many species of *Burkholderia* (Chen et al., 2012).

Burkholderia species are known for having versatile characteristics and variable genomes which are adapted to diverse ecological niches (Coenye et al., 1999). Each species of *Burkholderia* shows wide variation in genome structure within the strain level and an extraordinary adaptability in colonizing both plant and animal hosts (Francis et

al., 2013; Lessie et al., 1996). Recently, it was reported that significant phenotypic variations exist among strains of *B. glumae*, including variation in virulence, pigmentation and antifungal activities as well as genetic variations, and these were detected from DNA fingerprinting analyses by repetitive element sequence-based PCR (rep-PCR) (Karki et al., 2012b). Moreover, considerable amount of genome plasticity and unique genome regions were observed between two genomes of *B. glumae*, 336gr-1 and BGR1 which were isolated from two different environmental conditions and geographical locations (Francis et al., 2013). *Burkholderia glumae* has been isolated from many field crops such as eggplant, hot pepper, perilla, potato, tomato, sesame, and sunflower and showed wilting symptoms of bacterial wilts that is usually caused by *Ralstonia solanacearum* (Jeong et al., 2003). Similarly, the *B. glumae* strain isolated from a human patient having chronic granulomatus disease retained the capability of causing severe bacterial panicle blight in rice (Devescovi et al., 2007). These observations indicate that *B. glumae* is not only a pathogen of rice, but also of many non-traditional field crops and carries the possibility of being a human pathogen (Karki, 2010).

1.2. Bacterial panicle blight of rice

The disease caused by *B. glumae* was first described as a grain rooting, seedling and grain blight of rice in Japan (Goto and Ohata, 1956). Later, the disease was reported from other parts of the world including Asia, North America, Latin America and Africa (Cottyn et al., 2001; Jeong et al., 2003; Shahjahan et al., 2000b; Zeigler and Alvarez, 1989; Zhou, 2013). Louisiana has a long reported history of panicle blighting in rice and this disease was considered to be attributed to abiotic factors such as high

temperatures, water stress or toxic chemicals around plants (Groth et al., 1991). However, during 1996/97 it was reported that panicle blighting of rice was caused by the bacterium *B. glumae* (Shahjahan et al., 2000b). It was also reported that another bacterium, *B. gladioli*, can cause bacterial panicle blight of rice producing similar symptoms (Nandakumar et al., 2009). However, *B. gladioli* is less virulent and isolated less frequently from plants in naturally infected fields than *B. glumae* (Nandakumar et al., 2009). The bacterium, *B. glumae* is seed borne and can survive in seed storage for three years under room temperature (Tsushima et al., 1989). Infected seed is considered the major source of epidemics (Tsushima, 1996).

Bacterial seedling rot caused by *B. glumae* in young growing seedlings is due to the excessive growth of bacteria in the epidermis of pummels (Hikichi, 1993). Once the seedlings are transplanted into the paddy field, *B. glumae* colonizes the upper leaf sheath containing the flag leaf and invades the panicles (Hikichi et al., 1994). After rapid multiplication in the flag leaf, the pathogen forms a linear lesion which has a reddish brown border and a necrotic and gray area in the middle of the flag leaf which then extends to the leaf-blade collar (Hikichi et al., 1994). Afterwards, the developing seeds become blighted, unfilled, aborted, and the panicle branches stay green after the unaffected grain matures. The major visible symptoms of bacterial panicle blight of rice in the field and under greenhouse conditions are spikelet sterility, upward panicles and discoloration of the grains (Figure 1.2) (Nandakumar et al., 2009).

Burkholderia glumae lives on rice plants during the growing season, and the pathogen on the leaf sheath serves as a primary source of inoculum for disease

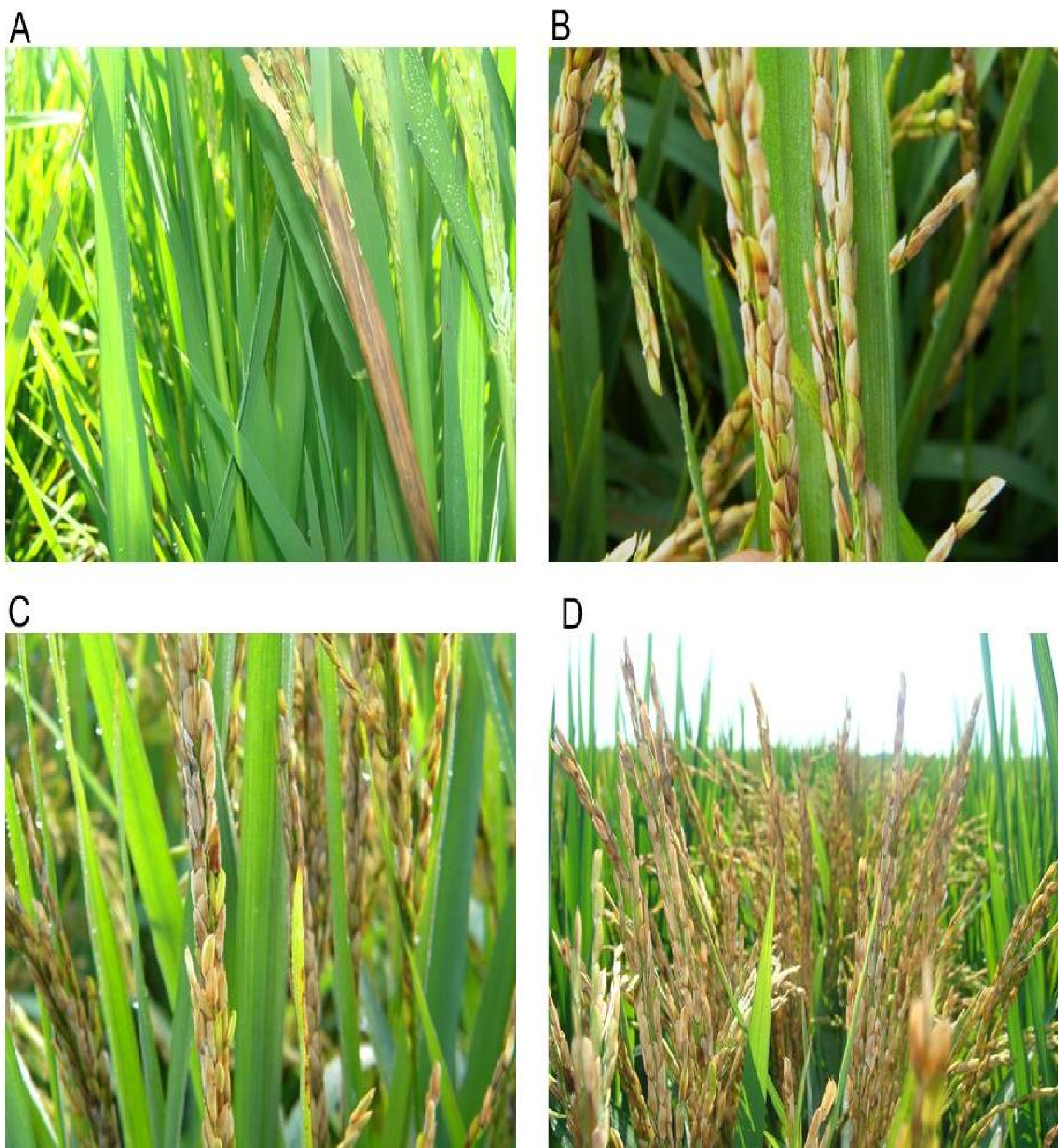

Figure 1.2. Bacterial panicle blight symptoms on rice. A) Grayish lesion developed in the flag leaf sheath. B) Typical panicle blight symptoms with each panicle showing discoloration. C) Secondary BPB infection in panicles. D) Typical symptoms of BPB, in which severely infected panicles remain upright with grains unfilled.

development (Tsushima et al., 1991) (Tsushima et al., 1991; Tsushima and Naito, 1991). *Burkholderia glumae* was isolated from the rice leaf blade only one day after inoculation however was isolated from sheath blades irrespective of sampling time (Tsushima et al., 1991). These findings suggest that *B. glumae* cannot live epiphytically on the leaf blade surface but survives in the leaf sheath latently. *Burkholderia glumae* moves from lower leaves to upper leaves as the rice plant develops (Tsushima et al., 1991). Populations of *B. glumae* on individual leaf sheath were reported to vary from one leaf sheath to another, showing a log normal distribution, and that the population on the upper leaf sheath decreased drastically over time (Tsushima, 1996). It was concluded that the *B. glumae* population in the flag leaf one week before heading is important for disease development (Tsushima, 1996).

A bacterial panicle blight (BPB) epidemic is the collective result of host susceptibility, virulent pathogen density and favorable environmental conditions. Rice is the only host of *B. glumae* causing BPB and susceptibility changes with time. Most of the rice cultivars grown in Louisiana and other southern states are susceptible to BPB; however Jupiter and LM-1 showed some partial level of resistance (Groth et al., 2007; Rush et al., 2007). Rice planted later in the season and fertilized with high nitrogen rates tends to have more disease (Personal communication, Dr. Donald Groth). High night temperatures and humidity is the major environmental factors that cause epidemics of BPB. It was reported that the night temperature above 32 C will result in outbreak of BPB (Nandakumar et al., 2009). Similarly, high relative humidity (RH) >90% at the flowering stage is critical for disease development (Tsushima, 1996; Tsushima, 2011). The percentage of diseased spikelets increases if the rice plants are kept under

high RH, and will be lower if kept under low RH. Moreover, the longer the incubation of diseased panicles in a moist chamber the higher the population of *B. glumae* (Tsushima and Naito, 1991). For the development of BPB, a high inoculum density of a virulent isolate of *B. glumae* is required and disease incidence will increase with an increase of density (Tsushima et al., 1985). *Burkholderia glumae* was recovered $<10^7$ colony forming units (cfu) from symptomless rice panicles and $>10^9$ cfu from visibly diseased grains from one gram fresh weight of leaf sheath during flowering stage (Tsushima and Naito, 1991). The stage of flowering of rice affects the development of BPB. The highest level of disease occurred when the pathogen is inoculated at 0-3 days of flowering; however, disease incidence became very low when the pathogen was inoculated 2 days before or 4 days after flowering (Tsushima et al., 1995). This suggests that BPB has very narrow window for development and that a high relative humidity at this stage is crucial for the disease development. Severely infected panicles served as a source of primary inoculum, and a higher disease severity developed closer to the source of primary inoculum (Tsushima and Naito, 1991). The earlier occurrence of diseased panicles and the higher disease severity of the panicles leads to the development of larger foci (Tsushima and Naito, 1991).

Despite the huge economic importance of BPB in rice producing areas around the world, few studies have been conducted to develop efficient control measures. Oxolinic acid is used as a chemical for controlling seedling rot and grain rot of rice in Japan; however, this chemical is not registered in US (Nandakumar et al., 2009). Pre-treatment of seeds before sowing, and spraying during heading stage of rice with oxolinic acid (OA) significantly reduced the population of *B. glumae*, and showed high

efficacy for the control of seedling rot and grain rot of rice (Hikichi, 1993; Hikichi and Egami, 1995). However, OA resistant *B. glumae* were isolated from OA treated fields (Hikichi et al., 1998). The incidence of seedling rot disease caused by a virulent strain of *B. glumae* was reduced by the pre-treatment of natural non-pathogenic strains of *B. glumae* (Furuya et al., 1991). Recently, the use of bacteriophages to lyse *B. glumae* cells and suppress the seedling rot disease of rice was reported in Japan (Adachi et al., 2012). One bacteriophage (BGPP-Ar), was more effective than existing chemical control method to control the seedling rot and seedling blight diseases of rice (Adachi et al., 2012). Apart from these control measures, no other methods have been reported.

CHAPTER 2
GENETIC BASIS OF DARK PIGMENTS PRODUCTION IN *BURKHOLDERIA GLUMAE*

2.1. Introduction

The bacterial genus *Burkholderia* not only has the capability to adapt to diverse ecological niches but also exhibits remarkable diversity and versatility in extracellular products (Vial et al., 2007). *Burkholderia* spp secrete a wide range of enzymes with proteolytic, lipolytic and hemolytic activities. Several species of *Burkholderia* also produce strain specific toxins, antibiotics, antifungal compounds, siderophores, melanin type pigments and dark pigments (Vial et al., 2007). There are some detail studies regarding the production of melanin type pigment in *Burkholderia* and their role in survival fitness against adverse environments (Zughaier et al., 1999). More specifically, melanin-producing strains of *B. cepacia* that could attenuate the oxidative burst of the human monocyte cell line MonoMac-6 (Zughaier et al., 1999) and melanin from *B. cenocepacia* is responsible for protecting the organism from oxidative stresses (Keith et al., 2007). Melanin is not required for growth and development of the species, but it offers a survival advantage over non-producing strains in adverse conditions, such as exposure to UV radiation, free radicals, and oxidants (Lopez-Serrano et al., 2004). In most cases, the color of melanin is brown or black and it can be produced by a variety of microorganisms, fungi and helminthes (Nosanchuk and Casadevall, 1997).

[1]

This chapter 2 (section 2.2) previously appeared as "[Karki, H. S., Barphagha, I. K., and Ham, J. H. A conserved two-component regulatory system, PidS/PidR, globally regulates pigmentation and virulence-related phenotypes of *Burkholderia glumae*. 2012. Molecular Plant Pathology 13, 785-794. It is reprinted by permission of [John Wiley and Sons—see the permission letter for proper acknowledgment phrase.]"

[1]

There are four classes of melanins, eumelanins, phaeomelanins, allomelanins, and pyomelanins, and that are derived from diverse sources. Eumelanins are derived from quinines and free radicals, phaeomelanins from tyrosine and cysteine, allomelanins from nitrogen-free precursors, and pyomelanins from catabolism of tyrosine through p-hydroxyphenylpyruvate and homogentistate (HGA) (Brandt and Warnock, 2003). Several genera of bacteria including *Aeromonas*, *Bacillus*, *Legionella Pseudomonas,* and *Streptomyces* can synthesize melanin, of which pyomelanin is the most common class synthesized from HGA intermediates (Alviano et al., 2004; Blasi et al., 1995). Similarly, homogentistate (HGA) is the essential precursor for the production of a melanin-like brown pigment in *B. cenocepacia*. It was reported that 4-hydroxyphenylpyruvic acid dioxygenase (*hppD*) activity, is encoded by the *hppD* gene, is necessary for melanin biosynthesis. A *hppD* mutant was resistant to paraquat challenge but sensitive to H_2O_2 (Keith et al., 2007). Synthesis of melanin is regulated by several melanogenic proteins like tyrosinase and tyrosinase related proteins (TRP-1 and TRP-2) and their transcription factors (Jimenez et al., 1991). Tyrosinase is a key enzyme of melanin biosynthesis. It catalyzes three different types of reaction; first is hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), second the oxidation of DOPA to DOPA quinone, and finally oxidation of 5,6-dihydroxyindole (DHI) to indole-quinone (Hearing and Tsukamoto, 1991).

Production of melanin is universal phenomenon for many micro-organisms to adapt to variable environmental conditions (Plonka and Grabacka, 2006). Melanin is reported as a virulence factor in several species of bacteria, and it is hypothesized that, melanin production was an evolutionary achievement needed for the development of for

free living bacteria. *Vibrio* spp. found in free living or parasitic contain both pyomelanogenetic and eu/pheomelanogentic pathways. Sometimes both pathways are active in one organism at the same time. The strains of *V. cholera* found in free living state are generally amelanotic or produce pyomelanin (Kotob et al., 1995). Some of the free living and pathogenic strains of *P. aeruginosa*, *Hypomonas* spp. and *Shewanelia colwelliana* produce pyomelanin (Shivprasad and Page, 1989). Ligiolysin (LLy) is one of the virulence factors of *Legionella pneumophilia* which causes legionnaire's disease (LD) and Pontiac fever, that is responsible for the fluorescent properties and pigment production of the pathogen (Wintermeyer et al., 1991). Interestingly, this protein LLy, is 80% similar to HPPD which is key for melanin synthesis in *Pseudomonas* spp. (Steinert et al., 2001) and *Burkholderia cenocepacia* (Keith et al., 2007). In *B. cepacia*, the production of melanin is related to increased virulence. *Burkholderia* species are known to produce large varieties of extracellular products that are correlated with an ecological diversity (Vial et al., 2007). Most of the studies have been done with members of the Bcc complex, mainly *B. cepacia*. Few studies has been done on *B. glumae* and the production of extracellular enzymes, toxin, antibacterial and antifungal compounds secreted.

Recently, we reported that some strains of *B. glumae* produced dark pigments in casamino acid-peptone-glucose (CPG) medium. The major objective of this study was to understand the genetic basis of dark pigment production in *B. glumae* by using transposon mutagenesis. Based on the above studies of what factors affect the production of dark pigments, enzymes, toxins, antibacterial and antifungal compounds of *B. glumae* that will provide a better understanding of whole genus *Burkholderia*.

A list of enzymes, toxins, antibacterial and antifungal compounds produced by the different species of *Burkholderia* are provided.

- Extracellular enzymes: Here are the lists of extracellular enzymes.
- Protease: A wide range of micro-organisms including the majority of Bcc isolates secrete proteases. These are involved in pathogenicity, regulation of metabolism, gene expression and enzyme modification (Rao et al., 1998).
- Lipase: Many *Burkholderia* spp. including *B. glumae* produce lipases that are involved in the breakage of cell wall components and act as a virulence factor (Devescovi et al., 2007).
- Polygalacturonase: *Burkholderia caryophylli*, *B. cepacia*, *B. gladioli* and *B. glumae* secrete, pectin-degrading enzymes called polygalacturonase, that are involved in invading the pathogen inside host tissues (Goh et al., 2004).
- Phospholipase C (PLC): *Burkholderia ambifaria*, *B. cenocepacia*, *B. multivorans*, *and B. vietnamiensis* isolates from cystic fibrosis (CF) patients secret phospholipase (D'Allicourt Carvalho et al., 2007) that are involved in cleaving the phosphodiester bond of phospholipids.
- Rhamnolipids: These are glycolipidic surface- active molecules produced by several species of bacteria. Rhamnolipids also are secreted by *Pseudomonas aeruginosa*, are involved in the swarming activity (Caiazza et al., 2005) and virulence factors (Zulianello et al., 2006). Rhamnolipids were recently reported to be secreted from a *B. glumae* strain BGR1 (Costa et al., 2011) but their role in virulence has not been investigated.
- Toxins: Here are the lists of toxins.

- Toxoflavin: Toxoflavin, a bright yellow pigment is a major virulence factor of *B. glumae* and is absolutely necessary for causing diseases in rice and other field crops (Jeong et al., 2003; Kim et al., 2004). Toxoflavin has antibacterial, antifungal and herbicidal effect as well as anti-cancer activities (Goh et al., 2004).
- Tropolone: Tropolone is a phytotoxin, non-benzenoid aromatic compound. It is produced by *Pseudomonas* and *Burkholderia* spp. including *B. glumae* and *B. plantarii*.
- Rhizobioxine: This is an enol-ether amino acid that is produced by the plant pathogenic bacterium *B. andropogonis* (Yasuta et al., 1999). Cholorotic symptoms produced by this pathogen are mainly due to the production of this compound in planta.
- Rhizoxin: *Burkholderia rhizoxina* produces rhizoxin that is responsible for rice seedling blight, and is also involved in the inhibition of mitosis and cell cycle arrest (Partida-Martinez and Hertweck, 2007).

❖ Antifungal and antimicrobial compounds: Here are lists of antifungal and antimicrobial compounds.

- Pyrrolnitrin: Pyrrolnitrin is a known antifungal as well as antibacterial compound produced by *B. cepacia* (Hwang et al., 2002) and other species of *Pseudomonas* (Burkhead et al., 1994). Biosynthesis of pyrrolnitrin originates from several steps of the tryptophan synthesis pathway (Kirner et al., 1998).
- Xylocandin Complex: Xylocandin, also known as cepacidines A and B is produced by *B. pyrrocinia* ATCC 39277(Bisacchi et al., 1987) and *B. cepacia* AF2001 (Lee et al., 1994). These families of compounds showed antifungal

activities against *Pythium ultimum* (Lee et al., 2000) and *Sclerotium rolfsii* (Kang et al., 2004).

- Quinoline derivatives: *Burkholderia cepacia* PCII produces several quinoline derivatives. Theses have promising antifungal activity against *Phytophthora capsici*, the cause phytophthora blight of red pepper (Moon et al., 1996).
- Glidobactins: *Burkholderia* sp. K481-B101 and *Burkholderia cepacia* have shown a wide range of inhibitory activities against fungi and yeast as well as antitumor activities due to these enzymes (Shoji et al., 1990).
- CF661: *Burkholderia cepacia* CF66 produces this antifungal compound against soil borne fungi such as *Aspergillus flavus*, *Fusarium oxysporum* and *R*. *solani* (Quan et al., 2006).
- Altericidins: These compounds produced by *B. cepacia* inhibit the conidial germination of *Alternaria kikuchiana* conindia KB-1(Kirinuki et al., 1984).
- Cepacins A and B: *B. cepacia* SC11 783 produces cepacins A and B that show antibacterial activities against *Staphylococci* and other Gram negative microorganisms (Parker et al., 1984).
- Hydrogen cyanide: Hydrogen cyanide is produced by several bacterial species as an inhibitor of cytochrome C oxidase (Blumer and Haas, 2000). The *Burkholderia* sp. strain MSSP produces hydrogen cyanide but its role has not yet been verified (Pandey et al., 2005).

❖ Phenazine: Phenazine, a secondary metabolite, is produced by several species of *Burkholderia*, *Pectobacterium* and *Pseudomonas* etc. This metabolite contributes to the ecological fitness as well as pathogenicity (Pierson and

Pierson, 2010) and is known for antibacterial, anti-tumor and anti-parasitic activities (Laursen and Nielsen, 2004).

- Phytohormones: Along with other genera (*Acetobacter, Azospirrrillum*, *Bacillus* and *Pseudomonas*), *Burkholderia* belongs to the plant growth promoting rhizobacteria (PGPR) group (Dobbelaere et al., 2003). This group of bacteria can promote the growth of plants by synthesis of phyto-hormones, mineralization, enhancing the uptake of nutrients, N_2-fixation etc. *Burkholderia vietnamiensis* was first reported as a N_2 fixing bacteria from the genus *Burkholderia* (Gillis et al., 1995). After wards other spp. of *Burkholderia* were reported to be involved in N_2 fixation (Burkhead et al., 1994). Several strains of *B. cepacia* and *B. vietnamiensis* isolated from rhizosphere can produce auxins and enhance the plant growth (Cornish and Page, 1995).

2.2. Materials and Methods

2.2.1. Bacterial culture and DNA manipulation

The bacterial strains and plasmids used this study are listed in Table 3.2 (Chapter 3). *Escherichia coli* and *B. glumae* strains were routinely grown in Luria Bertani (LB) broth media (Sambrook and Russell, 2001) at 37 and 30°C. KB and CPG agar media (Schaad et al., 2001) were used for testing toxoflavin and pigment production by *B. glumae*, respectively. Antibiotics were included in the media as necessary at the following concentration: ampicillin (100 µg/ml), gentamycin (20 µg/ml), kanamycin (50 µg/ml), and nitrofurantoin (100 µg/ml). Standard protocols (Sambrook and Russell, 2001) were used for general DNA manipulation procedures, including extraction, restriction digestion, ligation, PCR, and agarose gel electrophoresis. DNA

sequencing was performed by either the DNA sequencing facility (Gene lab) at the LSU Veterinary School (Baton Rouge, Louisiana, USA) or Macrogen Inc. (Seoul, Korea). Oligonucleotides for PCR and DNA sequencing were purchased from Bioneer Inc. (Alameda, CA, USA). Transformation of bacterial cells with plasmid DNA was made by either electroporation at 200 /1.5 kV using a GenePulser (Bio-Rad Laboratories, Hercules, CA, USA) or triparental mating (Figurski and Helinski, 1979).

2.2.2. Random mutation of *B. glumae* with mini-Tn*5gus*

Overnight cultures of *B. glumae* strain 411gr-6 and *E. coli* S17-1 pir (pUT:::mini-Tn*5gus*) were mixed in 3:1 ratio (v/v), and then 1 ml of the bacterial mixture was centrifuged at 13000 rpm for 1 min in a microcentrifuge tube. Following centrifugation, the supernatant was discarded and the pellet was dissolved in 50 µl LB broth, spotted on LB plates and incubated overnight at 30°C. The mated bacteria were then resuspended in 1 ml of LB broth, and 100 µl aliquots of the bacterial suspension were spread on CPG agar plates containing nitrofurantoin and kanamycin. After 2 days of incubation at 30°C, pigment-deficient or over sufficient mutants were screened on the basis of their pigmentation phenotypes on CPG agar medium.

2.2.3. Identification of genes disrupted by mini-Tn*5gus*

The Flanking sequences of mini-Tn*5gus* integrated into the mutant genomes were amplified following the method developed by Kwon et al. (Kwon and Ricke, 2000). Briefly, the Y-linker having cohesive end of *Nla*III digestion was made by annealing two oligonucleotides, 5’-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACATG-3’ and 5’-TGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT-3’. This Y-linker was then

ligated to the genomic DNA of a mutant digested with *Nla*III. The ligated DNA was subjected to PCR with the Y-linker primer, 5'-CTGCTCGAATTCAAGCTTCT-3', and the Tn*5* specific primer, 5'-GGCCAGATCTGATCAAGAGA-3', under the following reaction cycle: 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 58°C for 1 min, and 70°C for 1 min, and the final extension at 70°C for 5 min. A Y-linker for *Pst*I digestion made with two oligonucleotides, 5'-TTTCTGCTCGAATTCAAGCTTCTAACGATGTACGGGGACACTGCA-3' and 5'-GTGTCCCCGTACATCGTTAGAACTACTCGTACCATCCACAT-3' was used for mutants that could not identified with *Nla*III digestion scheme. The amplified PCR products from this procedure were purified using a QuickClean 5M PCR Purification Kit (GenScript, Piscataway, NJ, USA) and sequenced for identification of genes mutated by min-Tn5*gus* insertion. To identify the mutated genes of the screened *B. glumae* mutants, the flanking regions of the inserted mini-Tn*5gus* were amplified using a previously developed technique (Kwon and Ricke, 2000), sequenced, and then BLAST searched against the genome sequence of the fully sequenced *B. glumae* strain, BGR1 (Lim et al., 2009).

2.3. Results

We used transposon mutagenesis to identify genetic elements required for the pigmentation of *B. glumae* strain 411gr-6. Then the mutants showing altered pigmentation on CPG agar medium plates were compared to the parental strain and screened for further study. With this procedure, I screened about 30,000 mutants and 50 mutants showing no, less and over production of pigment were selected for further study. All of the mutant derivatives showing altered pigment production phenotypes

were confirmed at least twice in CPG agar plates. Disrupted genes were identified by sequencing the flaking regions of mini-Tn*5gus* insertion sites in eighteen mutants (Table 2.1). Among these pigment-deficient mutant LSUPB112 and LSUPB115 were found to be disrupted by insertion of mini-Tn*5gus* in an open reading frame (ORF) encoding a putative sensor histidine kinase (SHK) (Table 2.1). The other mutants showing no pigment production, LSUPB114 and LSUPB116, were found to have mini-Tn*5gus* insertion in ORFs encoding a putative 3-phosphoshikimate 1-carboxyvinyltransferase (EC 2.5.1.19) and a putative 3-dehydroquinate synthase (EC 4.2.3.4), respectively (Table 2.1). Interestingly, five mutants showing no pigment production LSUPB464, LSUPB465, LSUPB466, LSUPB468 and LSUPB470 had mini-Tn*5gus* inserted in the open reading framing encoding a putative LuxR family sensor regulator homolog TofR, a receptor of AHL signal molecules (Table 2.1). In addition, another mutant showing no pigment production, LSUPB467, was found to have mini-Tn*5gus* insertion in ORFs encoding a transcriptional regulator, AsnC family protein (Table 2.1). The mutant showing reduced pigments production than the parental strain 411gr-6, LSUPB118 and LSUPB119 and their disrupted ORFs were predicted to encode a quinoprotein glucose dehydrogenase and a succinate dehydrogenase iron-sulfur subunit, respectively (Table 2.1). The other mutants LSUPB461 and LSUPB463 showed reduced pigment production had the mini-Tn*5gus* inserted in the open reading framing encoding a succinylarginine dihydrolase and glycosyl transferase respectively. Another group of mutant showing over production of pigments compared to parental strain 411gr-6, LSUPB121, LSUPB122, LSUPB123 and LSUPB462 had mini-Tn*5gus* inserted in the ORFs encoding ATP-dependent Clp protease ATP-binding subunit clpA (Table 2.1).

Table 2.1. Description of mutated genes in the mini-Tn*5gus* derivatives of *B. glumae* 411gr-6 showing altered pigment production phenotypes.

Strain name	Gene ID of mutated gene	Gene name[a]	Putative product / function	Pigment production
LSUPB112 LSUPB115	bglu_1g00490	(Karki et al., 2012a)	Sensor histidine kinase / Signal perception and transduction	No
LSUPB114	bglu_1g08780	*aroA* (Duncan et al., 1984)	3-phosphoshikimate 1-carboxyvinyltransferase / Shikimic acid pathway	No
LSUPB116	bglu_1g03040	*aroB* (Millar and Coggins, 1986b)	3-dehydroquinate synthase / Shikimic acid pathway	No
LSUPB118	bglu_2g12650	*Gdh* (Cleton-Jansen et al., 1988)	Quinoprotein glucose dehydrogenase / Glucose metabolism	Reduced
LSUPB119	bglu_2g08260	*sdhB* (Darlison and Guest, 1984)	Succinate dehydrogenase iron-sulfur subunit / Tricarboxylic acid (TCA) cycle	Reduced
LSUPB461	bglu_1g10140	*astB* (Tocilj et al., 2005)	Succinylarginine dihydrolase/ Arginine catabolism	Reduced
LSUPB462 LSUPB121 LSUPB122 LSUPB123	bglu_1g28810	*clpA* (Gottesman et al., 1990)	ATP-dependent Clp protease ATP-binding subunit clpA/ Degradation of unfolded or abnormal proteins	Over
LSUPB463	bglu_1g06540	*wbdN* (Hayashi et al., 2001)	Glycosyl transferase/Glycosidic bond formation	Reduced
LSUPB467	bglu_1g00540	*asnC* (Kölling et al., 1988)	Transcriptional regulator, AsnC family/Cellular metabolism	No

(Table 2.1 continued)

Strain name	Gene ID of mutated gene	Gene name [a]	Putative product / function	Pigment production
LSUPB464 LSUPB465 LSUPB466 LSUPB468 LSUPB470	bglu_2g14470	*tofR* (Kim et al., 2004)	LuxR family sensor regulator/ Quorum sensing	No

[a]: All the listed gene names except *pidS* and *tofR* are from original studies with *E. coli*.

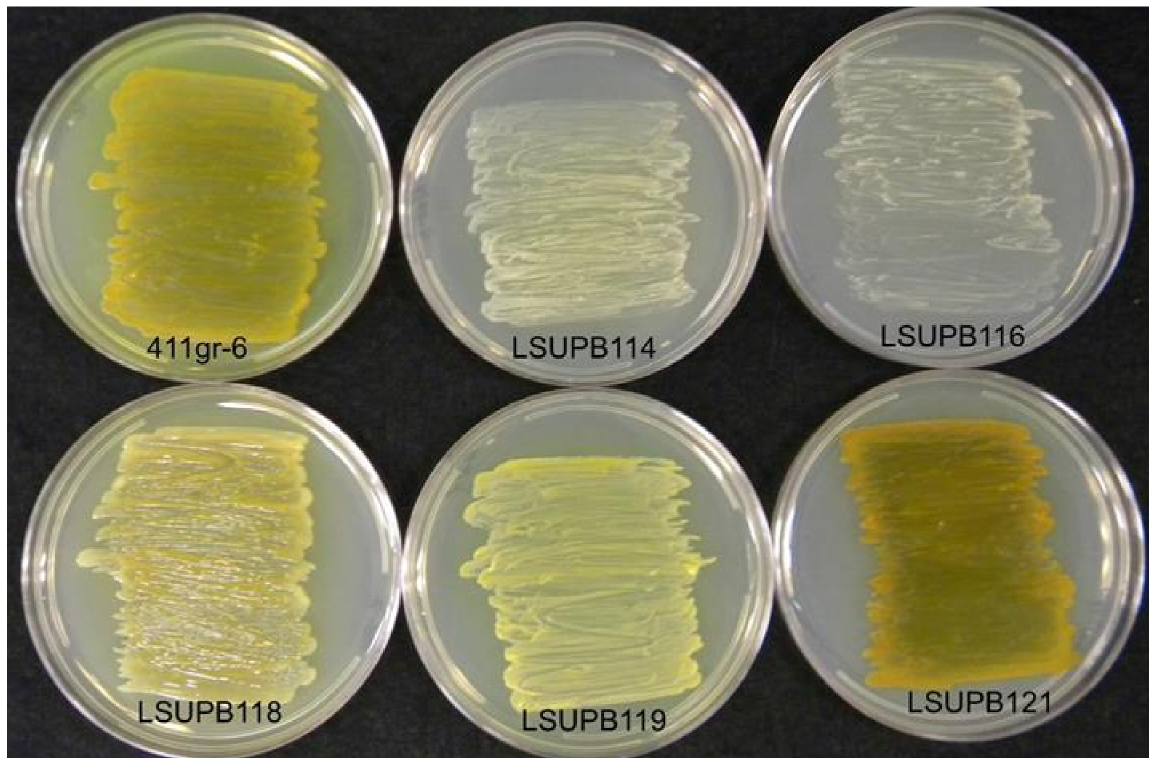

Figure 2.1. Pigmentation phenotypes of *B. glumae* strain 411gr-6 and its mutant derivatives. The pigment production of mutants were compared with wild type 411gr-6 and classified as no pigment (LSUPB114 and LSUPB116), reduced pigment (LSUPB118 and LSUPB119) and over production of pigment (LSUPB121). *B. glumae* strains were streaked on CPG plates and incubated at 30 °C for 48 h.

2.4. Discussion

Random transposon mutagenesis has been widely used to identify the genetic elements underlying the virulence mechanisms of different plant pathogenic bacteria. In this study, we used a mini-Tn*5gus* transposon to investigate the genetic basis of pigment production by *B. glumae* strain 411gr-6. We predicted some obvious mini-Tn*5gus* insertion bios during this study since five *tofR* and four *clpA* mutants were found. However, ten genes were identified as being involved in the pigment production phenotype of *B. glumae* strain 411gr-6. Among identified genes, some were already known to be related to the pathogenesis of this pathogen. So, it is of interest to know how pigment production and pathogenesis is related in *B. glumae*.

To identify the different genetic elements underlying the altered pigment production, pigment production phenotypes were categorized in three groups: 1) no pigment production 2) reduced pigment production and 3) over pigment production. In the category of mutant showing no production of pigments, two mutants LSUPB112 and LSUPB115 where mini-Tn*5gus* was inserted at two different locations within the ORF encoding putative sensor kinase of two component regulatory systems (TCRSs). Based on the initially observed pigment-deficient phenotypes of LSUPB112 and LSUPB115, the putative genes for the SHK and the RR were named ***pidS*** (***pi****gment-****d****eficient* ***S****HK*) and ***pidR*** (***pi****gment-****d****eficient* ***R****R*), respectively. In many prokaryotes, TCRSs play a pivotal role in signal perception and transduction for a wide range of cellular functions involved in metabolism, development, and pathogenesis (Laub and Goulian, 2007). This is the first report of TCRSs composed of PidS histidine kinase and the PidR response regulator being identified as essential regulatory systems for any type of phenotypes in

B. glumae. Similarly, LSUPB114 and LSUPB116 with mini-Tn*5gus* insertions in ORFs encoding two putative metabolic enzymes for the shikimate pathway (3-phosphoshikimate 1-carboxyvinyltransferase and 3-dehydroquinate synthase respectively), and these mutants were failed to produce pigments. Interestingly, both of these enzymes are components of the shikimic acid pathway (Duncan et al., 1984; Millar and Coggins, 1986a), suggesting that this metabolic pathway produces important precursor(s) for pigment production in *B. glumae*. Biosynthesis of pyrrolnitrin originates from several steps of the tryptophan synthesis pathway (Kirner et al., 1998). Two pigment deficient mutants had transposon insertion in genes in the aromatic compound synthesis pathway.

We identified five different pigment deficient mutants LSUPB464, LSUPB465, LSUPB466, LSUPB468 and LSUPB470 and these mutants had mini-Tn*5gus* inserted in the open reading framing encoding a putative LuxR family sensor regulator homolog TofR. The *tofR* is a co-gene of quorum sensing system of *B. glumae* which functions as a receptor for homoserine lactone. The quorum sensing system in *B. glumae* is a global regulatory system that controls most of the known virulence factors such as toxoflavin (Kim et al., 2004), lipase (Devescovi et al., 2007), flagella dependent motility (Kim et al., 2007) and catalase activity (Chun et al., 2009). It is very interesting to know that involvement QS in pigment production phenotype of *B. glumae*. In addition, another mutant showing no pigment production, LSUPB467 was found to have mini-Tn*5gus* insertion in ORFs encoding a transcriptional regulator, AsnC family protein. Proteins belonging to the Lrp/AsnC are widely distributed in prokaryotes, including bacteria and archaea (Charlier et al., 1997). Most of the genes that belong to the Lrp/AsnC regulon

are widely involved in the transport, degradation and biosynthesis of amino acids while few proteins also are involved in the production of porins, pili, sugar transporters and nucleotide transhydrogenases (Newman and Lin, 1995). The mutant LSUPB467 was also defective in toxoflavin production (data not shown). Based on this findings, understanding how the transcriptional regulatory, AsnC family protein regulates both toxoflavin and pigment production in *B. glumae* might be useful in developing novel ways to control this pathogen.

Meanwhile, four mutants LSUPB118, LSU119, LSUPB461 and LSUPB463, showed partial defects in pigmentation. LSUPB118 had a mini-Tn*5gus* insertion in a *gdh* homolog encoding a quinoprotein glucose dehydrogenase, while LSUPB119 had a *sdhB* homolog encoding a succninate dehydrogenase iron-sulfur subunit. Interestingly, both quinoprotein glucose dehydrogenase and succinate dehydrogenase are involved in electron transport for the oxidative phosphorylation producing ubiquitol by reducing ubiqinone during their enzymatic reactions (Elias et al., 2001; Hagerhall, 1997). We speculate that reduction power created by these oxidoreductases might contribute to pigment production in *B. glumae*. Similarly, other mutants showing reduced pigment phenotypes are LSUPB461 and LSUPB463 which had mini-Tn*5gus* insertion in *astB* homolog encoding succinylarginine dihydrolase and *wbdN* homolog encoding glycosyl transferase, respectively. Succinylarginine dihydrolase is the second enzyme of the arginine succinyltransferase pathway leading to the synthesis of arginine (Tocilj et al., 2005). Since arginine is an energy rich amino acid that can supply carbon, nitrogen and energy to various bacteria in different environment, understanding how arginine biosynthesis pathway is related to pigment production in *B. glumae* might help in better

understanding of physiology of this bacterium. Another enzyme, glycosyl transferase, is involved in biosynthesis of a variety of oligosaccharides, polysaccharides and glycoconjugates and mediates wide range of functions from structure and storage to signaling (Tarbouriech et al., 2001). Several QS and glycosyl transferase mutants are deficient in pigment production. Interestingly, both of these enzymes are part of signaling pathway in bacteria, so understanding of how QS and glycosyl transferase are involved in pigment production might help understanding a virulence related phenotype in *B. glumae.*

Another group of mutants, showing over production of pigments compared to the parental strain 411gr-6 were LSUPB121, LSUPB122, LSUPB123 and LSUPB462. These had a mini-Tn*5gus* inserted in the ORFs encoding ATP-dependent Clp protease ATP-binding subunit clpA. Clp is a protease composed of two units, ClpA and ClpP, that degrades casein and other proteins in the presence of ATP (Hwang et al., 1987; Katayama-Fujimura et al., 1987). Two subunits ClpA and ClpP are functionally different proteins and products of separate genes. Clp protease degrades protein into a large number of acid soluble peptides in a process completely dependent of ATP (Gottesman and Maurizi, 1992). Interestingly, our results showed that Clp protease is a negative regulator of pigment production in *B. glumae*, so Clp protease is involved in degradation of pigment, which might be useful for developing control measures.

In conclusion, I developed a lab protocol of transposon mutagenesis for *B. glumae* based on pigment production phenotype. I also report a genome-wide identification of genes related to pigment production this bacterium. I screened about thirty thousand mutants and fifty mutants were selected for further study. I uncovered

ten genes related to dark pigment production, of which most of them were previously unknown in any distinguishable phenotypes of *B. glumae*. I hope this study provide a new insight into the genetic determinants and regulation mechanisms of pigment production in *B. glumae*.

CHAPTER 3
ROLE OF TWO COMPONENT REGULATORY SYSTEM IN VIRULENCE OF *BURKHOLDERIA GLUMAE*

3.1. Introduction

To survive against fluctuating environmental conditions, bacteria have to sense and response to surrounding environments accordingly. They use their two-component signal transduction systems to sense, respond, and adapt to a wide range of environments, stressors, and growth conditions (Skerker et al., 2005). These systems are well known and characterized as one of the key signaling modalities in the bacterial kingdom, as well as being present in many other fungi, slime molds, and plants (Stock et al., 2000). Two component regulatory systems (TCRSs) primarily consists of membrane-integrated histidine kinases (HK) and a cytoplasmic response regulator (RR). HK perceives a wide range of signals from environments that includes temperature, nutrients, pH or presence of toxic compounds, sugars, peptides, antibiotics, and quorum-sensing signals (Laub and Goulian, 2007; Stock et al., 2000). HK also auto-phosphorylates on a conserved histidine residue (Laub and Goulian, 2007). The phosphoryl group is then transferred to a conserved aspartate residue of a cognate response regulator. The RR phosphorylates within the receiver domain and leads to the activation of an output domain that ensures the change of physiology of the

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This chapter 3 (section 4.2, 4.3 and 4.4) previously appeared as "[Karki, H. S., Barphagha, I. K., and Ham, J. H. A conserved two-component regulatory system, PidS/PidR, globally regulates pigmentation and virulence-related phenotypes of *Burkholderia glumae*. 2012. Molecular Plant Pathology 13, 785-794. It is reprinted by permission of [John Wiley and Sons—see the permission letter for proper acknowledgment phrase.]"

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bacterium by altering gene expressions (Laub and Goulian, 2007). The response regulator has been reported to bind DNA and function as a transcription factor due to its phosphorylation properties (Nikolskaya and Galperin, 2002). Interestingly, there are other types of output domains also that enable their response regulators to mediate protein–protein interactions or to perform enzymatic functions (Falke et al., 1997). Many HKs are bi-functional and possess both phosphorylation and phosphatase activity of their cognate RR. Phosphatase activity enables HK to dephosphorylate RRs (Lois et al., 1993). The bi-functional HKs are commonly present in phospho-transfer pathways where they need to shut down quickly. A variant of the two-component system is the phospho-relay system where a hybrid HK auto-phosphorylates, transfers the phosphoryl group to an internal receiver domain, instead to a separate RR protein. The histidine phosphotransferase (HPT) receives the phosphoryl group and subsequently, transfer to a terminal RR, which can induce the desired response (Hoch and Varughese, 2001; Varughese, 2002). HKs of both prokaryotes and eukaryotes share similar types of basic signaling components with diverse sensing domain and highly conserved kinase core that has a unique fold, distinct from that of the Ser/Thr/Tyr kinase superfamily (Stock et al., 2000). Input signals to sensing domain modulate the overall activities of kinase domain. HKs undergo an ATP-dependent auto-phosphorylation in kinase core of conserved His-residue. Then a phosphoryl group from the phospho-HK is transferred to a conserved Asp-residue in its regulatory domain. So, the control of TCRs is largely dependent upon the ability of HK to regulate the phosphorylation state of the downstream RR. Generally, HK usually have an N-terminal ligand-binding (sensing) domain and a C-terminal kinase domain. The ligand-binding domain varies from one HK

to another in length and amino acid sequences, indicating a detection of different environmental stimuli. The kinase domain shows higher sequence conservation and contains invariant histidine residues with short stretches of conserved amino acids. However, the larger HKs consist of five or six structurally and functionally unique domains. Despite the diversity, HKs can be roughly divided into two classes: named orthodox and hybrid kinases (Alex and Simon, 1994). Most of the orthodox HKs are divided into a periplasmic N-terminal sensing domain and a cytoplasmic C-terminal catalytic region that is designated as the kinase core which is best illustrated in *E. coli* EnvZ protein (Khorchid et al., 2005). Hybrid kinases are found in some prokaryotic and most all eukaryotic systems. Hybrid kinases contain multiple phosphodonor and phosphoacceptor sites and use multistep phosphorelay schemes. *E. coli* ArcB is a well-known representative of most hybrid kinases that is composed of two N-terminal trans-membrane regions followed by a kinase core and a second His-containing region termed as a His-containing phosphotransfer (HPt) domain (Ishige et al., 1994). A kinase core is the characteristics feature of the HK family which is composed of a dimerization domain and an ATP/ADP-binding phosphotransfer or catalytic domain (Stock, 1999). The length of the kinase core is about 350 amino acids and it is responsible for binding ATP and directing kinase transphosphorylation. The conserved His substrate is the central feature of the H boxis that is the part of the dimerization domain. A small number of two-component systems contain HPt domains. HPt domains are about 120 amino acids in length and are part of hybrid kinases in prokaryotes, whereas in eukaryotes, they are found as separate proteins. HPt domains contain a His residue which enable them to participate in phosphoryl transfer reactions.

In most of the prokaryotes, RRs are the terminal component of signal transduction systems that catalyzes the phosphoryl transfer from the phospho-His of the HK to a conserved Asp in its own regulatory domain. Many of the RRs consist of two or more domains named as a conserved N-terminal regulatory domain and a variable C-terminal effector domain. Most of the RRs act as transcription factors with DNA binding effector domains. The regulatory domain of RRs has three activities: first to phosphorylate HKs and transfer a phosphoryl group to Asp residue. Second, they catalyze auto-dephosphorylation and the third is regulation of effector domains (West and Stock, 2001). The regulatory domain often called a receiver domain also can be found in hybrid HKs. In this case, it is neither physically connected with an effector domain nor does have regulatory role in effector domain functions (West and Stock, 2001). The regulatory domain, of RR is well represented by chemotaxis protein CheY of *Salmonella typhimurium* that contains a cluster of conserved Asp residues, which bind to Mg^{2+} and form the active site for phosphoryl transfer (Sanders et al., 1989). There is great diversity in the effector domain of RR in respect to both structure and function. The majority of effector domains have DNA-binding activities that function as an activator or repressor of specific genes. OmpR, is a well-characterized member of the largest subfamily of RRs that functions as both an activator and repressor to regulate differentially the expression of the *ompC* and *ompF* genes that encode outer membrane porin proteins (Kenney, 2002; Pratt et al., 1996).

Interestingly, the number of TCRs differs greatly in different bacteria, in some cases they may contribute 2.5% of the genome (Mizuno et al., 1996). The number of TCRs in the prokaryote genome is positively correlated with the genome size and is

roughly proportional to the square of the total number of genes (Ulrich et al., 2005). Bacteria adopted for diverse environmental conditions and having diverse metabolic activities encode more TCRs than microorganisms adopted for uniform habitat (Beier and Gross, 2006). Till now, there have been more than 4,000 TCRs reported form 145 sequenced bacterial genome (Ulrich et al., 2005). There are a number of paralogous gene families of HKs and RRs that share significant homology at both sequence and structural level. These similarities between signaling proteins suggest the possibility of cross-talk (communication between pathways) between different pathways. In general, cross-talk between distinct pathways could be detrimental and should be kept minimal in order to detect stimuli effectively. However, sometimes it is beneficial for organisms to undergo cross-talk to detect diverse signals. Since, some bacteria can have up to as many as 200 two component systems, they need tight regulation to prevent unwanted cross-talk (Laub and Goulian, 2007). The availability of the complete genome sequence of *Burkholderia glumae* strain BGR1 has allowed us to identify two component proteins throughout the genome. In *B. glumae*, 11 HKs and 34 RRs are present (Wheeler et al., 2001). While in eukaryotes, TCRs are limited in numbers. For example *Saccharomycens cerevisicae* (yeast) contain only one phosphorelay system involved in osmoregulation (Maeda et al., 1994). While the pathogenic fungus *Candida albicans* contain at least two HKs that are involved in osmo regulation and hyphal development (Beier and Gross, 2006). Interestingly, TC proteins have been reported from plants such as *Arabidopsis thalianas* (Chang et al., 1993) and tomato (Yen et al., 1995) where they regulate ethylene-mediated fruit ripening.

Many pathogenic bacteria encounter different micro-environments during their infection cycles, and for successful infestation to the host, they need to cope with changing environments. Their ability to cope with different niches inside and outside hosts is meditated by TCRSs, which can therefore play important roles in virulence mechanisms of bacteria (Groisman and Mouslim, 2006). Similarly, pathogenic bacteria produce and secret virulence factors such as toxins, proteases, lipases and other factors involved in motility, adherence, colonization, biofilm formation and survival. In many cases, these virulence or virulence related factors are directly or indirectly under the control of TCRSs. In many cases, the role of TCRSs in bacterial pathogenicity is poorly understood. The role of TCRSs in bacteria may act as a regulatory to metabolic activities rather than a change in the expression of specific virulence factors (Beier and Gross, 2006). However, there are several well-studied TCRSs controlling pathogenicity of phyto-pathogenic bacteria. For example, *Erwinia carotovora* subsp. *carotovora* controls the virulence determinant with at least four different TCSs, named ExpS-ExpA (Eriksson et al., 1998; Frederick et al., 1997), PehR-PehS (Flego et al., 2000), PmrA-PmrB (Hyytiainen et al., 2003) and GacA-GacS (Cui et al., 2001). The ExpS-ExpA TCRS is required for the activation of the gene responsible for secretion of cell wall degrading enzymes. Similarly, PehR-PehS responds to extracytoplasmic levels of Ca and Mg which is required for the transcriptional activation of the endopolygalacturonase gene, as well as for virulence. PmrA-PmrB TCS is involved in controlling bacterial response to external pH and iron as well as production of extracellular enzymes that are crucial for bacterial virulence. GacA-GacS system controls the genes responsible for extracellular enzymes and harpin through global regulatory rsmB RNA. VirA/VirG TCRs

of *Agrobacterium tumefaciens* are involved in the expression of the vir region that is necessary for T-DNA transfer to plants in response to phenolic compounds secreted by wounded plants (Gelvin, 2006; Winans et al., 1994). In *Xanthomonas campestris* pv. *Campestris*, RavS/RavR, TCRs co-regulate virulence genes expression with quorum sensing (He et al., 2009). ColR/ColS, the two-component regulatory system of *Xanthomonas citri* subsp. *citri* is necessary for the expression *hrp* genes and other virulence factors such as, biofilm formation, catalase activity and LPS production (Yan and Wang, 2011). Recently we published that PidS/PidR, TCRSs of *B. glumae* is involved in attenuation of virulence by abolishing dark-pigments, reduction of toxoflavin and hypersensitive related phenotypes. Some of the important TCRSs present in bacteria are listed in Table 3.1.

The objective of this research is to study the role of a novel two-component regulatory system (TCRS) composed of the PidS sensor histidine kinase (SHK) and the PidR response regulator (RR) in pigmentation, hypersensitive response (HR) on tobacco and other virulence related phenotypes of *B. glumae*.

3.2. Materials and Methods

3.2.1. Bacterial culture and DNA manipulation

The bacterial strains and plasmids used this study are listed in Table 3.2. *Escherichia coli* and *B. glumae* strains were routinely grown in Luria Bertani broth (LB) media (Sambrook and Russell, 2001) at 37 and 30°C. KB and CPG agar media (Schaad et al., 2001) were used for testing toxoflavin and pigment production by *B. glumae*, respectively. Antibiotics were included in the media as necessary at the following concentrations: ampicillin (100 µg/ml), gentamycin (20 µg/ml),

Table 3.1. Known two-component regulatory systems present in bacteria

Organism	TCRS	Regulation or effect of inactivation	Reference
Escherichia coli	BarA/UvrY	Increased sensitivity to H_2O_2	(Nagasawa et al., 1992)
Salmonella enterica	BarA-SirA	Reduced motility	(Teplitski et al., 2003)
Erwinia carotovora	ExpS-ExpA	Pectate lyase, polygalacturonase, cellulase	(Eriksson et al., 1998)
Vibrio cholerae	ArcA-ArcB	Virulence regulator gene *toxT*	(Sengupta et al., 2003)
Organism	TCRS	Regulation or effect of inactivation	Reference
Pseudomonas spp	GacS-GacA	secondary metabolites and extracellular enzymes	(Heeb and Haas, 2001)
Pseudomonas fluorescens BL915	LemA/ GacA	Pyrrolnitrin, chitinase, cyanide, 2-hexyl-5-propyl-resorcinol, exoprotease	(Gaffney et al., 1994)
Pseudomonas viridiflava PJ-08-6A and SF312A	RepA/RepB	Pectate lyase, exoprotease, fluorescent siderophores, alginate	(Liao et al., 1994)
Salmonella enterica	BarA/ SirA	Reduction of swarming motility, reduction of gastroenteritis in bovine model, loss of type III secretion apparatus, loss of invasion endocytosis of epithelial cells	(Johnston et al., 1996; Mukhopadhyay et al., 2000)
Xanthomonas oryzae pv. *oryzae*	RaxR/RaxH	AvrXa21 activity	(Burdman et al., 2004)
P. syringae	CorS/CorR	Synthesis of coronatine, chlorosis	(Sreedharan et al., 2006)
E. amylovora	HrpX/HrpY	Type III secretion system	(Wei et al., 2000)

kanamycin (50 µg/ml), and nitrofurantoin (100 µg/ml). Standard protocols (Sambrook and Russell, 2001) were used for general DNA manipulation procedures, including extraction, restriction digestion, ligation, PCR, and agarose gel electrophoresis. DNA sequencing was performed by either the DNA sequencing facility at the LSU veterinary school, (Baton Rouge, Louisiana, USA) or Macrogen Inc. (Seoul, Korea). Oligonucleotides for PCR and DNA sequencing were purchased from Bioneer Inc. (Alameda, CA, USA).

Table 3.2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
B. glumae		
336gr-1	A virulent strain showing a pigment-deficient phenotype, Nit^R	This study
411gr-6	A virulent strain showing a pigment-proficient phenotype, Nit^R	This study
LSUPB112	A *pidS*::mini-Tn*5gus* derivative of 411gr-6, Nit^R, Km^R	This study
LSUPB115	A *pidS*::mini-Tn*5gus* derivative of 411gr-6, Nit^R, Km^R	This study
LSUPB133	A *pidR*::$pKNOCK_{Gm}$ derivative of 411gr-6, Nit^R, Gm^R	This study
LSUPB225	A *pidR*::$pKNOCK_{Km}$ derivative of 411gr-6, Nit^R, Km^R	This study
LSUPB302	A *pidR* deleted mutant derivative of 411gr-6, Nit^R	This study
Escherichia coli		
DH5	*SupE44* D*lac*U169 (f80 *lacZ*D*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Life Technologies

Table 3.2 Continued

Strain or plasmid	Description	Reference or source
XL1-Blue MR	*(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
<u>Plasmids</u>		
pUT::miniTn*5gus*	A suicide vector carrying mini-Tn*5gus*, Ap^R, Km^R	(de Lorenzo et al., 1990) (Fouts et al., 2002)
pKNOCK$_{Gm}$	A suicide vector, R6K *ori*, Gm^R	(Alexeyev, 1999)
pKNOCK$_{Km}$	A suicide vector, R6K *ori*, Km^R	(Alexeyev, 1999)
pBBR1MCS-5	A broad host range vector, Gm^R	(Kovach et al., 1995)
SuperCos1	A cosmid vector, Ap^R, Km^R	Stratagene
pSC-A-amp/kan	A PCR cloning vector, Ap^R, Km^R	Stratagene
pCL126	A cosmid clone harboring the *pidR/pidS* locus, Ap^R	This study
pPidi-1	A clone of *pidR* internal region in pKNOCK$_{Gm}$, Gm^R	This study
pPidi-2	A clone of *pidR* internal region in pKNOCK$_{Km}$, Km^R	This study
pPidRS-1	A subclone of pCL126 for the 3.9-kb *Hind*III/*Bgl*II fragment harboring both *pidR* and *pidS* in pBBR1MCS-5, Gm^R	This study
pPidRS-2	A subclone of pCL126 for the 3.9-kb *Hind*III/*Bgl*II fragment harboring both *pidR* and *pidS* in pBBR1MCS-2, Km^R	This study
pPidS	A *pidS* clone in pBBR1MCS-5 carrying the 3.3-kb *Eco*RI/*Spe*I fragment from pPidRS-2, Gm^R	This study
pPidS-1	A derivative of pPidS added with the 0.5-kb upstream region of *pidR*, Gm^R	This study

Transformation of bacterial cells with plasmid DNA was made by either electroporation at 200 /1.5 kV using a GenePulser (Bio-Rad Laboratories, Hercules, CA, USA) or triparental mating (Figurski and Helinski, 1979).

3.2.2. Generation of *pidR*::pKNOCK mutants (LSUPB133 and LSUPB225)

This work was done by Indergit Kaur and me. An internal region of *pidR* was initially amplified using DNA primers RR-int-F and RR-int-R (Table 3.3) and following PCR conditions (Table 3.4). The resultant PCR product was cloned into suicide vectors, $pKNOCK_{Gm}$ and pKNOCKKm (Alexeyev, 1999), generating pPidRi-1 and pPidRi-1. *Escherichia coli* S17-1 pir (Simon et al., 1983) was used for maintaining pKNOCK vectors and pPidRi-1 and pPidRi-2. pPidRi-1 *glumae* 411gr-6 via triparental mating with *E. coli* DH5 , which carries the helper plasmid, pRK2013 (Figurski and Helinski, 1979), and *pidR* mutants were selected on Luria Bertani (LB) agar containing nitrofurantoin and gentamycin (for LSUPB133) or nitrofurantoin and kanamycin (for LSUPB225). The mutation of *pidR* in LSUPB133 and LSUPB225 was confirmed by diagnostic PCR.

3.2.3. Generation of *pidR* deletion mutant (LSUPB302)

About 500 bp upstream and downstream of *pidR* were amplified using the primers (RR-UPF and RR-UPR and RR-DWNF and RR-DWNR respectively with desired restriction site added (Table 3.3) and PCR condition listed on Table 3.4. The PCR products were cloned into the PCR cloning vector, pSC-A-amp/kan, using a StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacture instructions resulting in pSC:: Upstream and pSC:: Downstream. First pSC:: Upstream was cloned to pKNOCKGm (Alexeyev, 1999) with the digestion of *BamHI* and *Spe*I generating pKNOCKGm::Upstream. The pSC:: Downstream was

digested with *BamHI* and *SmaI* and sub-cloned to the pKNOCKGm::Upstream generating pKNOCKGm::Upstream + Downstream. The fragment from pKNOCKGm::Upstream + Downstream was digested with *NotI* and *SpeI* and cloned to pKKSacB vector. *Escherichia coli* S17-1 pir (Simon et al., 1983) was used for maintaining pKKSacB vector. *E. coli* HB101, which carries the helper plasmid, pRK2013 (Figurski and Helinski, 1979), was used for the triparental mating to introduce pKNOCKGm::Upstream + Downstream to the virulent strain of *B. glumae*, 411gr-6. The selected conjugant colonies (single cross over mutants) from LB/Km/Nitro medium were inoculated into LB broth, and grown overnight at 30 °C. The overnight culture was then spread on LB agar plates containing 35% sucrose and incubated at 30°C for about a week. The colonies were picked and transferred to LB and LB/Km plates. Colonies that grew on LB plate but not on LB/Km plate were selected as mutants. Genomic DNAs of the mutants were extracted and the deleted region from the genome was confirmed by PCR with primers RR-whole-F and RR-whole-R (Table 3.3) and PCR conditions (Table 3.4).

3.2.4. Quantification of toxoflavin

Extraction and quantification of toxoflavin was done following the previously developed protocol (Iiyama et al., 1995) with some modifications. Briefly, an overnight culture of *B. glumae* in LB was washed twice with an equal volume of fresh LB broth and resuspended in 1/10 volume of LB broth. Then, 20 μl of the bacterial suspension (ca. 5 X 10^8 cells) was spread on a KB agar plate with three replications followed by incubation at 37°C for 48 h. Bacterial cells grown on the KB agar plates were then removed by flooding with sterile H_2O and the remaining KB agar media were cut into

small pieces. Toxoflavin diffused in the chopped KB agar media was extracted with an equal volume of chloroform. After the chloroform was completely evaporated under a fume hood, the residues were dissolved in 1 ml of aqueous 80% methanol and diluted five times in distilled water. Then absorbance was measured at 260 nm for each sample using a spectrophotometer (Biomate 3, Thermo Scientific, Pittsburgh, PA, USA).

Table 3.3. Primers used in this study

Primers Name	Primer sequence (5'to 3')	Amplified region	Purpose	Reference
RR-UP-F	ACTAGTACAAACCCGTAG	Upstream *pidR*	Deletion mutation	This study
RR-UP-R	GGATCCGAAACCTGCTTGTTC			
RR-DWN-F	GGATCCGCGATCGAAGTCTAC	Downstream *pidR*	Deletion mutation	This study
RR-DWN-R	GCGGCCGCGTAGGCGTAATG			
RR-whole-F	CCGCTGTACAATCAGCAATG	*PidR*	Confirmed deletion mutation	This study
RR-whole-R	TCGAACACGTAGGCGTAATG			
HK-int-F	GTTGTCCTCCACCACGATCT	*PidS*	Screening of cosmid library	This study
HK-int-R	CTGTCGAACCAGTTGCTGTC			
RR-int-F	AAGTGCGTCAGATGGTCT	*pidR*	Insertion mutation	This study
RR-int-R	AACTCGTGATCCTCGACCTG			

Restriction sites are manually introduced in primers: ACTAGT (*spe*I), GGATCC (*Bam*HI) and GCGGCCGC (*NotI*).

Table 3.4. PCR and electrophoresis conditions for tests conducted in this study

Test	PCR master-mix	PCR condition	Gel electrophoresis
Upstream of *pidR* amplification	25 µL volume: 1 µl of template DNA, 2.5 µl of GeneAmp® 10X PCR Buffer I, 1.0 µl each of primers RR-UP-F and RR-UP-R , 0.5 µl of dNTP mix, 1 µl of *Taq* polymerase	Initial denaturation at 95°C for 2 min; 29 cycles of denaturation at 94°C for 1 min , annealing at 50°C for 30 sec, and extension at 72 °C for 40 sec ; and final extension at 72°C for 7 min.	0.8% agarose gel
Downstream of *pidR* amplification	25 µL volume: 1 µl of template DNA, 2.5 µl of GeneAmp® 10X PCR Buffer I, 1.0 µl each of primers RR-DWN-F and RR-DWN-R , 0.5 µl of dNTP mix, 1 µl of *Taq* polymerase	Initial denaturation at 95°C for 2 min; 29 cycles of denaturation at 94°C for 1 min , annealing at 55°C for 30 sec, and extension at 72 °C for 40 sec ; and final extension at 72°C for 7 min.	0.8% agarose gel
Whole *pidR* amplification	25 µL volume: 1 µl of template DNA, 2.5 µl of GeneAmp® 10X PCR Buffer I, 1.0 µl each of primers RR-whole-F and RR-whole-R, 0.5 µl of dNTP mix, 1 µl of *Taq* polymerase	Initial denaturation at 95°C for 2 min; 29 cycles of denaturation at 94°C for 1 min , annealing at 55°C for 30 sec, and extension at 72 °C for 2 min ; and final extension at 72°C for 7 min.	0.8% agarose gel
Internal *pidS* amplification	25 µL volume: 1 µl of template DNA, 2.5 µl of GeneAmp® 10X PCR Buffer I, 1.0 µl each of primers HK-int-F and HK-int-R, 0.5 µl of dNTP mix, 1 µl of *Taq* polymerase	Initial denaturation at 95°C for 2 min; 29 cycles of denaturation at 94°C for 1 min , annealing at 55°C for 30 sec, and extension at 72 °C for 40 sec ; and final extension at 72°C for 7 min	0.8% agarose gel
Internal *pidR* amplification	25 µL volume: 1 µl of template DNA, 2.5 µl of GeneAmp® 10X PCR Buffer I, 1.0 µl each of primers RR-int-F and RR-int-R, 0.5 µl of dNTP mix, 1 µl of *Taq* polymerase	Initial denaturation at 95°C for 2 min; 29 cycles of denaturation at 94°C for 1 min , annealing at 50°C for 30 sec, and extension at 72 °C for 40 sec ; and final extension at 72°C for 7 min	0.8% agarose gel

3.2.5. Hyper sensitive response (HR) elicitation

Burkholderia glumae cells grown on LB agar media overnight at 37°C were resuspended in 10 mM $MgCl_2$ and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (ca. 2.5 X 10^8 colony forming units (cfu/ml). The bacterial suspension was infiltrated with a needle-less syringe into the fully expanded leaves of 10 to 12 month-old tobacco plants grown in a greenhouse. HR was observed at 18 h after infiltration.

3.2.6. Virulence assay with rice panicles

Bacterial suspensions for inoculation were prepared with the same method used for HR tests except that the bacterial cells were resuspended in sterile tap water instead of 10 mM $MgCl_2$ and their concentrations were adjusted to OD_{600} = 0.1 (ca. 5 X 10^7 cfu/ml). The rice variety Trenasse was grown in a greenhouse and sprayed with the bacterial suspensions at 20 – 30% heading stages. Two days after the first inoculation, a second inoculation was made in the same way and disease severity was evaluated 10 days after inoculation. Disease severity on rice panicles was determined by the following scale (Shahjahan et al., 2000a) with at least ten replications: No disease = 0, 1–10% symptomatic area = 1; 11–20% symptomatic area = 2, 21–30% symptomatic area = 3; 31–40% symptomatic area = 4, 41–50% symptomatic area = 5; 51–60% symptomatic area = 6, 61–70% symptomatic area = 7, 71–80% symptomatic area = 8, >81% symptomatic area = 9.

3.2.7. Virulence assay with onion bulb scales

Virulence phenotypes of *B. glumae* strains were also determined with the onion assay (Jacobs et al., 2008; Karki et al., 2012b). Briefly, scales of onion bulbs were cut into pieces (ca. 10 cm^2) with a sterile razorblade and placed in a wet chamber. Bacterial

suspensions of *B. glumae* strains were prepared with the same method for HR tests except that the bacterial concentrations were adjusted to OD_{600}= 0.2 (ca. 1 X 10^8 cfu/ml). Five μl of the suspension containing c. 5 X 10^5 cells were applied to a 2 mm-slit made on the center of each onion bulb scale with a micropipette tip, and the inoculated onion scales were incubated at 30°C in incubator for 48 h. The virulence of each *B. glumae* strain was determined by measuring the macerated area.

3.2.8. DNA constructs for genetic complementation tests

To generate pPidRS-1/pPidRS-2, a cosmid library of the genome of *B. glumae* 336gr-1, another highly virulent *B. glumae* strain, was screened by PCR using a *pidS* specific primer set (Table 3.3) to identify cosmid clones containing *pidS*. One of the screened cosmid clones, pCL126, was digested with *Hind*III and *Bgl*II to get the 3.9 kb fragment that contains both *pidR* and *pidS*. This fragment was then subcloned into a broad host range vector, pBBR1MCS-5 or pBBR1MCS-2 (Kovach et al., 1995), generating pPidRS-1 or pPidRS-2, respectively (Table 3.2). To generate pPidS-1, pPidRS-2 was digested with *Eco*RI and *Spe*I to obtain the 3.3-Kb fragment that includes *pidS* but not *pidR*. This fragment was subcloned into pBBR1MCS-5 using the same restriction sites, generating pPidS. To ensure the expression of *pidS* in pPidS, the *cis* elements that may be required for the transcription of the predicted *pidRS* operon were placed in front of *pidS* with the following procedure: the 507-bp upstream region of the *pidR* coding sequence was amplified with the primer set (Table 3.3) and subsequently cloned into pSC-A-amp/kan using a StrataClone PCR Cloning Kit (Stratagene). The resultant PCR clone was digested with *Eco*RI and the *Eco*RI-cut fragment containing the *pidR* upstream sequence was then ligated to the EcoRI-cut pPidS, generating

pPidS-1. The right orientation of the *pidR* upstream region in front of *pidS* in pPidS-1 was confirmed by a series of diagnostic restriction digestion.

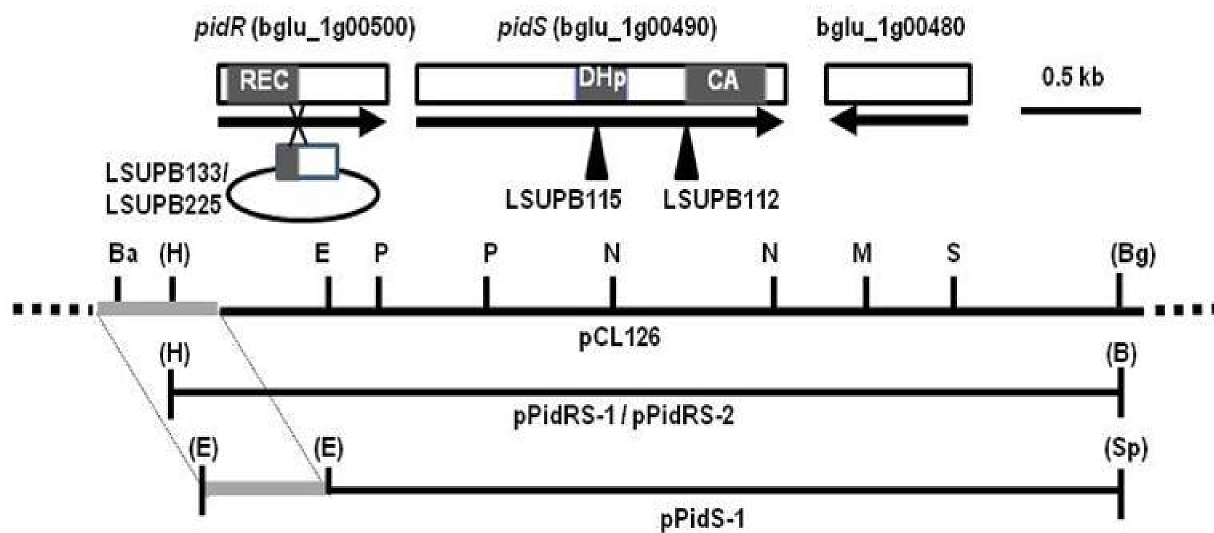
3.3. Results

3.3.1. Identification of genes involved in the pigmentation of *B. glumae*

The detail procedures and results for identification of genes involved in production of pigment by *B. glumae* were described in the previous (Chapter 2). Two of these pigment-deficient mutant derivatives, LSUPB112 and LSUPB115, were found to be disrupted by the insertion of mini-Tn5gus in an open reading frame (ORF) encoding a putative SHK (Figure 3.1).

3.3.2. Identification of a novel TCRS that controls the pigmentation of *B. glumae*

In LSUPB112 and LSUPB115, mini-Tn5gus was inserted at different genomic locations within an ORF encoding a putative SHK gene, indicating that the two mini-Tn5 derivatives of *B. glumae* are two independent mutants of the same SHK gene (Figure 3.1). This identified SHK gene corresponds to 'bglu_1g00490' of the BGR1 genome and forms a putative operon with the ORF located upstream which encodes a putative RR (bglu_1g00500) (Figure 3.1). Based on the initially observed pigment-deficient phenotypes of LSUPB112 and LSUPB115, the putative genes for the SHK and RR were named *pidS* (pigment-deficient SHK) and *pidR* (pigment-deficient RR), respectively. To determine the functionality of *pidR*, its ORF was disrupted through a single homologous recombination with its internal region (Figure 3.1). The derivatives of 411gr-6 generated from this procedure, LSUPB133 (Figure 3.1 and Table 3.2), also showed pigment-deficient phenotypes, like LSUPB112 and LSUPB115 (Figure 3.2[A]).



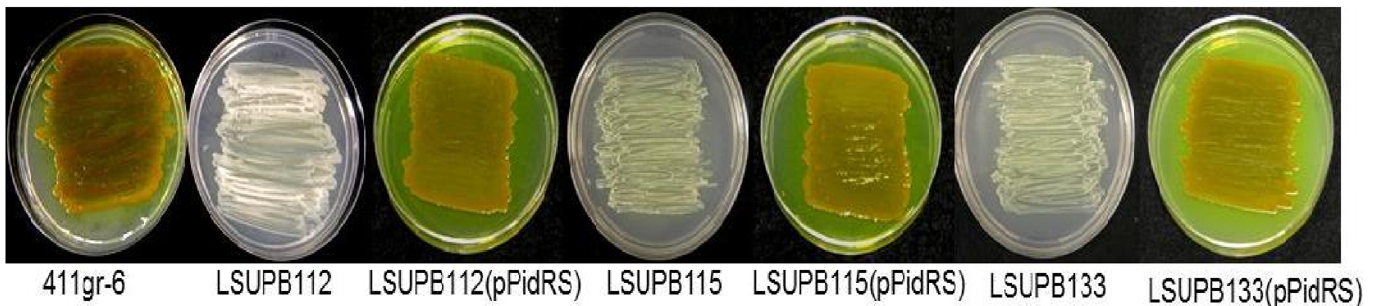
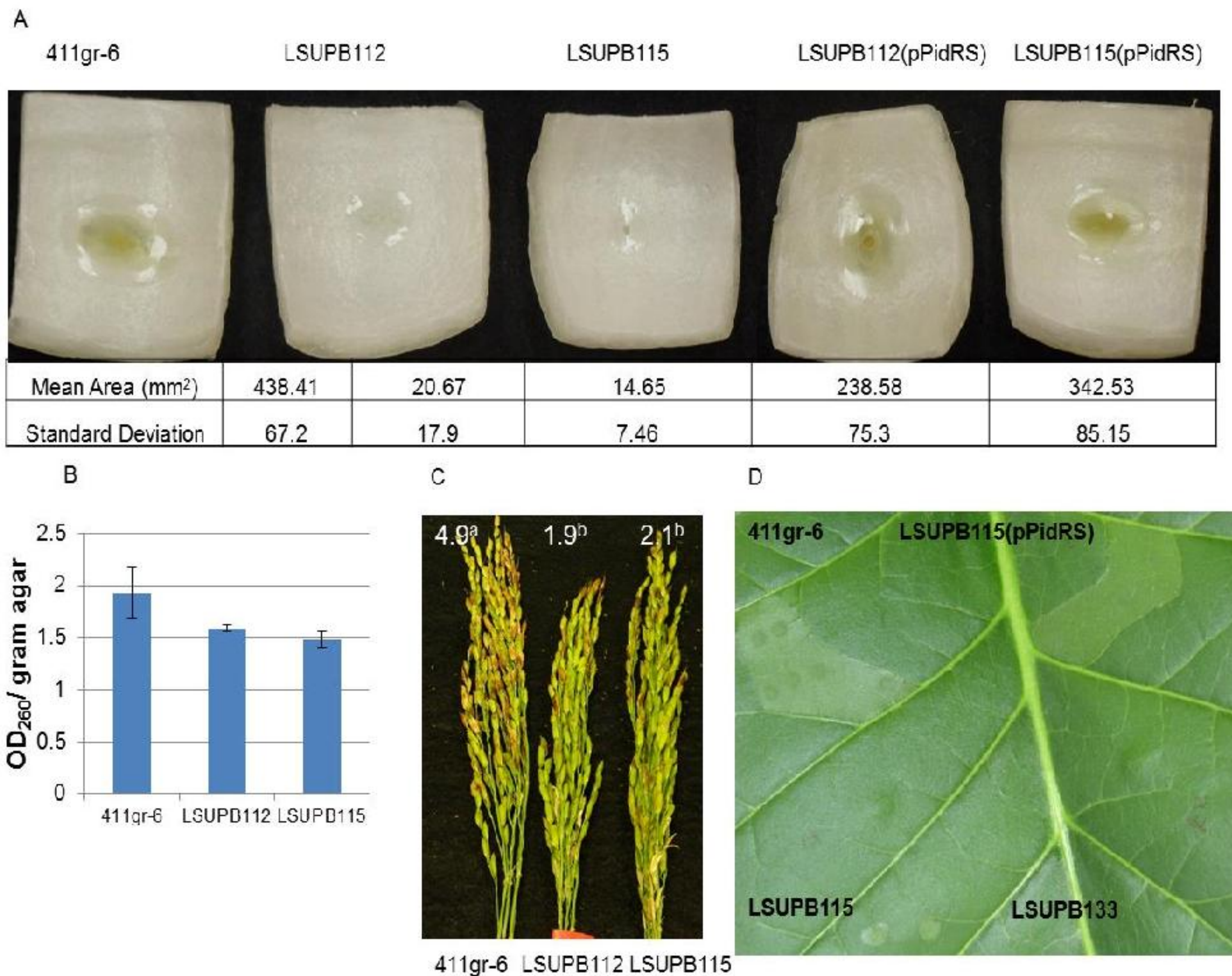


Figure 3.1. A physical map of the *pidS* and *pidR* genes as well as their clones, and the pigment production phenotypes of *pidS* and *pidR* mutants. This cartoon is based on the sequence information of the *B. glumae* BGR1 genome (GenBank: CP001503.2). ORFs are symbolized by rectangles and arrows, and their corresponding locus tags. Black triangles indicate the positions of mini-Tn*5gus* in the mutant strains, LSUPB112 and LSUPB115. Restriction sites in the map are represented by Ba, *Bal*I; H, *Hind*III; E, *EcoR*I; P, *Pst*I; N, *Not*I; M, *Mlu*I; S, *Sma*I: Bg, *Bgl*II; B, *BamH*I; and Sp, *Spe*I. Restriction sites used for subcloning of *pidS* and *pidR* are shown in parentheses. pCL126 is a cosmid clone harboring the *pidR/pidS* locus. The broad host range vectors for pPidRS-1 is pBBR1MCS-5 (Gm^R) (Kovach et al., 1994). Pigmentation phenotypes of virulent *B. glumae* strain, 411gr-6, and *pidS*$^-$ (LSUPB112 and LSUPB115) and *pidR*$^-$ (LSUPB133) derivatives of 411gr-6 as well as the complemented mutants with wild type pPidRS-1 clone (Mel1 on CPG agar plates.

However, it cannot be ruled out that the pigment-deficient phenotypes of the *pidR* mutants are caused by a polar effect of the *pidR* mutation on the downstream gene, *pidS*. To determine whether pidR alone is required for pigment production, the *pidR* mutant, LSUPB225, was tested for complementation with plasmids carrying either *pidS* only (pPidS-1) or both *pidR* and *pidS* (pPidRS-1) (Figure 3.1). Pigmentation of LSUPB225 was restored by pPidRS-1, but not by pPidS-1, indicating that *pidR* is also required for pigmentation, like pidS. pPidS-1 carries a functional copy of pidS because it can restore pigmentation of the pidS mutants, LSUPB112 and LSUPB115. To further validate this experiment a non-polar mutant of 411gr-6 (LSUPB302) was generated by the precise deletion of *pidR* (Figure 3.3). A series of complementation assays showed that pigment production is the bona fide function of *pidR* (Figure 3.3).

3.3.3. Role of the PidS/PidR TCRS in HR elicitation on tobacco leaves and in other virulence-related phenotypes

To determine the role of this TCRS in bacterial virulence, the pidS mutants, LSUPB112 and LSUPB115, were tested for several virulence-related phenotypes, including toxoflavin production, lipase activity, flagelum-mediated motility, HR elicitation in tobacco plants and virulence in rice. Remarkably, both LSUPB112 and LSUPB115 failed to elicit an HR on tobacco leaves and produced less toxoflavin (Figure 3.2) than the parental strain. Moreover, these mutant strains were significantly less virulent than the parental strain on rice panicles (Figure 3.2[C]). However, these mutants did not show significant differences in lipase activity or flagellum mediated motility (data not shown). Like the *pidS* mutants, the newly generated *pidR* strain, LSUPB133, could not elicit an HR on tobacco leaves (Figure 3.2[D]).



Mean Area (mm2)	438.41	20.67	14.65	238.58	342.53
Standard Deviation	67.2	17.9	7.46	75.3	85.15

Figure 3.2. Phenotypes of *Burkholderia glumae* strains in toxoflavin production, hypersensitive response and virulence in rice and onion. A) Symptoms on onion bulb scales caused by *B. glumae* strains, 411gr-6, LSUPB112, LSUPB115, LSUPB112 (pPidRS-1) and LSUPB115 (pPidRS-1). Numeric values indicate the average macerated area (mm^2) from three replications. B) Toxoflavin production of *B. glumae* strain, 411gr-6, LSUPB112 and LSUPB115, on King's B agar plates: Each error bar indicates the standard deviation from three replicates. C) Symptoms on rice panicles caused by *B. glumae* strains, 411gr-6, LSUPB112 and LSUPB11: Numbers indicate average scores of disease severity (DS) from ten replications evaluated using a 0-9 scale at 10 days post inoculation. Superscript letters indicate statistically significant differences ($P > 0.01$) among disease ratings. D) HR elicitation phenotypes of *B. glumae* strains, 411gr-1, LSUPB115, LSUPB115 (pPidRS-1) and LSUPB133 on a tobacco leaf.

3.3.4. Complementation of *pidS* and *pidR* mutant phenotypes

As described in the materials and method section, functional *pidS* and *pidR/pidS* clones were constructed for genetic confirmation of the pigment-deficient phenotypes of the *pidS* and *pidR* mutations (Figure 3.1). The recovery of virulence-related phenotypes, including HR elicitation on tobacco leaves and virulence on onion bulb scales, was also tested for *pidR* and *pidS* mutants complemented with a pidR/pidS clone. The complemented strains were selected on Luria–Bertani (LB) agar containing nitrofurantoin and gentamycin (for pPidRS-1) or kanamycin (for pPidRS-2), showed restored functions in pigment production on CPG agar plates (Figure 3.1) and in HR elicitation in tobacco (Figure 3.2[D]). In addition, complementation of the *pidS* and *pidR* mutants with pPidRS-1 resulted in a substantial increase in virulence in onion bulb assays (Figure 3.3[A]).

To further validate this experiment a non-polar mutant of 411gr-6, (LSUPB302) was generated by the precise deletion of *pidR* (Figure 3.3). A series of complementation assays showed that pigment production is the bona fide function of *pidR* (Figure 3.3). The results of all the complementation tests conducted in this study clearly indicate that the observed phenotypes of pidS and pidR mutants are bona fide.

3.4. Discussion

In this study, it was demonstrated that a newly found TCRS, PidS/PidR, is an essential regulatory component of *B. glumae* for pigmentation in CPG medium, HR elicitation in tobacco, and full virulence in rice and onion. At this time, little is known about the pigments produced by *B. glumae* 411gr-6. All the pathways, regulatory

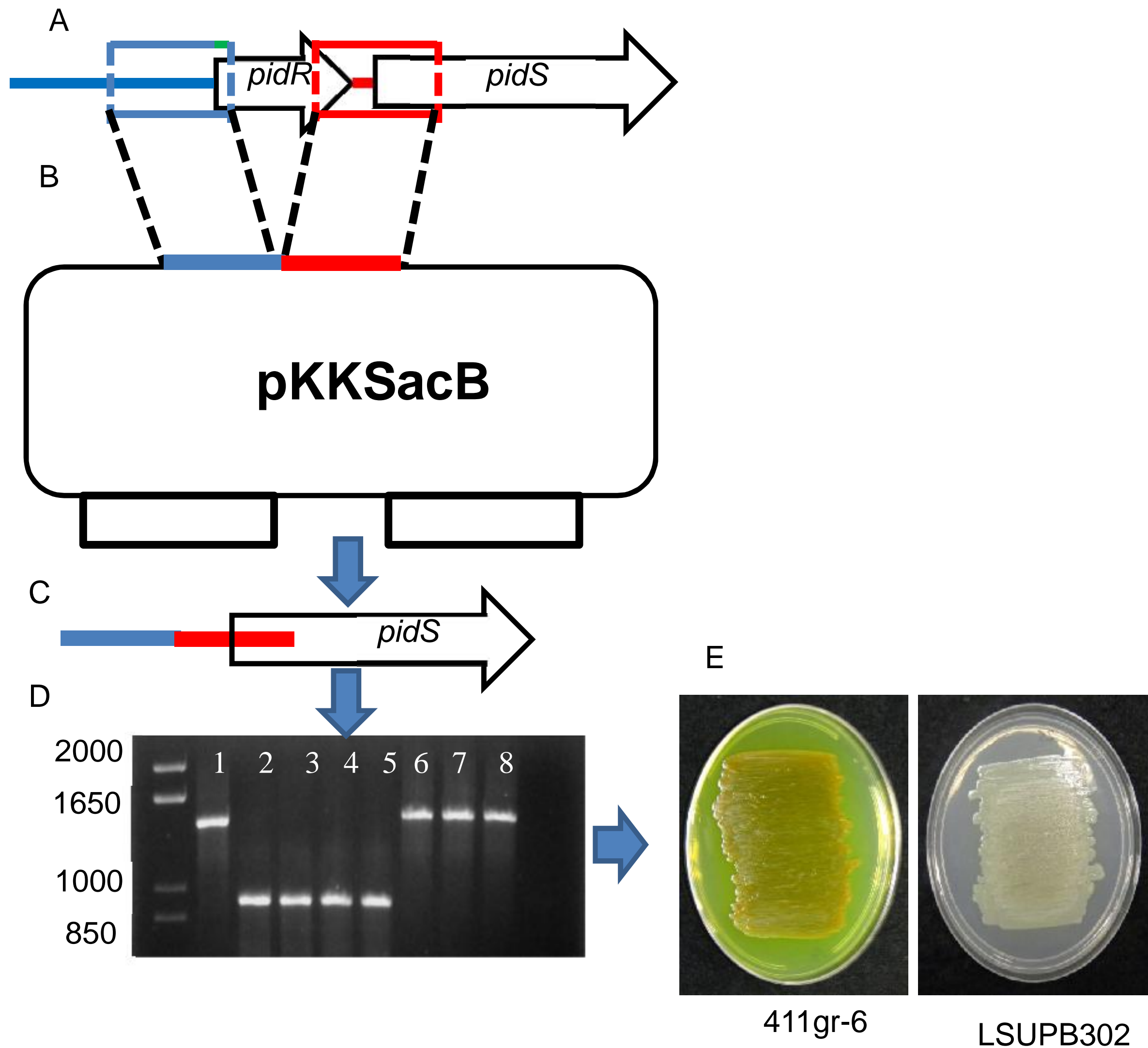


Figure 3.3. Deletion mutations of *pidR* in the genome of *B. glumae*. A) The cartoon is based on the physical location of *pidR* and *PidS* and the location of primers for amplification of upstream and downstream regions of *pidR*. B) Final stage of cloning upstream and downstream region of *pidR* into pKKSacB vector. C) Cartoon showing deletion of *pidR* form genome. D) Gel picture confirming *pidR* is deleted from the genome. 1; 411gr-6, 2, 3, 4 and 5 are *pidR* deleted mutant LSUPB302, 6,7 and 8; mutants did not show pigment deficient phenotypes. E) Non-polar mutant of *pidR* did not show pigment production phenotypes on CPG media.

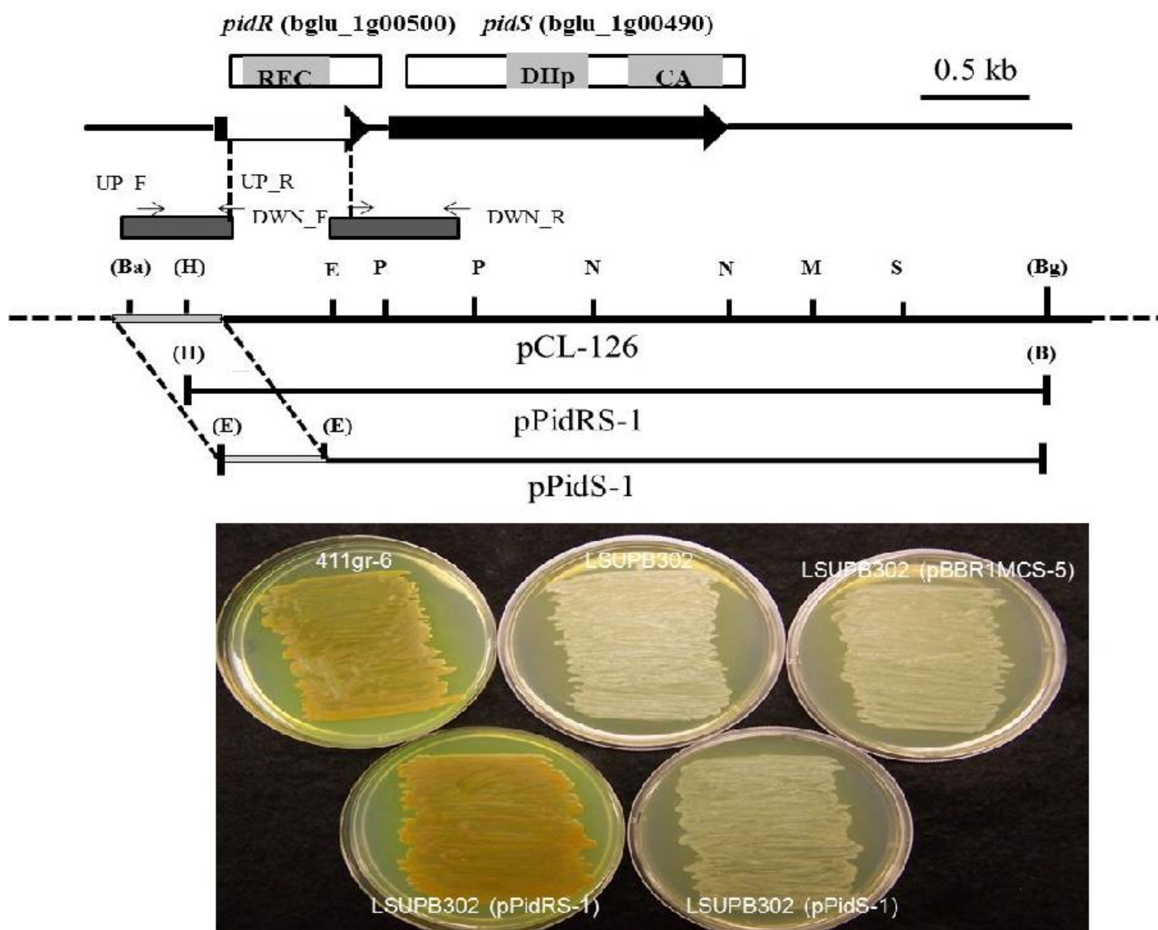


Figure 3.4. An overview of the *pidR* and *pidS* loci and the DNA constructs used for creation of the *pidR* deletion mutant and genetic complementation. This cartoon is based on the sequence information of the *B. glumae* BGR1 genome (GenBank: CP001503.2). ORFs are symbolized by rectangles and arrows, and their corresponding locus tags in the GenBank feature file of CP001503.2 are indicated above each ORF. The dark grey rectangles indicate the flanking regions cloned in pKKSacB for deletion of *pidR* while the white rectangle indicates the deleted region from *pidR*. Small arrows indicate the primers used for the amplification of each flanking region.. Restriction sites in the map are represented by Ba, *Bal*I; H, *Hind*III; E, *EcoR*I; P, *Pst*I; N, *Not*I; M, *Mlu*I; S, *Sma*I: Bg, *Bgl*II; B, *BamH*I; and Sp, *Spe*I. Restriction sites used for subcloning of *pidS* and *pidR* are shown in parentheses. pCL126 is a cosmid clone harboring the *pidR/pidS* locus. The 0.5-kb upstream region of *pidR*, which is predicted as the promoter region of the putative *pidRS* operon, is indicated as light gray line. CA = catalytic and ATP

binding domain; DHp = dimerization and histidine phosphotransfer domain; REC = receiver domain.

systems and genes involved in pigment production phenotypes of *B. glumae* were already discussed in previous chapter (Chapter 2).

HR elicitation by Gram-negative plant pathogenic bacteria in non-host plants, such as tobacco, is a hallmark of functional T3SS (Alfano and Collmer, 2004). The loss of ability to elicit HR in tobacco leaves caused by mutations in *pidS* and *pidR* indicates that the PidS/PidR TCRS is absolutely required for the expression of functional T3SS in *B. glumae.* In many plant pathogenic bacteria having narrow host ranges, such as *Pseudomonas* spp. and *Xanthomonas* spp., T3SSs encoded by *hrp* (***h****ypersensitive* ***r****esponse and* ***p****athogenicity*) genes are essential for both HR elicitation in non-hosts or resistant-hosts and pathogenicity in susceptible hosts (Alfano and Collmer, 2004). However, T3SSs are frequently dispensable for bacterial infection and only contribute to full virulence in certain types of plant pathogenic bacteria including *Pectobacterium* spp., which have broad host ranges and utilize extracellular enzymes secreted via a type II secretion system as primary virulence factors (Bauer et al., 1994; Holeva et al., 2004; Rantakari et al., 2001). In this study, *pidS* and *pidR* mutants could not elicit an HR in tobacco leaves but could produce symptoms in either rice panicles or onion bulbs even though at significantly reduced levels compared with the parental strain. These results indicate that the Hrp T3SS of *B. glumae* is not essential for pathogenicity even though it is required for full virulence. This speculation is consistent with the previous study by in which a T3SS-deficeint *B. glumae* mutant was still virulent although it showed significantly less virulence than full virulence in certain types of plant pathogenic bacteria including *Pectobacterium* spp., which have broad host ranges and

utilize extracellular enzymes secreted via a type II secretion system as primary virulence factors (Bauer et al., 1994; Holeva et al., 2004; Rantakari et al., 2001). In this study, *pidS* and *pidR* mutants could not elicit an HR in tobacco leaves but could produce symptoms in either rice panicles or onion bulbs even though at significantly reduced levels compared with the parental strain. These results indicate that the Hrp T3SS of *B. glumae* is not essential for pathogenicity even though it is required for full virulence. This speculation is consistent with the previous study by in which a T3SS-deficeint *B. glumae* mutant was still virulent although it showed significantly less virulence than its parental strain (Kang et al., 2008). In this regard, it is noteworthy that *B. glumae* also produces other more important virulence factors including the phytotoxin, toxoflavin (Kim et al., 2004), and lipase (Devescovi et al., 2007). In *B. glumae*, pathogenicity of T3SS-deficient mutants is retained probably because of these other major virulence factors.

In prokaryotes, TCRSs play a pivotal role in signal perception and transduction for a wide range of cellular functions involved in metabolism, development, and pathogenesis (Laub and Goulian, 2007). In plant pathogenic bacteria, several TCRSs are also known to have global effects on virulence. For example, GacS/GacA of *Pseudomonas syringae* controls the production of every known virulence factor, including coronatine, extracellular polysaccharides (EPS), and the T3SS and its effectors, via positive regulation of the QS system (Mole et al., 2007). The HrpX/HrpY TCRSs control the genes encoding T3SSs in plant pathogenic bacteria belonging to *Enterobacteriaeae* including *Erwinia amylovora* (Wei et al., 2000) and *Pantoea stewartii* (Merighi et al., 2003). RpfC/RpfG of *Xanthomonas campestris* is known to regulate

multiple virulence factors, including extracellular enzymes and EPSs, via the degradation of the signal molecule 3',5'-cyclic diguanylic acid and the interconnection with the cell-to-cell communication mediated by diffusible signal factor (DSF) (Ryan et al., 2006). Recently, it was reported that the ColS/ColR TCRSs of *X. campestris* pv. *campestris* (Zhang et al., 2008) and *X. citri* subsp. *citri* (Yan and Wang, 2011) play important roles in tolerance to environmental stresses, T3SS and virulence (specifically $ColS_{XC1050}/ColR_{XC1049}$ of *X. campestris* and $ColS_{XAC3249}/ColR_{XAC3250}$ of *X. citri*). Interestingly, PidR shows more than 31% amino acid sequence identity with both ColR response regulators of the two *Xanthomonas* spp., while PidS has less than 20% identity with the corresponding ColS sensor kinases. The apparent similar functions between PidS/PidR and ColS/ColR TCRSs in virulence and the significant sequence homology between PidR and ColR response regulators strongly suggest that both TCRSs act on common regulatory pathways for bacterial pathogenesis in different host environments. Remarkably, it was found that orthologs of PidS and PidR are highly conserved among many *Burkholderia* spp. *B. gladioli*, another bacterium causing BPB in rice (Ham et al., 2011), contains PidR and PidS orthologs showing the highest homology with PidR and PidS. Remarkably, amino acid sequences of PidR *B. gladioli* and PidR *B. glumae* were identical to each other (Table 3.5). The next closest orthologs of PidS were present in *B. pseudomallei* and *B. mallei*, and showed more than 84% amino acid sequence identity to that of PidS. The first 200 amino acids from the N-termini, which correspond to the signal input domain, showed higher homology to PidS (> 92% identity) than to the remaining regions containing dimerization and histidine phosphotransfer (DHp) and catalytic and ATP binding (CA) domains.

Table 3.5. Comparison of the PidS/PidR two-component regulatory system with their representative orthologs of *Burkholderia* spp. and other plant pathogenic bacteria.

PidS (YP_002909958.1: 517 aa)			PidR (YP_002909959.1: 230 aa)		
Protein ID	Organism	Amino acid sequence identity (%)[a]	Protein ID	Organism	Amino acid sequence identity (%)
YP_004358720.1	*B. gladioli* BSR3	90.6	YP_004358721.1	*B. gladioli* BSR3	100
YP_001068298.1	*B. pseudomallei* 1106a	84.3	YP_001068297.1	*B. pseudomallei* 1106a	99.6
YP_104430.1	*B. mallei* ATCC23344	84.2	YP_104431.1	*B. mallei* ATCC23344	99.6
ZP_02465317.1	*B. thailandensis* MSMB43	83.9	ZP_02465316.1	*B. thailandensis* MSMB43	99.6
ZP_02357567.1	*B. oklahomensis* EO147	83.6	ZP_02357566.1	*B. oklahomensis* EO147	99.6
ZP_02890082.1	*B. ambifaria* IOP40-10	82.9	ZP_02890081.1	*B. ambifaria* IOP40-10	98.7
YP_002229170.1	*B. cenocepacia* J2315	82.9	YP_002229171.1	*B. cenocepacia* J2315	98.7
ZP_02380348.1	*B. ubonensis* Bu	82.7	YP_001578267.1	*B. multivorans* ATCC17616	98.7
NP_643557.1 ($ColS_{XAC3249}$)	*Xanthomonas citri* subsp. *citri* 306	19.4	NP_643558.1 ($ColR_{XAC3250}$)	*Xanthomonas citri* subsp. *citri* 306	31.9
YP_242140.1 ($ColS_{XC1050}$)	*Xanthomonas campestris* pv. *campestris* 8004	18.3	YP_242139.1 ($ColR_{XC1049}$)	*Xanthomonas campestris* pv. *campestris* 8004	31.5
AAD24682.1 (HrpX)	*Erwinia amylovora* Ea321	17.4	AAD24683.1 (HrpY)	*Erwinia amylovora* Ea321	19.1

Similar patterns were also observed in other PidS orthologs of *Burkholderia* spp. In general, N-terminal signal input domains of different SHKs tend to be more variable in amino acid sequence because they are responsible for sensing different signals. Higher amino acid sequence homology among PidS and its orthologs at the N-terminal signal input domain strongly suggests that these SHKs recognize the same or similar signals and, further, have similar biological functions in different species of *Burkholderia*. Orthologs of PidR from other *Burkholderia* species showed even higher levels of similarity to PidR. In particular, the PidR orthologs of *B. pseudomallei* and *B. mallei* were almost identical (99.6% identity) to PidR. Other PidR orthologs of *Burkholderia* spp. showed more than 96% identity, indicating that PidR and the PidR orthologs of *Burkholderia* spp. may exert a common regulatory function. It would be very interesting to investigate if the mutation of *pidS* or *pidR* orthologs would also result in impaired T3SS function and reduced virulence in animal pathogenic *Burkholderia* spp. such as *B. mallei* and *B. pseudomallei*. If the regulatory mechanisms and virulence function of PidS and PidR proteins are conserved among pathogenic *Burkholderia* species, further in depth research on the PidS/PidR TCRS of *B. glumae* would provide valuable information for the study of animal/human pathogenic *Burkholderia* species, which is often hindered by strict legal barriers and limitations.

Conclusively, we have demonstrated that the newly discovered PidS/PidR TCRS is required for the pigmentation of *B. glumae* under a certain nutritional condition, grown on CPG medium, and for HR elicitation on tobacco leaf by this bacterium. The PidS/PidR TCRS was also shown to contribute to the virulence of *B. glumae* in pathogenicity assays on rice panicles and onion bulbs. *pidS* and *pidR* orthologs are

highly conserved among other *Burkholderia* species suggesting that they might play similar roles in bacterial pathogenesis. Further studies on this new regulatory factor would expand our knowledge on global regulatory systems of *B. glumae* and other important *Burkholderia* species.

CHAPTER 4
GROWTH, VIRULENCE AND PIGMENT PRODUCTION OF SHIKIMATE PATHWAY MUTANTS OF *BURKHOLDERIA GLUMAE* STRAIN 411GR-6

4.1. Introduction

The bacterium, *Burkholderia glumae* causes bacterial panicle blight of rice as well as the previously reported grain rotting and seedling blight in Japan (Goto and Ohata, 1956). Now this pathogen been reported from the Southern United States (Nandakumar et al., 2009), Latin America (Zeigler and Alvarez, 1989) and other Asian countries (Cottyn et al., 2001; Jeong et al., 2003). A characteristic feature of pathogenic strains of *B. glumae* is the production of bright yellow pigment, toxoflavin that is essential for pathogenicity (Kim et al., 2004; Suzuki et al., 2004). Lipase, a cell wall degrading enzyme produced by *B. glumae* is crucial for pathogenesis (Devescovi et al., 2007) and also has several superior biotechnological values (BoekeMa et al., 2007). Flagella driven motility is important for virulence in *B. glumae* because it allows the bacteria to re-locate at the infection sites of the host (Kim et al., 2007). Toxoflavin, lipase, flagella dependent motility are considered the primary virulence factors, and the production of these pathogenic determinants are dependent on *N*-acyl homoserine lactones (AHLs) mediated quorum sensing (Devescovi et al., 2007; Kim et al., 2007; Kim et al., 2004). Some strains of *B. glumae* produce dark pigments on CPG media and show diversity in pigmentation phenotypes (Karki et al., 2012b). In recent study, it was shown that a two-component regulatory system (TCRS) composed of the PidS, sensor histidine kinase and the PidR response regulator is essential for dark pigment production, elicitation of HR in tobacco leaves and pathogenicity in rice panicles (Karki et al., 2012a). Apart from these virulence factors, the development of bacterial panicle

blight symptoms is largely dependent on prevailing weather conditions especially high temperature and humidity (Nandakumar et al., 2009; Tsushima, 2011).

Microbes produce a variety of pigments, and these are the characteristics used in the name of a species as well as provide important clues for diagnostics. Bacteria that belong to the genus *Xanthomonas* produce a unique class of carotenoid-like pigments (xanthomonadins) whose yellow color was recognized early as a major characteristic of the genus (Starr and Stephens, 1964). The major pigment-producing microorganism are *Bacillus* spp. - brown pigments, *Pseudomonas* spp.- yellow pigments, *Streptomyces*- yellow, red and blue pigments. These pigments provide a number of useful functions to these bacterial sp. such as protection against UV radiation (Romero-Martinez et al., 2000), heat and cold (Paolo et al., 2006), oxidants (Liu et al., 2005) and antimicrobial compounds (Van Duin et al., 2002). Pigmentation might also play a role in the virulence of microbes by killing the host immune response or providing inflammatory damage to cells and tissues (Liu and Nizet, 2009). Recently, it was shown that *B. glumae* strain 411gr-6 produces a purple and yellow-green pigments other than toxoflavin on CPG agar plates (Karki et al., 2012b). However their role in pathogenesis is still not clear.

The shikimate acid pathway is present in bacteria, fungi, yeasts, algae, plants and certain apicomplex parasites while absent in metazoans including humans. The shikimate acid pathway consists of seven enzyme-mediated steps yielding chorismic acid as a final product, which is the direct precursor for aromatic amino acids, folate, ubiquinones, and other isoprenoid quinines (Knaggs, 2003). The shikimate pathway mutant of *Burkholderia pseudomallei* was unable to grow in minimal media; however, the growth of the mutant was restored with the exogenous application of tryptophan,

tyrosine, phenylalanine, PABA, and 2,3-dihydroxybenzoate (Cuccui et al., 2007). Several studies conducted have shown a disruption of genes in shikimate acid pathway in a wide range of bacteria and this revealed the mode of infection (Günel-Özcan et al., 1997; Simmons et al., 1997; Stritzker et al., 2004). In the plant pathogenic bacterium, *Xanthomonas oryzae* pv. *oryzae* the shikimate acid pathway is associated with pigment production and pathogenicity (Goel et al., 2001; Park et al., 2009). In this study we investigate the role of shikimate acid pathway, a pathway for the production of aromatic amino acid, in dark pigment and toxoflavin production as well as growth, and virulence of *B. glumae*.

4.2. Materials and Methods

4.2.1. Bacterial strains and plasmids

All the bacterial strains and plasmids used in this study are listed in Table 4.1. *Burkholderia glumae* 411gr-6, is a pigment producing highly virulent strain isolated from the rice (Karki et al., 2012b) and was used for mutagenesis. *E. coli* DH10B, pKNOCK$_{Gm}$ (Alexeyev, 1999) and pBB1MCS-5 (Kovach et al., 1995) were used as a helper strain, suicide vector and broad host range vector, respectively. *B. glumae* and *Escherichia coli* strains were routinely grown and maintained in the Luria Bertani (LB) broth and agar media (Sambrook and Russell, 2001), minimal M9 media (Sambrook and Russell, 2001), King's B and CPG agar media (Schaad et al., 2001) at 30 to 37°C unless otherwise described and depending upon the experimental conditions. Antibiotics were added at the following concentrations: ampicillin (Amp), 100 µg/ml; kanamycin (Km), 50 µg/ml; nitrofurantoin (Nit), 100 µg/ml; and gentamycin (Gm), 20 µg/ml.

Table 4.1. Plasmids and *Burkholderia glumae* strains used in the study

Strain or plasmid	Description	Reference or source
<u>*B. glumae*</u>		
336gr-1	A virulent strain showing a pigment-deficient phenotype, Nit^{R}	(Nandakumar et al., 2009)
411gr-6	A virulent strain showing a pigment-proficient phenotype, Nit^{R}	(Nandakumar et al., 2009)
LSUPB114	A *aroA*::mini-Tn*5gus* derivative of 411gr-6, Nit^{R}, Km^{R}	(Karki et al., 2012a)
LSUPB116	A *aroB*::mini-Tn*5gus* derivative of 411gr-6, Nit^{R}, Km^{R}	(Karki et al., 2012a)
LSUPB303	A *pidR*::pKNOCK$_{Gm}$ derivative of 411gr-6, Nit^{R}, Gm^{R}	This study
LSUPB304	A *pidR*::pKNOCK$_{Km}$ derivative of 411gr-6, Nit^{R}, Km^{R}	This study
<u>*E. coli*</u>		
DH5	*SupE44* D*lac*U169 (f80 *lacZ*D*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Life Technologies
S17-1 pir	*recA thi pro hsdR* [res– mod+][RP4::2-Tc::Mu-Km::Tn*7*] *pir* phage lysogen	(Simon et al., 1983)
XL1-Blue MR	*(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
<u>Plasmids</u>		
pUT::miniTn*5gus*	A suicide vector carrying mini-Tn*5gus*, Ap^{R}, Km^{R}	(de Lorenzo et al., 1990)
pKNOCK$_{Gm}$	A suicide vector, R6K *ori*, Gm^{R}	(Alexeyev, 1999)
pBBR1MCS-5	A broad host range vector, Gm^{R}	(Kovach et al., 1995)
SuperCos1	A cosmid vector, Ap^{R}, Km^{R}	Stratagene
pSC-A-amp/kan	A PCR cloning vector, Ap^{R}, Km^{R}	Stratagene
pCL317	A cosmid clone harboring the *aroL/aroB* locus, Ap^{R}	This study
pBBaroLB	A subclone of pCL317 for the 2.5-kb *SmaI* fragment harboring both *aroL* and *aroB* in pBBR1MCS-5, Gm^{R}	This study

4.2.2. Recombinant DNA techniques

Procedures for routine DNA cloning and amplification were conducted as per Sambrook and his colleagues (Sambrook and Russell, 2001).

4.2.3. Screening of mini-Tn5*gus* mutants and identification of genes

Screening mini-Tn5*gus* mutants and identification of genes were performed as described previously (Karki et al., 2012a) and in previous chapter 2.

4.2.4. Generation of 4-hydroxyphenylpyruvic acid dioxygenase (*hppD)* and rhamnotransferase (*rhlC*) mutants

Internal regions of *hppD* (bglu_1g02320) and *rhlC* (bglu_2g05690) were amplified using the primer set, 5’ GCATCTTCGACGAGAACGAG 3’ and 5’ CACAGCACGATCTGGATGAT 3’ and 5’GCATCTTCGACGAGAACGAG 3’ and 5’CACAGCACGATCTGGATGAT 3’, respectively. The amplified PCR products were cloned into the PCR cloning vector, pSC-A-amp/kan, using a StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, CA, USA) following the manufacture’s instruction. The insert of the resultant PCR clone, pSC::hppD and pSC:: *rhlC* were digested with *XhoI* and *PstI* and ligated to the *XhoI*/*PstI*-cut pKNOCK$_{Gm}$ (Alexeyev, 1999), generating pKNOCK$_{Gm}$::*hppD* and pKNOCK$_{Gm}$::*rhlC*, respectively. *Escherichia coli* S17-1 pir (Simon et al., 1983) was used for maintaining pKNOCK vectors and pKNOCK$_{Gm}$::*hppD* and pKNOCK$_{Gm}$:: *rhlC*. *E. coli* HB101, which carries the helper plasmid, pRK2013 (Figurski and Helinski, 1979) , was used for the triparental mating to introduce pKNOCK$_{Gm}$::*hppD* and pKNOCK$_{Gm}$:: *rhlC* to the virulent strain of *B. glumae*, 411gr-6. *hppD and rhlC* mutants from homologous recombination were selected on LB agar containing nitrofurantoin and gentamycin, and their phenotypes of pigment production were checked on CPG agar plates.

4.2.5. Genetic complementation of shikimate acid pathway mutant, LSUPB116

To generate a broad host range vector, pBBR1MCS-5 containing whole region of 3-dehydroquinate synthase (*aroB*), cosmid library of the genome of *B. glumae* 336gr-1, was screened by using a *aroB* specific primer set, 5'-AGGTCGATACCGTGGTGCT-3' and 5'-CCTCGATCCAGTCGAAGAAC -3' to indentify cosmid clones containing the *aroB* gene. One of the screened cosmid clones, pCL317 was digested with *SmaI* (New Engald biolabs) to obtain a 2.5-kb fragment containing *aroL* (Shikimte kinase) and *aroB*. This fragment was then sub-cloned into broad-host-range vector pBBR1MCS-5 (Kovach et al., 1995), with the same restriction digestion as the cosmid clone, generating pBBaroLB. For the complementation assay, pBBaroLB was introduced into LSUPB116, *aroB* mutant through triparental mating as described previously (Karki et al., 2012a). The complemented *B. glumae* strains were selected on LB agar plates containing kanamycin (Km), 50 µg/ml; nitrofurantoin (Nit), 100 µg/ml; and gentamycin (Gm), 20 µg/ml as selection markers.

4.2.6. Toxoflavin production assay

Extraction and quantification of toxoflavin was done following the previously developed protocols (Iiyama et al., 1995) with some modifications. Briefly, *B. glumae* strains were grown in 3 ml of LB broth overnight, washed twice with equal volumes of the same media, and re-suspended in 100 µl of LB broth. Then, 20 µl of the bacterial suspension was used as starter inoculum to grow in 15 ml of LB broth in a 250 ml flask for 24 h at 37°C, and afterwards bacterial growth was measured. Toxoflavin was extracted from 1 ml of LB broth media with an equal volume of chloroform. Then the chloroform was evaporated under a fume hood, and the residues were dissolved in 1 ml

of aqueous 80% methanol. Absorbance was measured at 393 nm using a spectrophotometer (Biomate 3, Thermo Scientific, Pittsburgh, PA, USA). The final toxoflavin value was calculated based on bacterial growth.

4.2.7. UV light sensitivity assay

Bacterial cells were streaked on CPG agar plates and incubated at 30°C for 48 h. Bacterial colonies were dissolved in sterile water with cotton swab then OD_{600} was adjusted to 0.1 and diluted 1/10 volume. 100 µl of diluted *B. glumae* cells were spread on a CPG plate, which was subsequently exposed to the UV light from G15T8 germicidal fluorescent bulb,15W (General electric, USA) in laminar hood (EdgeGARD® EG3252, The Baker Company, ME, USA) at the distance of 71.43 cm with 253.7nm wave length for various time durations. Colonies obtained from the surviving cells were counted following 48 h of incubation at 30°C. Data were transferred to percentage of survival at different times of UV exposure.

4.2.8. Growth assay of shikimate pathway mutants

Burkholderia glumae strains were grown in LB broth overnight, washed twice with equal volumes of fresh LB broth, and re-suspended in 1/10 volume of LB broth. Then, 25 µl of the bacterial suspension, having equal starter inoculums were cultured on LB broth, minimal M9 media (Sambrook and Russell, 2001). Minimal M9 media consisted of 0.1 g (each) of L-tryptophan, L-phenylalanine, and L-tyrosine/liter at 37°C on a rotary shaker at 180 rpm. The growth of *B. glumae* strains were observed by measuring the OD_{600} at 12, 16, 20, 24 and 36 h intervals.

4.2.9. Virulence assay with rice panicles

Detail procedure of virulence assay is described in Chapter 3 (Karki et al., 2012a).

4.2.10. Virulence assay with onion bulb scales

Virulence phenotypes of *B. glumae* strains on onion bulbs were determined as previously developed method which is described in Chapter 3 (Karki et al., 2012a).

4.3. Results

4.3.1. Screening, characterization and identification of pigment deficient mutants

Two of the pigment deficient, shikimate acid pathway mutants, LSUPB114 and LSUPB116 were investigated for their virulence related phenotypes. Previously, it was shown that in the mutants, LSUPB114 and LSUPB116, a mini-Tn5*gus* transposon was inserted in the ORFs and encoded a putative 3-phosphoshikimate 1-carboxyvinyltransferase and a putative 3-dehydroquinate synthase, respectively (Figure 4.1[B]).

4.3.2. Role of the *aroA* and *aroB* genes in pigment production and virulence-related phenotypes

To determine the role of *aroA* and *aroB* genes in pigment production phenotype, mutant of respective genes LSUPB114 and LSUPB116 were grown on CPG agar plates at 30 C. Both of the mutants failed to produce pigment, remarkably, the pigment production of *B. glumae* strain 411gr-6 is dependent upon the temperature (Figure 4.2) whose production is only visible at 30 C. Moreover, these mutant strains were significantly less virulent than the parental strain on rice panicles (Figure 4.3[A]) and onion scales (Figure 4.3[A]).

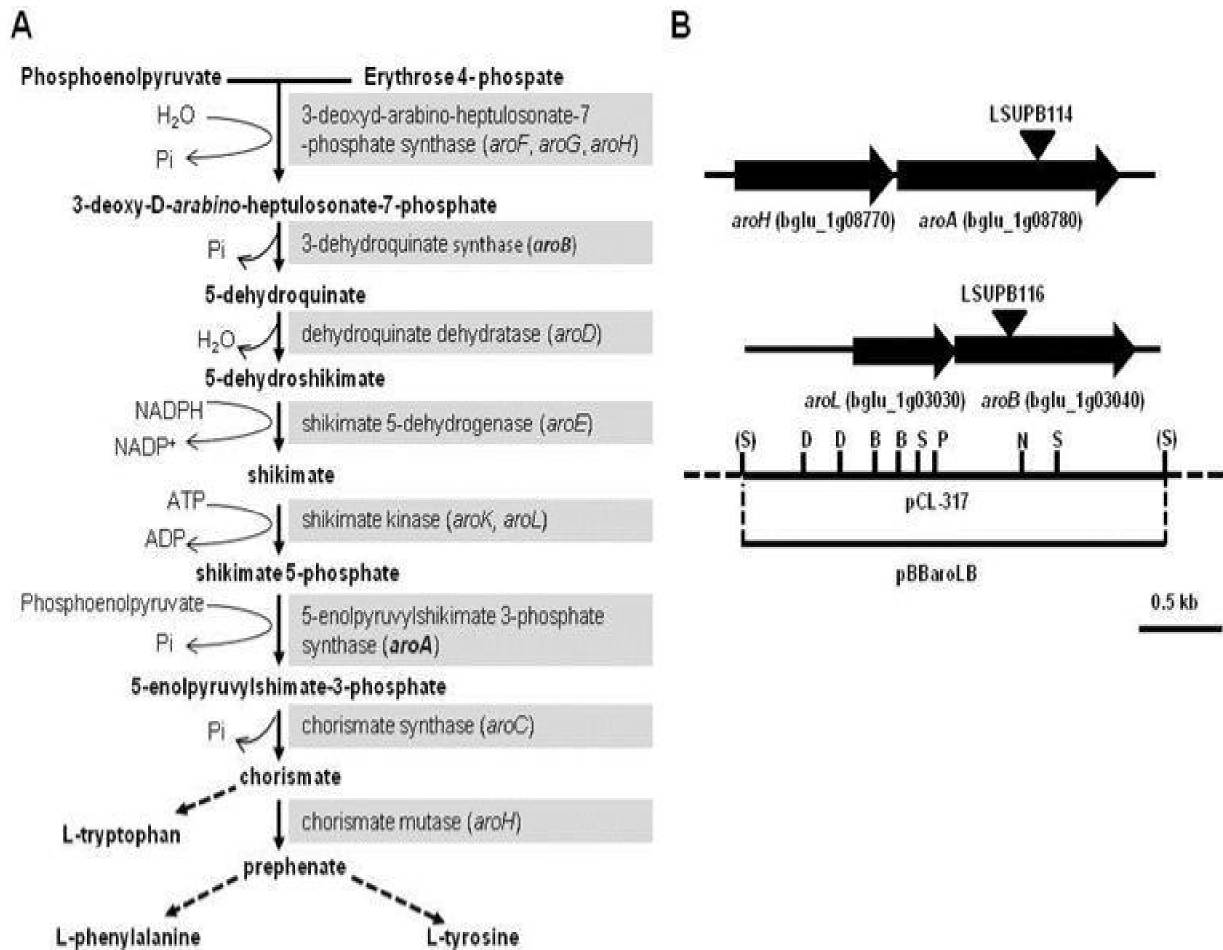


Figure 4.1. The shikimate pathway and the *aroA* and *aroB* loci. A) A schematic description of the shikimate pathway and the enzymes involved in each step of the pathway adopted from Tzin et al. and Baez-Viveros et al. (Baez-Viveros et al., 2007; Tzin and Galili, 2010).The genes encoding the metabolic enzymes are indicated in parentheses. B) A physical map of *aroA* and *aroB* and their respective mutants LSUPB114 and LSUPB116. This map is based on the sequence information of *Burkholderia glumae* BGR1. The locus tag featured in gene bank for each gene is indicated in parenthesis. Black inverted triangles indicate the site of miniTn5*gus* insertion in each mutant. Restriction sites in the map are represented by: D, *Dra*III; B, *Bam*HI; S, *Sac*I; P, *Pst*I; N, *Not*I; and (S), *Sma*I. *Sma*I was used for sub-cloning the 2.5 kb-fragment containing *aroL* and *aroB* from the cosmid, pCL317, into the broad host range vector pBBR1MCS-5 (Gm^R), creating pBBaroLB.

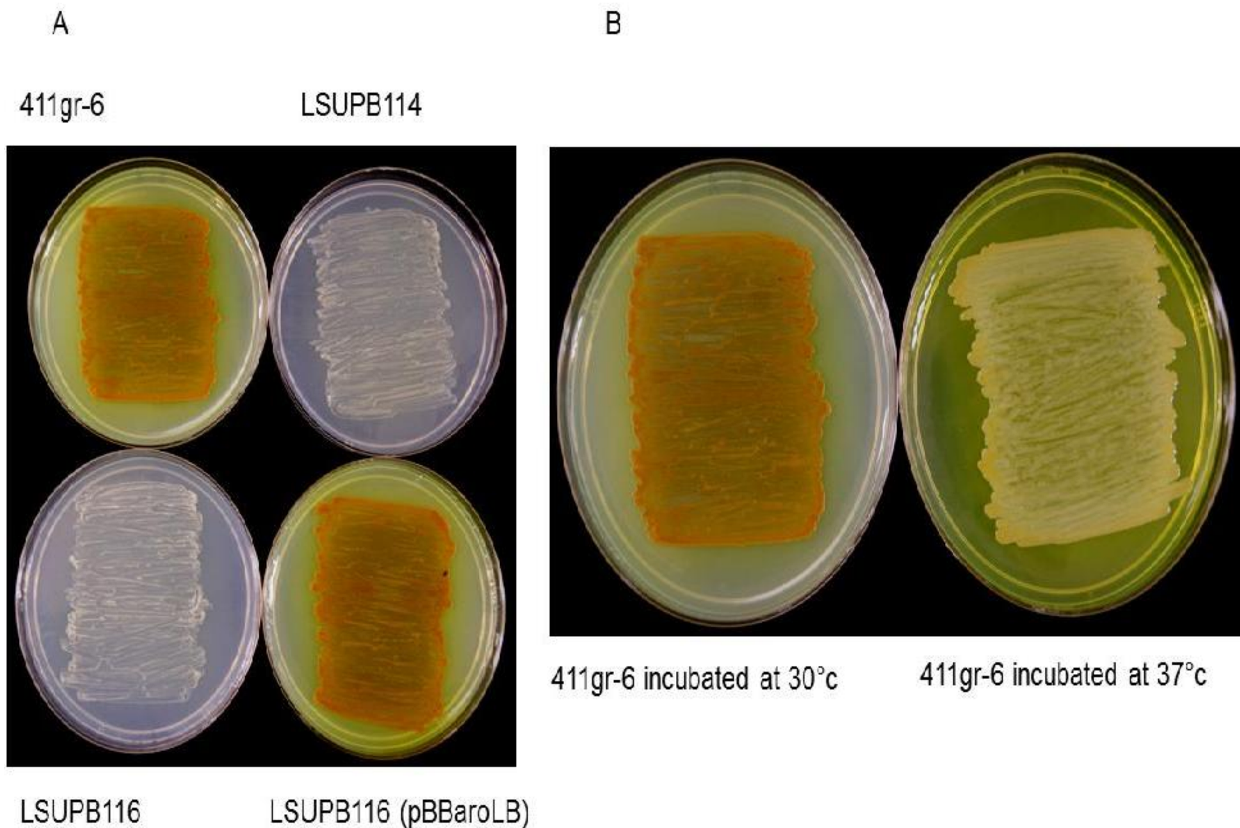


Figure 4.2. Dark pigment production of *B. glumae* strains. A) Pigmentation phenotypes of wild type *B. glumae* strain, 411gr-6, shikimate acid pathway mutants, LSUPB114 and LSUPB116 and complemented, LSUPB116(pBBaroLB) on CPG agar plates. Freshly grown bacterial colonies were streaked on CPG agar plates, which were incubated at 30°C, and photographed 48h after inoculation. B) Wild-type strain, 411gr-6, incubated at 30°C (left) and 37°C (right) for 48 h after streaking on CPG agar plates.

4.3.3. Intact toxoflavin production by *aroA* and *aroB* mutants

To determine if near non-pathogenic phenotypes of LSUPB114 and LSUPB116 were caused by an impaired toxoflavin production, LSUPB114, LSUPB116 a parental wild type 411gr-6 and a complemented strain LSUPB116(pBBaroLB) were examined their the ability to produce toxoflavin. There was no significant difference in toxoflavin production among 411gr-6, LSUPB114, LSUPB116 and LSUPB116(pBBaroLB) (Figure 4.3 [C]).

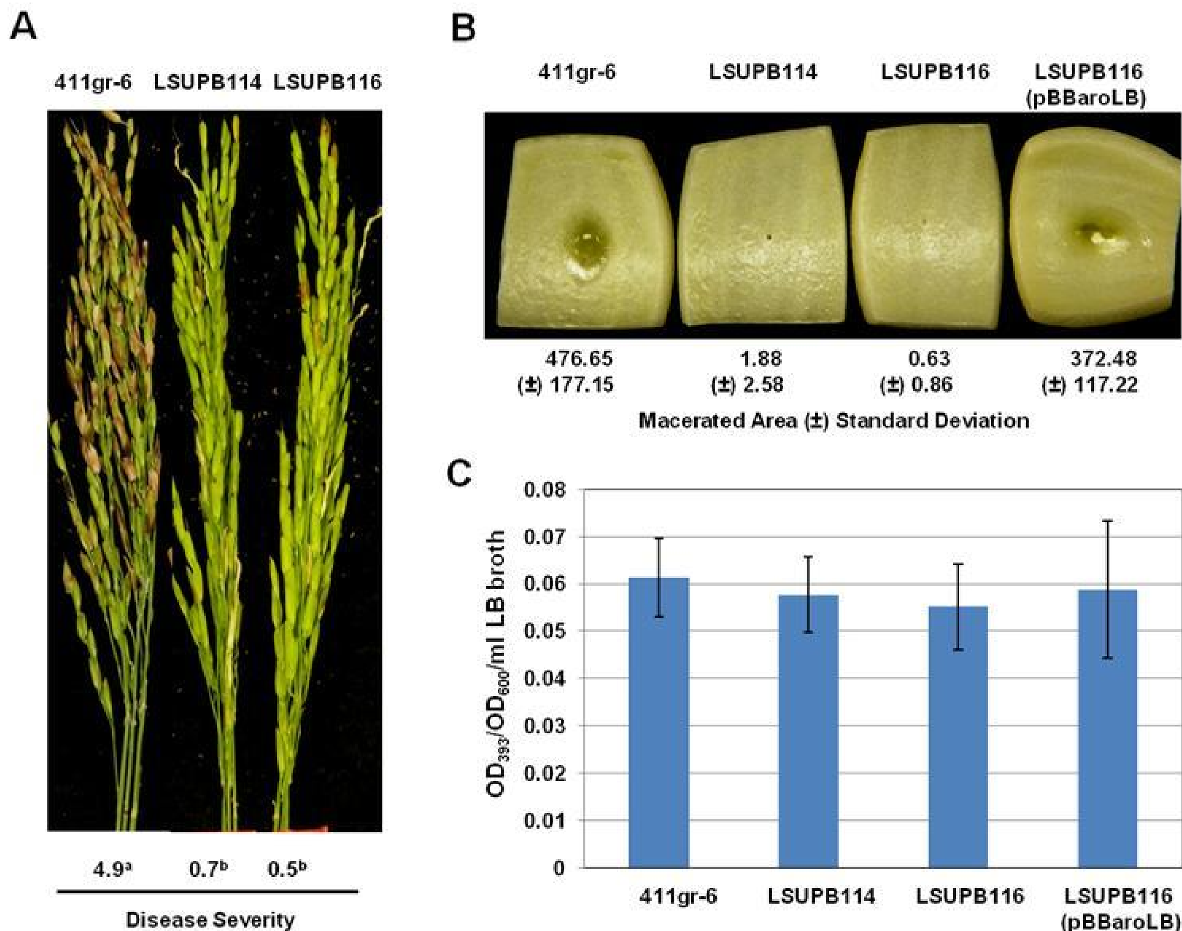


Figure 4.3. Virulence and toxoflavin production of *B. glumae* strains. A) Representative bacterial panicle blight symptoms on rice panicles caused by the *B. glumae* strains, 411gr-6, LSUPB114 and LSUPB116. The number below each picture indicates the average value of disease severity (DS) from ten replications evaluated using a 0-9 scale at 10 days post inoculation. In this scale, 0 = no symptoms and 9= more than 90% of the panicle is symptomatic. Superscript letters indicate statistically significant differences ($P > 0.01$) among different strains. B) Maceration symptoms on onion bulb scales caused by the *B. glumae* strains, 411gr-6, LSUPB114, LSUPB116, and LSUPB116(pBBaroLB). Onion scales were inoculated with *B. glumae* strains and incubated at 30° C for 48 h. Numeric values indicate the average macerated area (mm^2) from six replications. The photograph was taken at the time of symptom evaluation. C) Toxoflavin production of *B. glumae* strains 411gr-6, LSUPB114, LSUPB116 and LSUPB116 (pBBaroLB). Each error bar indicates the standard deviation from three replications.

4.3.4. Growth defects of *aroA* and *aroB* mutants in M9 minimal medium

There was no significant difference between the mutants and the wild type in growth rate in LB broth media at 12, 16, 20, 24 and 36 h after inoculation (Figure 4.3 [A]). In contrast, LSUPB114 and LSUPB116 did not grow in M9 medium, while 411gr-6 and LSUPB116 (pBBaroLB), did (Figure 4.3 [B]). The growth defect of the *aroA* and *aroB* mutants could be restored in M9 medium supplemented with the three aromatic amino acids (phenylalanine, tryptophan and tyrosine) (Figure 4.3 [C]), but not with M9 media supplemented with only one of the three aromatic amino acids (data not shown).

4.3.5. Growth inhibition of *B. glumae* strains by glyphosate

Consistent with the requirement of the shikimate pathway genes *aroA* and *aroB* for growth in M9 medium, the growth of *B. glumae* 411gr-6 was suppressed by the herbicidal compound, glyphosate (Roundup®), which inhibits 5-enolpyruvylshikimate 3-phosphate synthase (*aroA*) (Figure 4.3 [D]) (Steinrucken and Amrhein, 1980). The growth of *B. glumae* strain 336gr-1, a non-pigmenting wild type, was also inhibited by glyphosate in M9 minimal medium like *B. glumae* 411gr-6 (Figure 4.3[D]). However, growth of *B. glumae* strain 336gr-1 was more inhibited by glyphosate than 411gr-6 (Figure 4.3[D]).

4.3.6. Impaired UV tolerance of *aroA* and *aroB* mutants

The wild type strain 411gr-6 and its pigment-deficient mutants, LSUPB114 and LSUPB116, were tested for their tolerance to UV light along with the non-pigmenting wild type strain 336gr-1. The pigment-deficient shikimate pathway mutants, LSUPB114 and LSUPB116, showed substantially reduced tolerance to UV light compared with the pigment-producing wild type strain, 411gr-6 (Figure 4.4). 411gr-6 showed about 45%

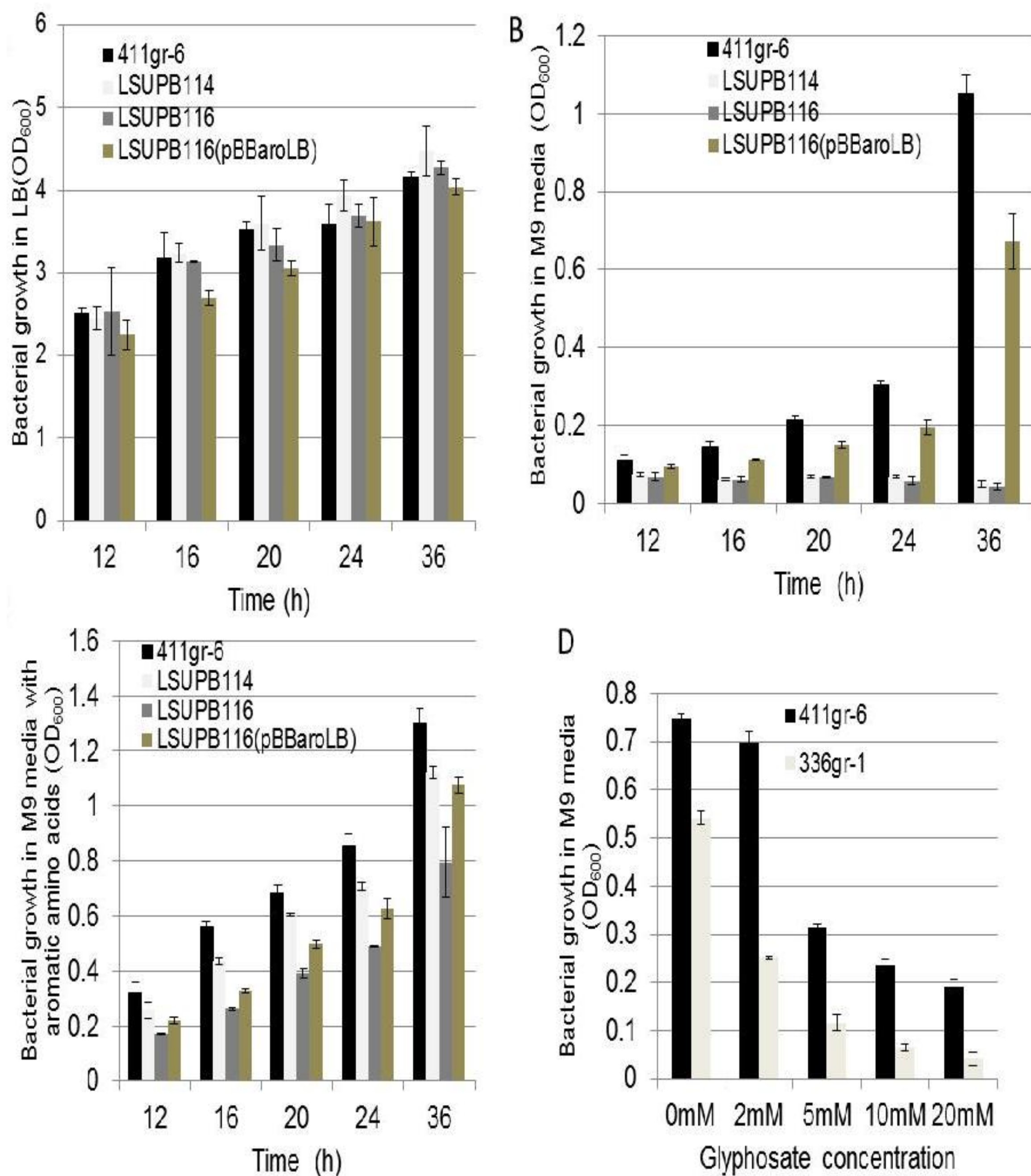


Figure 4.4. The growth of *B. glumae* strains on different media; A) LB, B) M9, C) M9 supplemented with aromatic amino acids, and D) M9 amended with glyphosate. Each error bar indicates the standard deviation from three replications.

average survival rate at 0.5-min UV exposure and survived a 3-min-UV exposure (Figure 4.4). In contrast, LSUPB114 and LSUPB116 showed only about 12% and 7% average survival rates, respectively, at 0.5-min-UV exposure and could not survive a 3-min-exposure (Figure 4.4). The non-pigmenting wild type strain, *B. glumae* 336gr-1, also showed reduced tolerance to the UV light compared with *B. glumae* 411gr-6 like LSU114 and LSUPB116 (Figure 4.4). *B. glumae* 336gr-1 seems to have a functional shikimate pathway unlike LSUPB114 and LSUPB116 because it could grow well in M9 medium without any supplemented aromatic amino acid (Figure 4.3[D]).

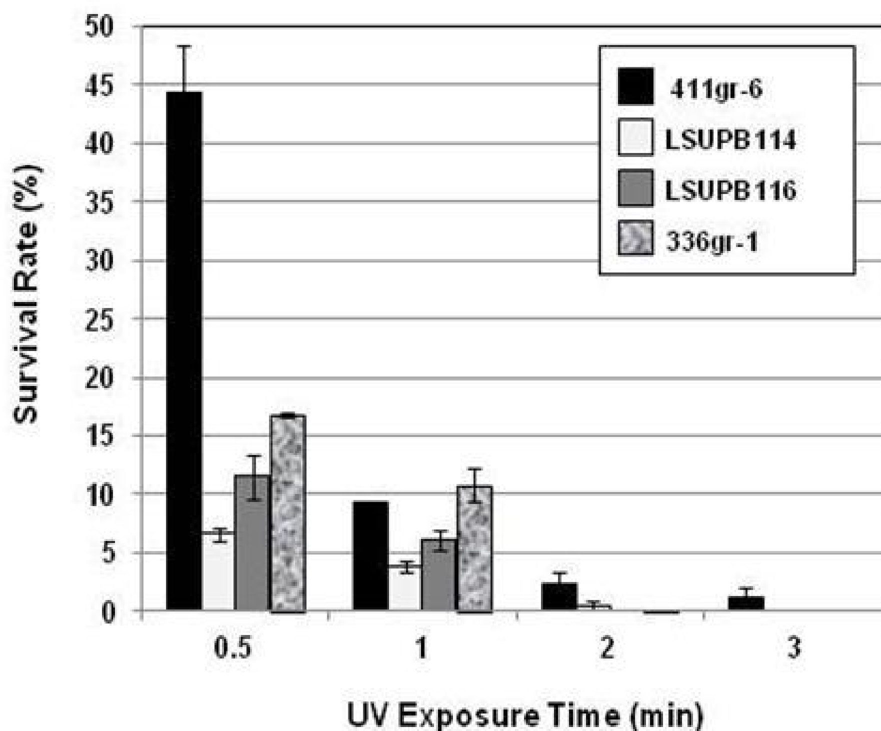


Figure 4.5. Effects of UV radiation on the survival of *B. glumae* strains. The assay was repeated at least three times independently and the representative results are shown. Error bar indicates the standard deviation from three replications.

4.3.7. Screening of *B. glumae* BGR1 genome for the presence of shikimate acid pathway genes

The whole genome of *B. glumae* strain BGR1 form NCBI data base were scanned for the presence of shikimate acid pathway genes. The first enzyme of the shikimate pathway is 3-deoxyd-arabino-heptulosonate-7-phosphate synthase (DAHPS) (converting PEP and E4-P into 3- dehydroquaianate. As we searched in the *B. glumae* genome we found phospho-2-dehydro-3-deoxyheptonate aldolase with three genes ID namely bglu_1g06220, bglu_2g20130 and bglu_2g11470 (Table 4.2.). The second enzyme of the shikimate pathway is 3-dehydroquinate synthase (*aroB*), which converts 3-deoxy-d-arabino-heptulosonate-7-phosphate into 3-dehydroquinate the first cyclic compound of this pathway as shown in Table 4.2. *B. glumae* has 3-dehydroquinate synthase in the genome. The third and fourth enzymatic steps are catalyzed by 3-dehydroquinatedehydratase (*aroD*) /shikimate 5-dehydrogenase (*aroE*), and are well conserved in *B. glumae* genome leading to the formation of shikimate (Table 4.2). Shikimate kinase (*aroL*) catalyzes the fifth enzymatic step of the shikimate pathway that converts shikimate to shikimate 3-phosphate which is present in both chromosome of BGR1 genome (Table 4.2). The sixth enzymatic step leads to the formation of enolpyruvyl shikimate 3-phosphate which is catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (*aroM)* and is reported in the second chromosome of BGR1 genome (Table 4.2).

Table 4.2. The genes for the shikimate acid pathway found in the genome of *Burkholderia glumae* BGR1.

Shikimate pathway step	Gene ID	Gene name	Gene product
1	bglu_1g06220 bglu_2g20130 bglu_2g11470	*aroG*	phospho-2-dehydro-3-deoxyheptonate aldolase
2	bglu_1g03040	*aroB*	3-dehydroquinate synthase
3	bglu_1g05080 bglu_2g03860	*aroD*	3-dehydroquinate dehydratase
4	bglu_1g05140 bglu_2g03870	*aroE*	shikimate 5-dehydrogenase
5	bglu_1g03030 bglu_2g20020	*aroL*	shikimate kinase
6	bglu_2g08950	*aroM*	5-enolpyruvylshikimate-3-phosphate synthase
7	bglu_1g21620	*aroC*	chorismate synthase

4.4. Discussion

In this study, we provide the first evidence that mutation in the genes involved in shikimate acid pathway (*aroA* and *aroB*) of *B. glumae* strain 411gr-6 resulted in a lack of pigment production, failed to grow in M9 medium and were almost non-pathogenic to rice. Also, these mutants showed reduced tolerance to UV light. To the best of our knowledge this represents the first report of involvement of shikimate acid pathway in virulence related phenotypes of the plant pathogenic bacterium, *B. glumae*.

B. glumae is primarily associated with seedling rot in rice nurseries and panicle blight symptoms in rice (Goto and Ohata, 1956; Goto, 1987; Ham et al., 2011; Nandakumar et al., 2009). Outbreaks of bacterial panicle blight and its pathogen (*B. glumae*) is associated with warmer and dry environmental conditions because the optimal temperature for the growth of *B. glumae* ranges from 35 to 40° C (Nandakumar et al., 2009). Also higher relative humidity (≧95 is) absolutely required for the symptoms development in spikelet (Tsushima, 2011).

Dark pigment production in *B. glumae* strain 411gr-6 is temperature dependent, dark pigment production is only visible after incubation at 30° C, while incubation at 37° C showed toxoflavin production. Similarly, the production of toxoflavin, the most important virulence factor of this pathogen is hugely dependent upon temperature; the maximum amount of toxoflavin is produced at 37° C while no detecTable amount is produced 25-28° C (Matsuda and Sato, 1988). It wold be interesting to know how *B. glumae* co-regulated toxoflavin and pigment production in a temperature dependent manner.

The shikimate acid pathway is essential for the synthesis of aromatic compounds in prokaryotes, fungi and plants (Knaggs, 2003). Metazoans including humans lack the shikimate acid pathway, so need to obtain aromatic compounds from intake from food. Feedback inhibition (an enzyme that is involved in catalysis of the production of a certain substance in the cell is inhibited when that substance has accumulated to a certain level) is controlling the expression of shikimate acid pathway through intermediates and downstream products (Krämer et al., 2003). This could be the reason for the loss of pathway genes due to positive selection force, if exogenous availability of

products and its component enzymes (Zucko et al., 2010). Previously, it was shown that most of the tested free-living bacteria contained a complete shikimate acid pathway or were just missing one or two enzymes, while more than one quarter of the tested host-associated bacteria having incomplete pathway or missing one or more enzymes (Zucko et al., 2010). From this result, it was speculated that these host-associated bacteria might obtain their essential aromatic compounds from the host. Many members of the *Burkholderia* are free living bacteria; however, there is a lot of diversity among them. Screening the whole genome sequence of *B. glumae* strain BGR1from NCBI data base for the presence of previously described shimkimate acid pathway genes (Zucko et al., 2010) revealed that the *B. glumae* genome contains complete sets of genes for shikimate acid pathway. The first enzyme of the shikimate pathway is 3-deoxyd-arabino-heptulosonate-7-phosphate synthase (DAHPS) which is synonymous with phospho-2-dehydro-3-deoxyheptonate aldolase. In *E. coli,* there are three DAHP synthase isoforms indicated by *aroF*, *aroG* and *aroH* while some bacteria like *Mycobacterium tuberculosis* contain only one DAHP synthase (Parish and Stoker, 2002). *B. glumae* genome has phospho-2-dehydro-3-deoxyheptonate aldolase with three genes ID namely bglu_1g06220, bglu_2g20130 and bglu_2g11470. Accordingly, the second, third and fourth steps of the pathway are catalyzed by 3-dehydroquinate synthase (*aroB*), dehydroquinatedehydratase (*aroD*) and shikimate 5-dehydrogenase (*aroE*) respectively, and these are well conserved in the BGR1 genome. The fifth step of pathway is catalyzed by shikimate kinase which is present as two isoforms in *E. coli* as shikimate kinase I encoded by the *aroK* gene and shikimate kinase II encoded by the *aroL* (Whipp and Pittard, 1995). In *B. glumae* genome there are two shikimate kinases

present in different chromosomes. The sixth and seventh steps are catalyzed by 5-enolpyruvylshikimate 3-phosphate synthase (*aroM)* and chorismate synthase (*aroC*) respectively leading to the production of chorismate and this step is also well conserved in the BGR1 genome.

B. cenocepacia strain C5424 produces a brown melanin like pigment, and disruption of the *hppD* gene resulted in a non-pigmented mutant (Keith et al., 2007). Initially by observing the dark pigments phenotype of strain 411gr-6, we speculated that these pigments might be melanin. After a series of biochemical analysis, we confirmed that these dark pigments are not melanin (Karki et al., 2012b). Recently, we reported that *B. glumae* strains produce diverse types of dark pigments and our preliminary studies showed that 411gr-6 produces at least purple and yellow-green pigments (Karki et al., 2012b). Similarly, we mutated the *hppD* gene of *B. glumae* and observed the phenotype of pigment production. Our result showed that mutation of *hppD* gene did not abolish pigment production (data not shown). Various types of pigments such as melanin, scytonemin and carotenoids have been well studied for protection of micro-organisms against UV light. The pigments accumulated in cell-surface of micro-organism confer protection against UV by observing a wide range of wave length (Cockell and Knowland, 1999). For instance, melanin pigment-producing mutant of *Vibrio cholera* was more resistant to UV irradiation than the wild type *V. cholerae* (Valeru et al., 2009). Similar with previous findings, our data show that the pigment producing strain of *B. glumae* is more tolerant to UV light than pigment non-producing strain. We found that pigment producing wild-type strain; 411gr-6 could survive after 3

min of UV exposure while other non-pigment producing strains including 336gr-1, LSUPB113 and LSUPB116 could not.

The *aro* mutants of *Salmonella enterica* showed reduced virulence due to the inability to produce aromatic metabolites such as phenylalanine, tyrosine, and tryptophan, and these mutants could not grow in minimal media unless aromatic amino acids or their precursors were added (Sebkova et al., 2008). Previously, it was shown that *aro* mutants of *Salmonella* grow like wild-type in nutrient rice BHI media, showing that essential aromatic compounds can be supplied through culture media (O'Callaghan et al., 1988). Similarly, our results showed that there is no significant difference in the growth pattern between *aro* mutants and wild-type in nutrient rich, LB broth media. The *aroB* mutant of *Burkholderia pseudomallei* was unable to grow in minimal media but growth was restored with the addition of tryptophan, tyrosine, phenylalanine, PABA, and 2,3-dihydroxybenzoate (Cuccui et al., 2007). Our results showed that in contrast, with the wild-type and complemented strains, the shikimate acid pathway mutants could not grow in minimal M9 media. This supports the fact that *aro* mutants of *B. glumae* could not grow in minimal media .The minimal media supplemented with individual aromatic amino acids separately could not restore the growth of the mutants (data not shown) but, minimal media supplemented with all the three aromatic acids restored the growth of mutants as the wild type. However, the growth of *aro* mutants was observed to be less than the wild-type in minimal media supplemented with aromatic amino acids. We believe that, increasing the amount of aromatic amino acids increases the growth of mutants and mutants may have exhaust the exogenous supply of aromatic compounds.

The genes involved in the shikimate acid pathway are associated with pigment production and virulence in rice pathogenic bacteria *Xanthomonas oryzae* pv. *oryzae* (Goel et al., 2001; Park et al., 2009). For instance, mutation of *aroE* gene (encodes shikimate dehydrogenase) and *aroK* (encodes for shikimate kinase) in *Xanthomonas oryzae* pv. *oryzae* affects xanthomonadin production and virulence (Goel et al., 2001; Park et al., 2009). In this study, it was found that the shikimate pathway genes *aroA* and *aroB* are essential for the pigment production, growth of *B. glumae* in M9 medium and the virulence of this pathogen. It will be also an important future study to elucidate how this temperature-dependent differential production of toxoflavin and the pigments is co-regulated and plays a role in the parasitic fitness of *B. glumae* in nature.

CHAPTER 5
BREEDING AND GENETIC STUDIES TO UNDERSTAND BACTERIAL PANICLE BLIGHT AND SHEATH BLIGHT RESISTANCE IN RICE

5.1. Introduction

Rice is an important crop because it is a staple food for more than 50% of the world's population mostly in developing countries. The production and productivity of rice has increased over time due to several traditional and molecular approaches. Unfortunately, the demand of rice is ever increasing due to the rapid growth of population mainly in rice feeding countries. To meet the global food demand, grain production should be increased by 50% by 2025 (Khush, 2001). In the rice production, about 37% rice yield is reduced due to several yield reducing factors, most importantly rice diseases. Bacterial blight, blast, sheath blight, brown spot, stem rot and sheath rot are some of the major rice diseases around the world although more than 40 rice diseases were documented (Latif et al., 2011). It is very difficult for farmers to manage these diseases one at a time. The best way to provide crop protection against these diseases is through plant resistance. That's why plant pathologists are working with plant breeders to incorporate disease resistance genes. Besides, plant disease resistance, many economically important characteristics, such as yield, height, salt tolerance, heading date and cold tolerance are associated with quantitative inheritance. Quantitative traits are controlled by few or many genes that interact with each other and are affected by environment. Due to the development of genetic markers, it became possible to draw marker saturated linkage maps and genotype of individuals in a population and locate quantitative trait loci (QTL) in a chromosome contributing phenotypes. To overcome the problems of classical breeding and to expedite the

process, marker assisted selection has become an important tool in plant breeding for moving genes from one varietal background to another, ultimately developing disease resistant cultivars.

Molecular markers have changed the prospects of plant breeding. The efficiency and effectiveness of breeding program has been greatly increased due to molecular markers as compared to the conventional breeding program. Molecular markers that are tightly linked to the characters are used for screening the phenotypes in marker assisted selection. With the help of alleles of a DNA marker, plants having particular genes or QTL governing certain characters can be identified based on their genotype rather than their phenotype. There are tandem repeated mono to hexa nucleotide motifs abundantly found in eukaryotic genomes known as simple sequence repeats (SSR) or microsatellites. Due to their abundance and variability, SSRs are use as genetic markers (Wang et al., 1994). SSR are abundant, occur frequently and randomly are distributed in eukaryotic genome (Tautz and Renz, 1984). In different organisms, the frequencies of SSR vary greatly (Wang et al., 1994). Dinucleotide repeats $(AC)_n$ and $(GA)_n$ are the most common. The chloroplast DNA, also has microsatellites but their frequency is low (Wang et al., 1994). Hybridization and PCR based categories have been used to exploit microsatellites sequence for the study of DNA polymorphism. In a hybridization based approach, genomic DNA is digested with individual restriction enzymes and hybridized with radio labelled synthetic oligonucleotide probes, complementary to SSR motifs (Weising et al, 1995) whereas in a PCR based approach, DNA polymorphisms are detected by amplification of the flanking region of SSR (Tautz, 1989; Weber and May, 1989) or by using primers complementary to SSR motifs (Wu et

al., 1994). SSR markers are technically efficient and have the multiple potential for high throughput mapping, genetic analysis, and marker assisted crop improvement strategies. The evaluation of genetic diversity in narrowly defined gene pool by using other kinds of molecular markers such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) is not efficient due to the unavailability of polymorphism (Powell et al., 1996). So, SSR markers could be a valuable tool for genetic variability study in a narrowly defined gene pool. SSR markers are commonly used in fingerprint accessions, diversity analysis, identifying introgressions in inter-specific crosses, trace pedigrees, located genes, QTLs identification, and in marker assisted selection (Motley, 2004). These SSR markers are co-dominant, multi-allelic and can be used for Indica and Japonica germplasm as well as wild germplasms (Motley, 2004). Publicly available sequence data of the rice genome provides the basis for high throughput in silicon identification of SSR loci. AT-rich microsatellites tend to show more variation than motifs that are GC-rich, so these could be the best SSRs for rice (Akagi et al., 1996). But, later it was reported that long SSRs are more polymorphic than shorter ones regardless of the motifs, so the length of the SSR repeat unit is important for overall polymorphisms between genotypes (Cho et al., 2000). Therefore, SSRs are classified into two groups based on the length of repeated motifs. Class I (hyper variable markers) consists of 20 nt in length and class II (potential variable) consists of 12 nt and 20 nt in length (McCouch et al., 2002). The comparison of relative frequency of SSRs in coding and non-coding sequence based on rice ESTs showed that SSRs are abundant in coding regions. The rice genome sequence also showed that the regions which are richer in

expressed genes are also tending to be richer in SSR sequences. This has further implications for SSRs as genetic markers. GC-rich tri-nucleotide SSRs concentrated in coding region whereas AT di-nucleotide are scare in EST sequences but abundant in inter-genic region (Cho et al, 2000). Monsanto Rice Genome (MRG) released 6655 SSR containing sequences in rice genome (McCouch et al., 2002). Now, 18,828 SSR markers are publicly available in www.gramene.org. The frequency of most abundant motifs of microsatellites in the rice genome are GA, AT and CCG, respectively (McCouch et al., 2002). Earlier, there were several reports that microsatellites are not clustered in certain regions of the genome but are uniformly. But in the rice genome, there is local clustering of SSR that are non-randomly distributed in GC rich genic region, often with specific components or genes.

Scientists are able to find thousands of QTLs governing certain characteristics but only a few of them are cloned and validated by functional analysis. Among them, very few QTLs governing quantitative disease resistance have been cloned (St Clair, 2010). For example, quantitative resistant loci (QRLs) governing resistance for blast resistance in rice (Fukuoka et al., 2009) and two other for slow rust resistance in wheat (Fu et al., 2009) have been cloned. But for other characteristics of plants, several QTLs have been cloned and validated by function analysis such as fruit weight in tomato (*fw2.2*) (Frary et al., 2000) and grain protein content in wheat (*Gpc-B1*) (Uauy et al., 2006). In rice, photo period response (*Hd1*) (Yano et al., 2000), grain number (*Gn1a*) (Ashikari et al., 2005) , grain length and weight (*GS3*) (Fan et al., 2006), heading date (*Ehd1*) (Doi et al., 2004), plant height (*sd1*), (Sasaki et al., 2002), salt tolerance (*SKC1)* (Ren et al., 2005), cold tolerance *qLTG3-1* (Fujino et al., 2004), submergence tolerance

(*Sub1A*) (Xu et al., 2006) and seed shattering (*sh4*) (Li et al., 2006) have been cloned and characterized.

Bacterial panicle blight (BPB) caused by *Burkholderia glumae* showed spikelet sterility, discoloration of developing grains, panicles extending upward due to unfilled grains (Karki, 2010), and discoloration in sheath in the case of severe infection (Jeong et al., 2003). Environmental conditions such as high temperatures and humidity occurring during the panicle initiation growth stage are favorable for disease development, causing more than 40% yield reduction (Nandakumar et al., 2009). Incidence of BPB around major rice producing areas of the world is increasing possibly due to the current global warming and lack of control measures (Karki, 2010). Because of high yield loss, BPB is the second most important rice disease of Louisiana after sheath blight (Karki, 2010). In spite of its major economic impact and global emergence, the pathogen has not been studied much with regards to its epidemiology, virulence mechanisms and host-pathogen interaction (Ham et al., 2011). Out-breaks of the disease depend upon the occurrence of favorable environmental conditions. There are no reports of effective control measures for this pathogen so far in the United States. Thus, the future prospect of controlling BPB relies on cultivating disease-resistant cultivars. Currently, most of the rice varieties are susceptible to BPB and only few show partial resistance. Three is a need to explore for BPB resistance related QTLs, genes, and transcription factors to improve the control of BPB.

Sheath blight (SB) caused by *Rhizoctonia solani* is one of the major diseases in rice producing countries around the world that affects both the quantity and quality (Wu et al., 2012). The fungus, *Rhizoctonia solani*, has a broad host range that affect about

200 species of weed and cultivated crops including carrot, cotton, rice, maize, potato, soybean, tomato and wheat (Lehtonen et al., 2008). There are fungicides available in the market to control this fungus, but problems associated with field scouting, proper timing of application, fungicide resistance and cost associated with application hinder the success of chemical control for sheath blight. So far, there are no reports of rice germplasm that have immunity to this disease; however, only partial resistance has been reported in a few varieties. Wide variation has been observed among rice varieties for resistance to this disease (Groth and Nowick, 1992) possibly due to the lack of a single gene resistance. However, there are several known resistance genes involved in resistance to this disease. Most importantly, disease response is not consistent even within plots, locations and time of replications. SB development largely depends upon non-genetic factors, such as surrounding environment, temperatures and humidity, plant density, tiller numbers, leaf angle and length, lodging, rate and time of inputs application.

Despite the huge economic importance of BPB and SB in the rice production system of Southern United States, not much is understood about the genetic basis of resistance to these diseases. This study was designed to provide valuable information concerning on the genetic basis of BPB and SB resistance in rice.

5.2. Materials and Methods

5.2.1. Selection of parents and rice population advancements

Out of several released rice varieties in Louisiana, partial resistance line LM-1 and susceptible varieties Trenasse and Bengal were chosen as respective parents for developing mapping populations. Each mapping population was comprised of 300

recombinant inbred lines (RILs) derived from a cross between Bengal and LM-1 and Trenasse and LM-1. These mapping populations were maintained and developed each year. However, for the final genetic study, and phenotyping only RILs developed from Bengal and LM-1 were chosen.

5.2.2. Layout and experiment design

The RILs derived from LM-1 and Bengal were evaluated for BPB and SB severity at the Rice Research Station, Crowley in 2012 and 2013. Experiments were design each year with two replications of single-row plots consisting of about 15 plants per row for sheath blight and about 25 plants for BPB. Purple rice was planted at 4-12 row intervals for proper demarcation of RILs. Along with these, both parents LM-1 and Bengal were included in each replication.

5.2.3. *B. glumae* inoculation and bacterial panicle blight assessment

Panicles of RILs were sprayed with *B. glumae* (OD_{600}= 0.1) at 20 – 30% heading stage in the field. Second, third and fourth inoculations were made at 3 days interval to capture the appropriate stage of panicle initiation among RILs. BPB ratings were done as explained on Chapter 3.

5.2.4. *R. solani* inoculation and disease assessment

RILs were sown at the Rice Research Station, Crowley during mid-March (done by Dr. Donald Groth). One month after sowing, about 15 rice plants were maintained in each row by thinning and transplanting. A virulent strain of *R. solani*, LR172, originally isolated from LA was used to inoculate the rice plants at the panicle differentiation stage, about 45 of planting. Inocula of *R. solani* were produced by using a moist, autoclaved grain/hull mixture (1:2 vol/vol). The mixture with the initial fungal inocula was

incubated at 30°C for 12 to 14 days and further propagated with 1:2 volumes of the fungal mixture and grain/hull mixture and incubating at room temperature for 24 h. Fungus inocula was evenly distributed in each row by hand. Each RIL was evaluated for sheath blight severity approximately 2 weeks before ripening stage. Disease rating was done based a on 0-9 scale, with 0 = no infection and 9 = plants were killed or collapsed (Li et al., 1995a). For example, a rating of 5 indicated, 50% of the height of the plants above water line was infected.

5.2.5. DNA extraction

Plant genomic DNA was extracted by using Cetyltrimethyl Ammonium Bromide (CTAB) method (Clarke, 2009). Briefly, 200 mg of young growing leaves were collected from 10 rice seedlings of about 1 month old and ground into a fine paste with the help of liquid N_2. The fine paste was dissolved in 500 µl of CTAB buffer, RNaseA (1 µl per ml) and mixed. The CTAB/plant extracts mixture was incubated for about 15 min at 55°C in a water bath. After incubation, CTAB/plant extract mixture was span down at 12000 g for 5 min. The supernatant was transferred into a microfuge tube, and 250 µl of chloroform: Isoamyl alcohol (24:1) was added. The solution was mixed by inversion followed by centrifugation at 13000 rpm for 1 min. The upper aqueous phase was transferred to a clean microfuge tube and 50 µl of 7.5 M ammonium acetate was added, followed by 500 µl of ice-cold absolute ethanol. The tube was inverted several times to precipitate the DNA and centrifuged for 1 min at 13000 rpm to precipitate the DNA. The DNA pellet was washed twice with 70% ethanol and allowed to dry at room temperature. The DNA was dissolved in 500 µl H_2O.

5.2.6. Phenotypic and agronomic data collection from RILs

The RILs were phenotyped for bacterial panicle blight (BPB) severity, sheath blight (SB) severity, heading date, plant height, panicle length and flag leaf area. All the data were collected from plants grown in the field at Rice Research Station, Crowley, LA with standard cultivation practices. Heading date from each RIL was recorded when about 50% of the plants in each row were headed. Flag leaf area was calculated by measuring the length and width of each flag leaf about 1 week before the ripening stage. Similarly, plant height and panicle length were measured during the maturity stage. Each trait was taken from three replications.

5.2.7. Polymorphic marker survey between parents

SSR markers were selected from the list of available primers at random covering all the regions of chromosomes form the web site http://www.gramene.org/markers/. These selected primers were used for the polymorphism survey between parents.

5.2.8. Breeding of Lemont/LM-1

Both parents were grown and maintained at Rice Research Station, Crowley, LA. However, about one week before heading they were brought to the green house and male and female parents were grown separately in a pot. Emasculation of the female parent “Lemont” was done by vacuum emasculator (Sha, 2013). Briefly, panicles which had just emerged from the flag leaf sheath or before anthesis were selected for emasculation. About one third of the selected spikelets were cut by scissors to expose anthers, and all the anthers were completely removed with the vacuum emasculator. Immediately after emasculation, the panicles were covered with glassine paper bags. Fully developed panicles were selected from the male parent (LM-1), and hand

pollination was performed from11 am to 1 pm. At peak of blooming stage, the entire part of the flowering panicle was cut and taken to the female plants. The top of the glassine paper bag, which was used for protecting the emasculated spikelets was cut and the male panicle was inserted inside and rotated vigorously to shed pollen on to the emasculated female panicles. The glassine bag was resealed, and this process was repeated another 2-3 times. After a week of pollination, the success of crossing was evaluated by observing the seed set in the panicle. The mature seeds (F1 seeds) from crosses were harvested after one month after pollination. The F1 seeds were planted and crossing between two parents was confirmed by running PCR of two polymorphic SSR primers.

5.3. Results

5.3.1. Screening of SSR markers

350 SSR markers were screened and this covered the whole region of chromosome 2, 10, 11, and 12 among four rice lines namely Jupiter, Trenasse, LM-1 and. The polymorphic survey results showed only 63 polymorphic markers between LM-1 and Bengal as well as 64 polymorphic markers between Jupiter and Trenasse (Appendix A).

5.3.2. Advancement of mapping population

The partially resistant line LM-1 was crossed with the highly susceptible cultivars Trenasse and Bengal to produce breeding and mapping populations. (done by Dr. Xueyan Sha). After the F_2 population, recombinant inbred lines (RILs) were generated following the single seed descent method (Figure 5.1). RILs derived from Bengal and

LM-1 were evaluated for two important rice diseases of Louisiana, bacterial panicle blight and sheath blight, and other important agronomic characters in field every year (since 2009) and harvested for continuation of generation advancements. However, RILs derived from Trenasse and LM-1 were only grown for the generation advancements. Currently 300 RILs exit from each cross at F6 generations.

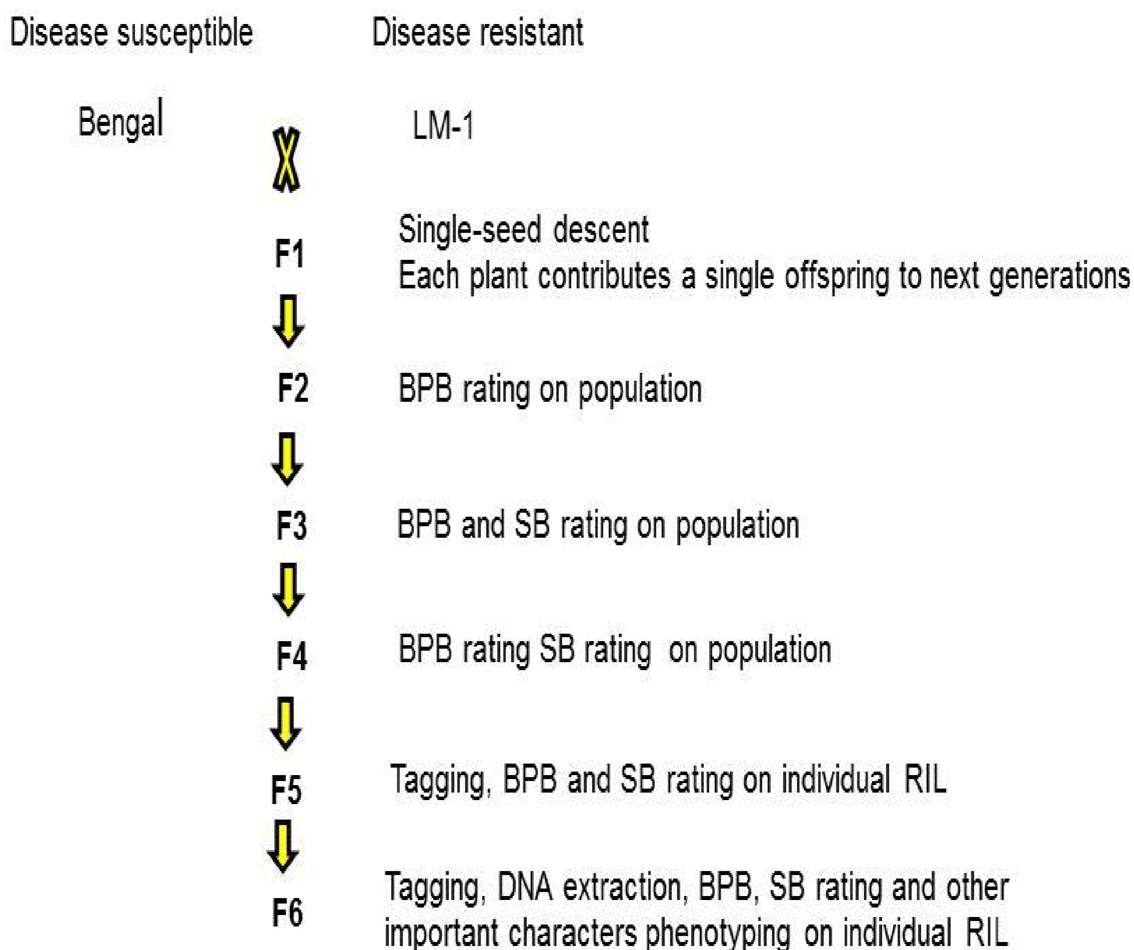


Figure 5.1. A schematic view describing the rice inbred line generation advancement and phenotyping.

5.3.3. Bacterial panicle blight assessment in the mapping population

BPB evaluation is a difficult task due to the fact that susceptible lines may escape infection by BPB, and be regarded as a resistance line. This happens since *B. glumae* is highly sensitive to the temperature and humidity and infection taken place at a certain stage of panicle initiation. So it is very important to design experiments having enough replications and repetitions each year. During 2009, 2010 and 2011 we studied BPB infection at the population level (Figure 5.3) The mean BPB ratings for parents were 3.2 and ratings of 517 F2 segregating lines were 3.1 (Figure 5.3) in 2009. Similarly, in 2010 the mean BPB ratings of 300 segregating lines and parents were 1.7 and 2.2 respectively, (Figure 5.3). In 2011, the average disease ratings of parents as well as segregating lines were slightly increased compared to the previous years (3.7 for parents vs. 3.3 for segregation lines (Figure 5.3). During 2012 and 2013, BPB was assessed for individual RILs with two replications. The average phenotypic distribution of BPB ratings per year and each replication were shown in Figure 5.4. The parental lines slightly differed for BPB response in years with LM-1 ranging from 2.1 to 3.1 and Bengal ranging from 5 to 6.6 respectively, in 2013 and 2012 (Figures. 5.4 and 5.5) The average BPB ratings of RILs were 3.8 and 3.58 during 2012 and 2013, respectively (Figures 5.4, 5.5 and Appendix E). The results showed that some RILs are immune and some are highly susceptible to BPB (Figures 5.4, 5.5 and Appendix E) during the 2 year experiment.

A

B

Figure 5.2. Diverse disease susceptibility/resistance phenotypes in the mapping population for bacterial panicle blight (A) and sheath blight (B). Different levels of infection were shown.

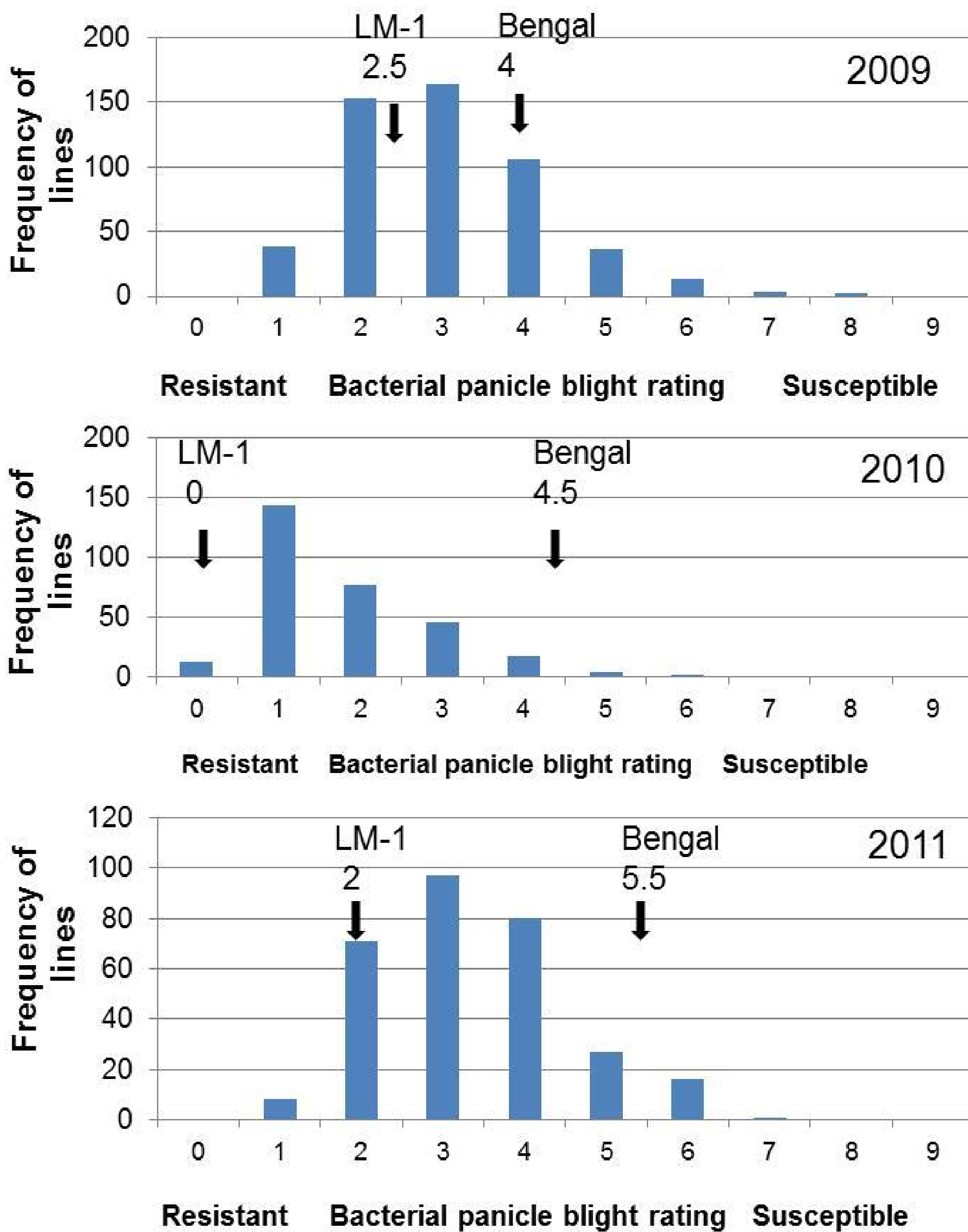


Figure 5.3. The distributions of the segregating lines of the F2 (2009), F3 (2010) and F4 (2011) mapping populations for the cross between Bengal and LM-1 based o the disease ratings for bacterial panicle blight.

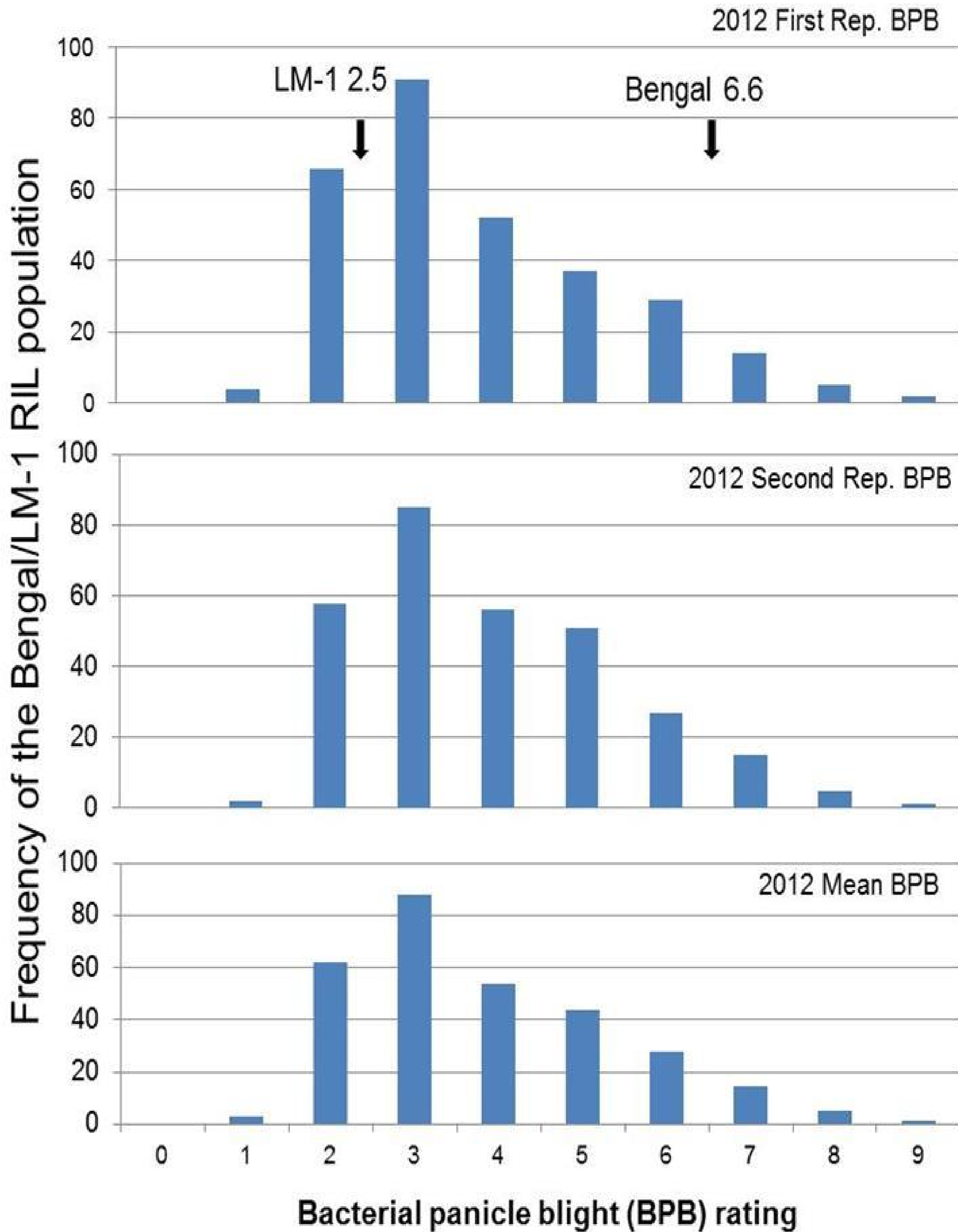


Figure 5.4. The distributions of the F5 recombinant inbred lines from the Bengal/LM-1 cross based on bacterial panicle blight ratings.

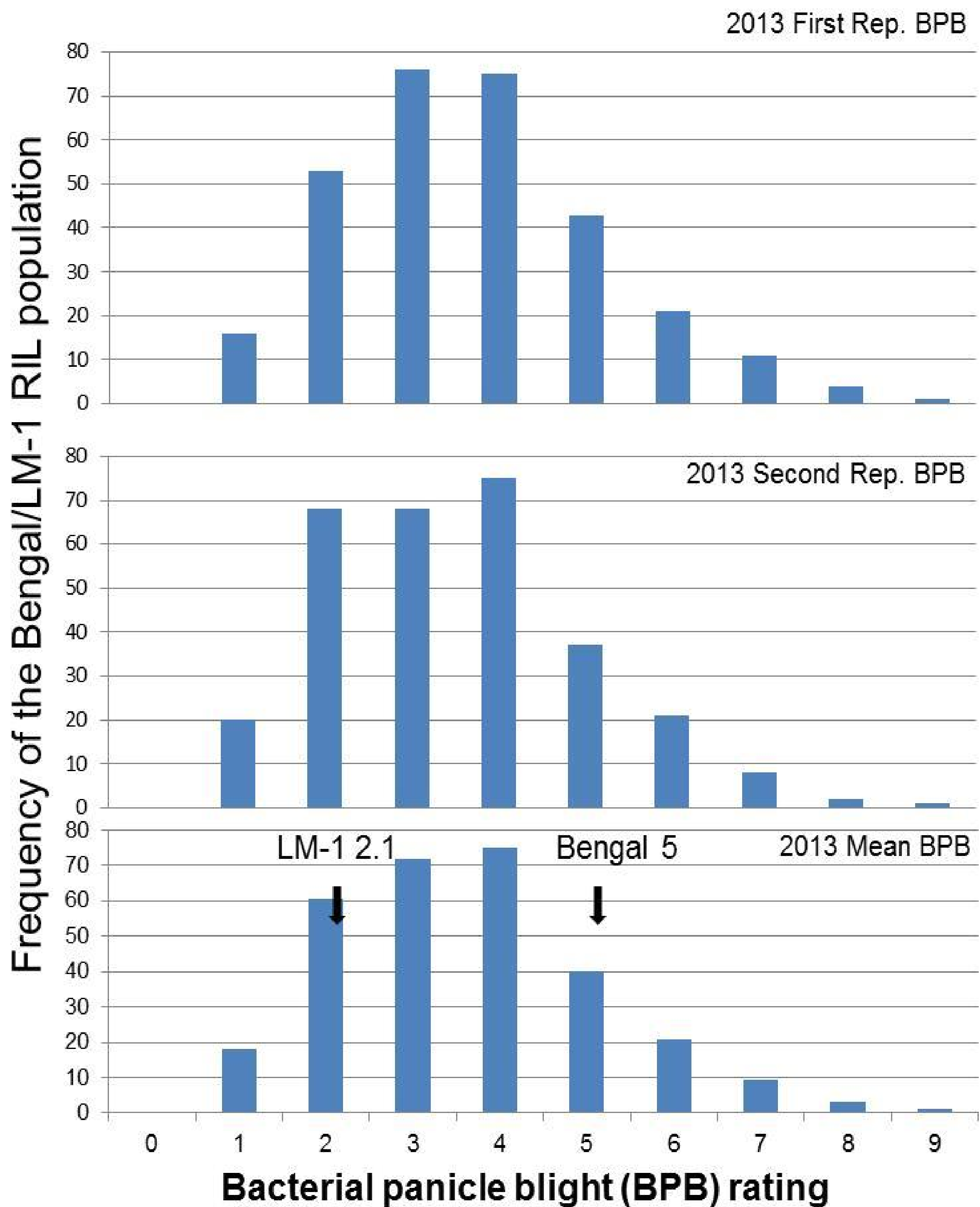


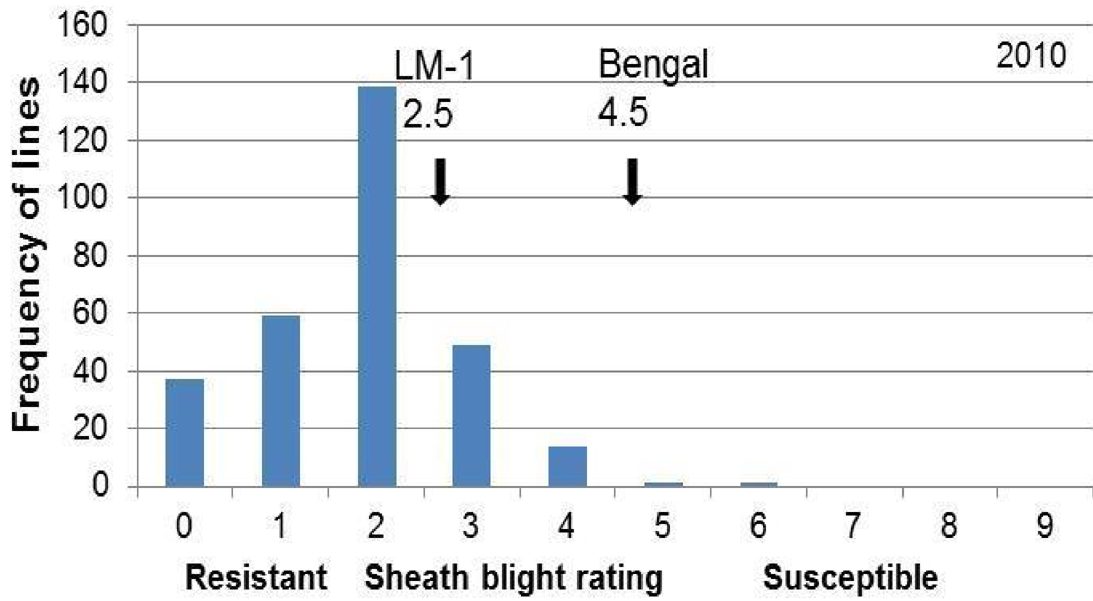
Figure 5.5. The distributions of the F6 recombinant inbred lines from the Bengal/LM-1 cross based on bacterial panicle blight ratings.

5.3.4. Sheath blight assessment in the mapping population

Sheath blight severity was evaluated during 2010, 2011 and 2012 at the Rice Research Station, Crowley, LA. Equal numbers of plants and homogenous spacing between plants were maintained. A sheath blight phenotype assessment was made in each RIL in 2012 however, infection at the population level was studied during 2010 and 2011 (Figure 5.6).The mean SB ratings of parents were 3.5 and the mean rating for 300 F3 segregating lines was 1.18 in 2010 (Figure 5.6). Similarly, in 2011 the mean SB ratings of 300 segregating lines and parents were 3.1 and 4.4, respectively (Figure 5.6). In 2012, the mean disease ratings of RILs were increased compared to the previous years, while the mean parent's ratings did not change significantly over the years (4.3 for parents vs. 4.1 RILs) (Figure 5.7 and Appendix E).

5.3.5. Phenotypic characteristics

The phenotypic values of four traits plant height, heading date, panicle length and flag leaf areas, were scored in 300 RILs along with both parents (Bengal and LM-1) during 2012 and 2013. Considerable variations were detected among RILs phenotypes. Heading date is an important character associated with BPB of rice. Mean heading date of RILs was 113 and 93 days in 2013 and 2012, respectively (Appendix B). In 2012, LB_105 had the shortest (82 days) and LB_46 has the longest (100 days) heading date as compared to LM-1(98 days) and Bengal (93 days) (Appendix B). The average number of heading days for Bengal was 115 days and 120 days for LM-1. LB_58 (104 days) and LB_178 (100 days) were two RILs which had shortest and longest days for heading, respectively in 2013 (Appendix B). Plant height is an important quantitative character associated with sheath blight resistance; it was measured during the maturity



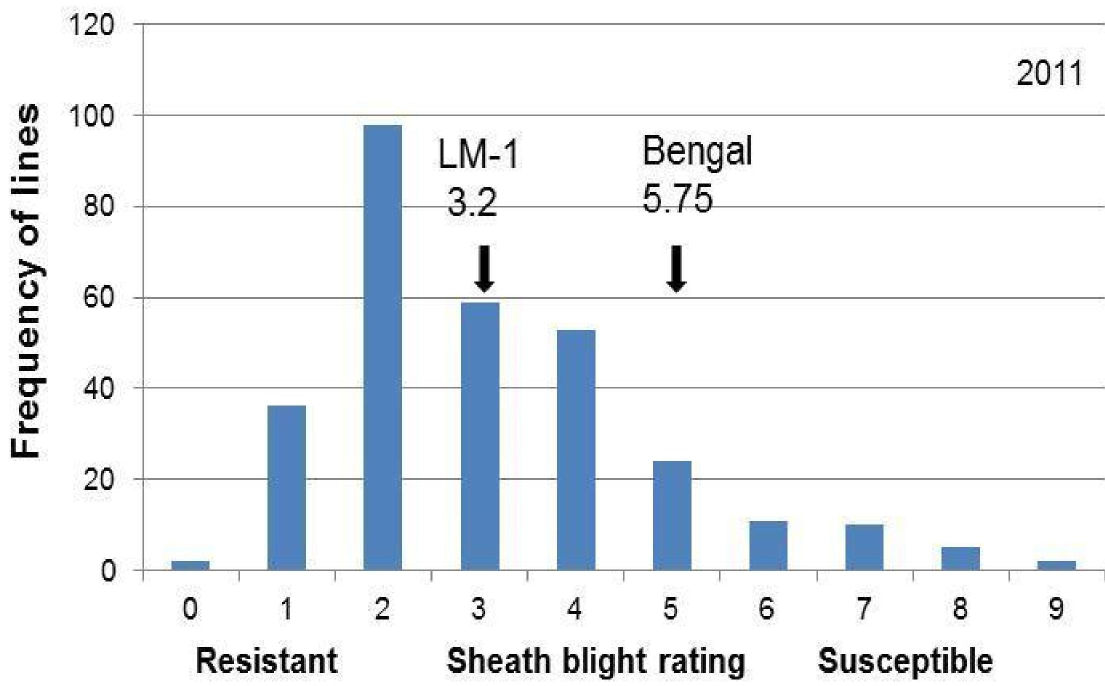


Figure 5.6. The distributions of the segregating lines of the F3 (2010) and F4 (2011) mapping populations for the cross between Bengal and LM-1 based o the disease ratings for sheath blight.

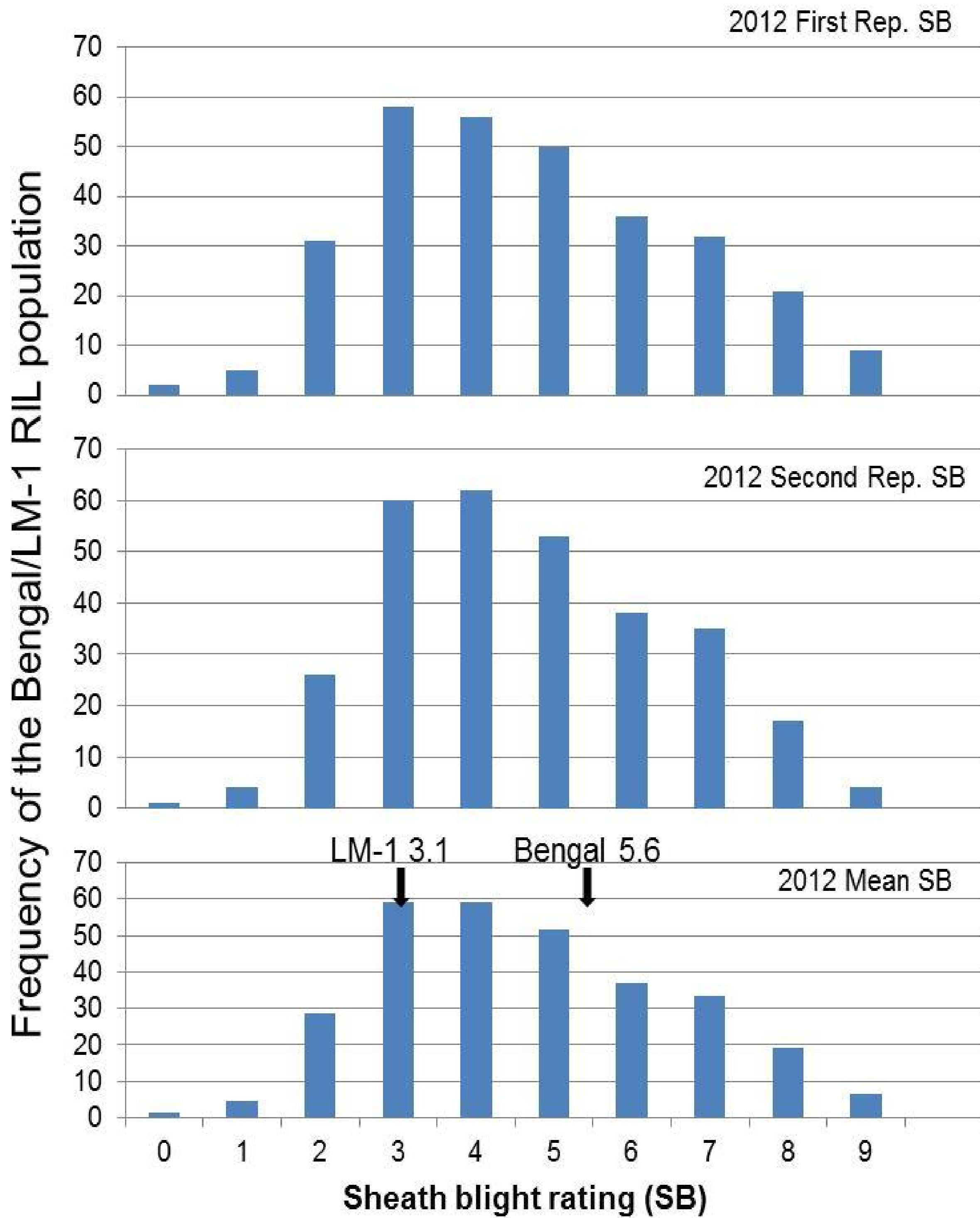


Figure 5.7. The distributions of the F6 recombinant inbred lines from the Bengal/LM-1 cross based on sheath blight ratings.

stage. Between the two parents, Bengal was taller than LM-1 (89.6 cm for Bengal vs. 81.3 cm for LM-1). The tallest RIL was LB_253 (116 cm), the shortest was LB_71 (66.3cm) and the mean height was 93.6 (Appendix C). Panicle length and flag leaf area are two important characters associated with yield. The average panicle length of RILs was 20 cm. LM-1 had a slightly longer panicle than Bengal (19.3 cm for LM-1 vs. 18 cm for Bengal) while LB_184 had the longest panicle (25.6 cm) and LB-55 had the shortest panicle (15.6 cm) (Appendix C). The mean flag leaf area of RIL was 40.4 cm^2. Bengal and LM-1, had flag leaf areas of 43.7 and 38.4 cm^2, respectively. LB_137 has the largest flag leaf area (74.5 cm^2) and LB-109 has the smallest area (20.9 cm^2) among the 300 RILs (Appendix D).

5.3.6. Crossing of LM-1 with Lemont and generation advancement

The cross pollination between two rice lines Lemont and its gamma ray mutant derivatives LM-1 was made during 2012 (Figure5.8). About 550 F1 seeds were obtained from the cross of those parents. 10 F1 plants were selected randomly from 20 F1 plants grown in green the house and the crossing of Lemont/LM-1 was confirmed by polymorphic markers in 5 plants. From those 5 F1 plants, about 500 F2 seeds were harvested and preserved for generation advancement.

5.4. Discussion

Mapping populations have several uses, such as QTL identification, verification and measurement of QTL effects, breeding values, and introgression of interesting characters to an adopted genetic background. The line partially resistant to BPB and SB, LM-1, was crossed to the susceptible cultivar Bengal to produce breeding and mapping population at the Rice Research Station. 300 RILs derived from Bengal and

LM-1 were evaluated for BPB and SB resistance and other important agronomic characters in the field each year from 2009.

Figure 5.8. F1 seeds obtained from the cross between Lemont and its mutant derivative LM-1.

We studied response of individual RIL to BPB in 2012 and 2013. We found that BPB developed more profusely in 2012 than 2013 which was evident form the BPB response of both parents and RILs in 2012 and 2013. Since development of BPB is highly dependent upon existing weather conditions, we experienced considerably low temperatures during rice growing season in 2013, and that might be the reason for low

occurrence of BPB**.** A day to head is an important trait of rice which plays significant role in adaption to different cultivation areas and cropping seasons (Yamamoto et al., 2000). Previous, study showed that days to heading is correlated with BPB severity and the putative QTLs mapped for heading date is also mapped with QTLs for BPB resistance (Pinson et al., 2010). It might be possible that late heading rice plants are exposed to the cooler temperature which is not conducive for pathogen growth. We scored heading date of each RILs during 2012 and 2013 and found that days to heading in 2013 took considerably longer days than previous growing season. The rice growing season was accompanied with relatively low temperature in 2013, so heading days might take relatively longer days.

Previously, it was reported that, heading days, plant height and panicle number is associated with sheath blight resistance (Li et al., 1995b). Rice cultivars with late heading days are more resistant to sheath blight than those with early heading days (Park et al., 2008). Most of the current US rice cultivars are high yielding and having good agronomic characters but they are susceptible to SB (Zhu et al., 2000). The results of the sheath blight severity assay of 300 RILs under the field condition during multiple years showed a considerable amount of variation among RILs and some of these lines could be useful for rice breeding program.

The other important agronomic characters, such as plant height panicle length and flag leaf area were scored in the 300 RILs along with both parents (Bengal and LM-1) during 2012 and 2013. There was considerable amount of variation in these characters among RILs. Rice plant height is an important agronomic characteristic

associated with yield that is regulated by quantitative genes (Wang and Li, 2005). Plant height is also associated with sheath blight resistance (Zou et al., 2000).

Panicle length is an important yield component of rice which is highly influenced by depth of flooding water, plant density, and weather conditions such as light and temperature (Ahamadi et al., 2008) and fertilizer level**.**

Genetic diversity among crop cultivars is a possible solution for disease management (Browning and Frey, 1969). Planting genetic diverse rice cultivars provides a greater disease suppression than monoculture of uniform rice lines (Zhu et al., 2000). SSR polymorphism study between four cultivars namely, Jupiter, Trenasse, LM-1 showed that less polymorphism exit among those rice lines. All the above mentioned rice varieties (parents) are well adapted US rice cultivars, since they have narrow genetic base. Rice cultivars released after 1994 in the United States were developed from crosses from older cultivars or breeding lines (Personal communication Dr.Sha). That might be the excellent reason for the low polymorphic SSR markers between our parents.

The most important and challenging tasks in rice breeding program is to combine all agronomically important and disease resistance characteristics into a single cultivars (Jia et al., 2012). This study identified rice lines that were resistance to BPB and SB along with superior agronomic characteristics. These rice lines will be useful resource for developing new cultivars as well as for studying the genetic basis of quantitative disease resistance.

CHAPTER 6
GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF LB-33, A HIGH YIELDING RICE LINE WITH SHEATH BLIGHT AND BACTERIAL PANICLE BLIGHT RESISTANCE

6.1. Introduction

Rice is one the most important cultivated crops in the world, since about 50% of the total population depends on it for their calorie supply. The United States only produces 2% of the world rice but is the fourth largest exporter only behind Thailand, Vietnam and India. The major rice breeding effort in the United States is concentrated on the further improvement of yield potential, quality, disease resistance, seedling vigor and other agronomic characters. Bacterial panicle blight (BPB) caused by *Burkholderia glumae* and sheath blight (SB) caused by *Rhizoctonia solani* are the major diseases of rice in the Southern United States contributing significant yield loss. Under favorable conditions for disease development, BPB can cause up to 70% yield reduction (Nandakumar et al., 2009) and SB can cause up to 50% yield reduction (Marchetti and Bollich, 1991) when highly susceptible cultivars are grown. Current commercial rice cultivars grown in Louisiana are early-maturing, semi-dwarf, resistant to lodging, upright plant type, high tillering and high yielding but they are susceptible or very susceptible to BPB and SB (Groth et al., 2007; Rush et al., 2011; Rush et al., 2007). For the control of BPB, no chemicals are registered in the United States. Some chemical control methods are available for SB but this approach is associated with risk to environment and costs. The best way to control diseases is incorporation of resistance genes into adapted cultivars. The objective of this project was to develop BPB and SB resistance, early-maturing, high yielding rice lines with potential for release a Southern US Cultivar.

6.2. Materials and methods

6.2.1. Cross and generation selection

LB-33 was selected from a cross between Bengal (Linscombe et al., 1993) and LM-1 (Groth et al., 2007) that was made by Dr. Xueyan Sha in 2008 at the Louisiana State University Agricultural Center, Rice Research Station in Crowley, LA. Bengal was used as a female parent which is early maturing, high yielding, medium grain cultivar, developed at Rice Research Station at Crowley, LA in 1992 with the pedigree of 'MARS'//'M201'/MARS made at the Rice Research Station in 1983 (Linscombe et al., 1993). LM-1 was used as a male parent, is a mutant germplasm line developed at the Rice Research Station at Crowley, LA by irradiation of the US cultivar Lemont (Bollich et al., 1985; Groth et al., 2007). LM-1 has general agronomic characteristics similar to Lemont and improved disease resistance to sheath blight, bacterial panicle blight and narrow brown leaf spot (Groth et al., 2007).

F1 seeds were planted in the greenhouse, and after close examination of possible selfed plants, F1 plants were bulk-harvested in spring 2008. Then, F_2 population of about 1500 plants was space planted in 100 rows each containing 15 plants. From that population, about 300 plants were selected representing each row and panicles were harvested in 2008. Since then generation advancements were done at the Rice Research Station, Crowley, LA in 2009, 2010, 2011 and 2012. Each year, one plant was selected from a row containing about 15-20 plants, and seeds from a single plant were planted in a row the next year. During 2012, LB-33 was identified as an outstanding line that showed enhanced disease resistance to sheath blight and bacterial

panicle blight as well as interesting agronomic traits associated with high yield. Advance genotypic and phenotypic studies were conducted on LB-33 in 2013.

6.2.2. Methods for agronomic and botanical trait characterizations

LB-33 was characterized in comparison with its parents Bengal and LM-1 in two different environments, i.e. in the greenhouse and field. For greenhouse experiments, rice seeds were germinated in plastic pots (15 cm diameter by 20 cm height) containing a soil mixture of clay, Jiffy MixH (Ferrry-Morse Seed Co, Fulton, KY, USA) and sand in a 3:2:1 ratio. After 24 days, four rice seedlings were transplanted in each pot. Then the rice plants were fertilized with Nitrogen containing fertilizer (Urea) at tillering and 1 week before heading stage. For field experiments at the Rice Research Station (Crowley, Louisiana, USA), all the three rice lines were grown in rows (12 to 15 plants per row) with ca. 30 cm intervals between rows. The characteristic features of soil at LSU AgCenter Rice Research Station are silt loam soil (fine, mixed, thermic Typic Albaqualfs) with 1.1% organic matter and pH 5.8 (Linscombe et al., 2004). Nitrogen (as urea) was applied at 68 kg N per acre prior to permanent flooding. A permanent flood was established about 4 weeks after seedling emergence and about ten cm water level was throughout the season. In both experimental conditions, ten rice plants were randomly chosen and phentoyped for, plant height, heading date at vegetative and maturity stage, leaf area, flag leaf area and panicle length. Plant height at vegetative stage was measured when the plants reached to the 5th leaf growth stage, heading date was recorded when about 50% of plants were headed and other characters were recorded at maturity stage. Characters associated with seed and milling quality were performed after harvest in the under laboratory.

6.2.3. DNA extraction and genotyping with SSR polymorphic markers

About 30 representative seeds were planted in greenhouse selected from 30 different plants of LB-33 in F6 generation and one young leaf was collected from each seedlings. Total DNA was extracted from leaves by the CTAB method (Murray and Thompson, 1980) and described in previous chapter 5.

The polymorphism survey between Bengal and LM-1 was conducted using publicly available SSR markers of rice from www.gramene.org and some of the primers were previously associated with sheath blight resistance. Then, 33 polymorphic SSR markers were selected randomly (Table 6.1) and used for genotyping of LB-33 in the F6 generation. PCR amplifications were performed in 25 µl reaction mixtures containing 1.0 µl (70 ng) DNA, 2.5 µl of 10X PCR buffer, 0.5 µl of 10 mM dNTPs, 1.25 µl of each forward and reverse primers (10 pM of each primer), 0.2 µl of Paq 5000 DNA polymerase (Agilent Technologies, Inc. CA, USA) and 18.4 µl H_2O. PCR consisted of an initial denaturation for 5 min at 95 °C; 34 cycles of 4 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C; followed by a final extension for 5 min at 72 °C. PCR products were separated by electrophoresis in 4 % Agarose SRF^{TM} gel (AMRESCO LLC, OH, USA) at 180 V for 4 h.

6.2.4 Statistical analysis

Data were analyzed using SAS software, version 9.2 (SAS Institute). Analysis of variance for grain yield, milling yield, plant height, leaf area, flag leaf area, panicle's characteristics, and days to 50% heading were performed separately for field and greenhouse data with at ten replications.

6.3. Results

6.3.1. Genotyping with polymorphic markers

Electrophoresis analysis of PCR products derived from genomic DNA of LM-1, Bengal and LB-33 was performed, and homozygosity of LB-33 was confirmed by the polymorphic primers between LM-1 and Bengal. The results from 33 polymorphic primers showed that only six primers, (RM104, RM3870, RM20612, RM5414, RM16459, and RM5961), corresponded to LM-1 and LB-33, while the rest of the primers corresponded to LB-33 and Bengal (Table 6.1).

6.3.2. Grain description

Bengal has typical U.S. media grain rice characteristics (Linscombe et al., 1993). LB-33 had the following seed dimensions: 8.6 mm length, 2.7 mm width, 1.9 mm thickness and 28.43 mg weight of each seed. Seed length of LB-33 is intermediate between the two parents i.e. shorter than LM-1 but longer than Bengal however they are not statistically different with each other (Table 6.2). In terms of width, thickness and weight LB-33 was statistically closely related to Bengal than LM-1 (Table 6.2).

6.3.3. Agronomic and botanical description

LB-33 possesses a height taller than both parents LM-1 and Bengal which are considered to be semi-dwarf rice cultivars. During the vegetative growth stage, Bengal was taller than LB-33, however, as the plants reached reproductive stage, Bengal was overtaken by LB-33 under all experimental conditions. LB-33 had an erect flag leaf orientation, and leaves are dark green. LB-33 had a longer and wider flag leaf than both of the parents under both greenhouse and field growing conditions which are significantly different from each other (Table 6.3). The mean flag leaf area of LB-33 was

80.22 cm^2 whereas it was 45.45 and 40.26 cm^2 for LM-1 and Bengal, respectively. From vegetative to reproductive stages, LB-33 had were bigger and wider leaves than its parents.

Table 6.1. Polymorphic simple sequence repeat used for genotyping.

Markers	LM-1	Bengal	LB-33	Chr.	References
RM5389	L	U	U	1	(McCouch et al., 2002)
RM104	L	U	L	1	(Akagi et al., 1996)
RM14304	U	L	L	2	(Matsumoto et al., 2005)
RM3838	U	L	L	5	(McCouch et al., 2002)
RM1237	L	U	U	5	(McCouch et al., 2002)
RM3870	L	U	L	5	(McCouch et al., 2002)
RM19387	L	U	U	6	(Matsumoto et al., 2005)
RM527	U	L	L	6	(Temnykh et al., 2001)
RM20216	L	U	U	6	(Matsumoto et al., 2005)
RM20612	U	L	U	6	(Matsumoto et al., 2005)
RM20774	U	L	L	6	(Matsumoto et al., 2005)
RM527	U	L	L	6	(Temnykh et al., 2001)
RM1093	U	L	L	7	(McCouch et al., 2002)
RM3710	U	L	L	8	(McCouch et al., 2002)
RM152	L	U	U	8	(Akagi et al., 1996)
RM22899	L	U	U	8	(Matsumoto et al., 2005)
RM3710	U	L	L	8	(McCouch et al., 2002)
RM152	L	U	U	8	(Akagi et al., 1996)
RM22899	L	U	U	8	(Matsumoto et al., 2005)
RM23959	U	L	L	9	(Matsumoto et al., 2005)
RM5414	L	U	L	9	(McCouch et al., 2002)
RM16459	L	U	L	9	(Matsumoto et al., 2005)
RM23982	U	L	L	9	(Matsumoto et al., 2005)
RM24779	L	U	U	9	(Matsumoto et al., 2005)
RM24702	U	L	L	9	(Matsumoto et al., 2005)
RM27080	L	U	U	9	(Matsumoto et al., 2005)
RM257	L	U	U	9	(Chen et al., 1997)
RM5961	L	U	L	11	(Chen et al., 1997)
RM27080	L	U	U	11	(Matsumoto et al., 2005)
RM28230	L	U	U	11	(Matsumoto et al., 2005)
RM202	L	U	U	11	(Chen et al., 1997)
RM167	L	U	U	11	(Wu and Tanksley, 1993)
RM224	U	L	L	11	(Chen et al., 1997)

The leaf area of second leaf counting from the flag leaf in LB-33 was not significantly different with its parents under greenhouse conditions; however it was significantly different than both parents under field conditions (Table 6.3). The panicle length of LB-33 was significantly longer than both parents under both greenhouse and field conditions (Table 6.5). Panicle length for LM-1 and Bengal were not significantly different from each other. The mean panicle length of LB-33 from the greenhouse and field was 27.8 cm, compared to 21.6 and 22.2 cm for LM-1 and Bengal, respectively. LB-33 had the highest number of secondary panicles in a panicle than both parents. Under greenhouse, the number of secondary panicles per panicle of LB-33 was statistically similar to Bengal but different than LM-1. However under field conditions, LB-33 had statistically different number secondary panicles per panicles than both parents. The average number of secondary panicles per panicle of LM-1, Bengal and LB-33 were 12, 13 and 15, respectively (Table 6.5). The number of filled grains per panicle of LB-33 was significantly higher than both parents under both growing conditions. Under field conditions LM-1 and Bengal had statistically similar numbers of seeds per panicle while in the greenhouse they had statistically different seeds number per panicle (Table 6.5). Overall, LB-33 had average of 208.4 seeds per panicle while LM-1 and Bengal had 147.47 and 174.40 seeds per panicle, respectively. The weight of each panicle of LB-33 was significantly higher than both parents under both growing conditions.

Table 6.2. Grain dimensions and weights of LM-1, Bengal and LB-33.

Cultivar	Length	Width	Thickness	Length/Width ratio	Weight
		mean ±SD(mm)			mg
LM-1	8.69±0.29a	2.45±0.08b	1.92±0.11b	3.544	23.55±2.06b
Bengal	8.47±0.30a	2.96±0.11a	2.08±0.07a	2.862	28.65±3.27a
LB-33	8.66±0.28a	2.72±0.125a	1.99±0.9a	3.177	28.43±2.24a

Table 6.3. Leaf and flag leaf areas of LM-1, Bengal and LB-33.

	Leaf area (cm^2)			Flag leaf area (cm^2)		
	LM-1	Bengal	LB-33	LM-1	Bengal	LB-33
Green house	50.48±10.48a	45.85±10.05a	47.17±14a	52.63±9.37c	39.89±9.44b	89.6±14.80a
Field	46.24±8.52b	40.71±5.96b	59.71±5.61a	38.18±4.34b	40.71±5.96b	70.71±9.63a
Mean	**51.07**	**43.28**	**54.69**	**45.45**	**40.26**	**80.22**

Table 6.4. Plant heights of LM-1, Bengal and LB-33 at the vegetative and reproductive stages

	Height (cm) at vegetative stage			Height (cm) at reproductive stage		
	LM-1	Bengal	LB-33	LM-1	Bengal	LB-33
Green house	49.65±2.89c	75.33±3.22a	68.58±6.47b	88.82±6.55c	91.69±4.97b	114.06±7.59a
Field	54.35±3.81c	80.26±4.8a	67.69±6.59b	79.2±1.93b	83±4.78b	103.1±5.27a
Mean	**52**	**77.79**	**68.13**	**84.01**	**87.39**	**108.58**

A

B

LM-1 LB-33 Bengal

Figure 6.1. Plant heights of LM-1, Bengal and LB-33 at the vegetative and reproductive growth stages. A) Vegetative stage. B) Reproductive stage

The average weights of each panicle of LM-1, Bengal and LB-33 were 3.066, 3.56 and 5.07 grams, respectively (Table 6.5). Consistent with weight of panicles, LB-33 had significantly higher yield per panicle than both parents under the different growing conditions. The average weights of seed from each panicle of LM-1, Bengal and LB-33 were 2.59, 3.04 and 4.58 g respectively (Table 6.5).

Table 6.5. The panicle associated characteristics of LM-1, Bengal and LB-33.

LM-1

	Panicle L (cm)	Secondary panicle	Seed/panicle	Wt/panicle (gm)	Yield/panicle (gm)
Greenhouse	23.5±2.05b	11.1±1.44b	137.9±22.28c	2.87±0.48c	2.46±0.48c
Field	19.8±1.22b	13.9±0.99b	157±18.80b	3.26±0.41b	2.724±0.59 b
Mean	**21.65**	**12.5**	**147.45**	**3.065**	**2.592**

Bengal

	Panicle L (cm)	Secondary panicle	Seed/panicle	Wt/panicle (gm)	Yield/panicle (gm)
Green house	24.55±2.3b	13.2±1.39a	176.2±33.08b	3.59±0.61b	3.23±0.56b
Field	20±1.33b	14.5±2.27b	172.6±23.20b	3.53±0.76b	2.84±0.75b
Mean	**22.28**	**13.85**	**174.40**	**3.56**	**3.04**

LB-33

	Panicle L (cm)	Secondary panicle	Seed/panicle	Wt/panicle (gm)	Yield/panicle (gm)
Green house	30.7±2.52a	14.3±1.7a	209.4±17.10a	5.1±0.66a	4.619±0.71a
Field	25±1.49a	16.9±1.19a	207.4±27.69a	5.04±0.71a	4.55±0.61a
Mean	**27.85**	**15.6**	**208.4**	**5.07**	**4.5845**

The days to 50% heading were different for parent cultivars and LB-33. The days to 50% heading for LM-1, Bengal and LB-33 were 98, 93 and 96 days in 2012, and 120, 118 and 115 days in 2013, respectively, under field (Table 6.6). All the rice lines headed early in under the green house with 89, 81, 86 and and for LM-1, Bengal and LB-33, respectively (Table 6.6).

Table 6.6. The milling qualities and heading days of LM-1, Bengal and LB-33.

Rice	Milling %*		Days to 50% heading	Days to 50% heading	Days to 50% heading
	Head rice	Total rice	Field (2012)	Field (2013)	Greenhouse(2013)
LM-1	56.2	66.75	98	120	89
Bengal	65.15	68.58	93	115	81
LB-33	64.61	67.09	96	118	86

* Measured by Dr. Donald Groth and his lab members

6.3.4. BPB and SB resistance and impacts on yield

Sheath blight and bacterial panicle blight resistance were evaluated in 2012 and 2013. LB-33 had mean SB ratings of 1.66 (Table 6.7). Parental lines LM-1 and Bengal had sheath blight ratings 2.74 and 5.97 (Table 6.7). In regards to resistance to bacterial panicle blight of rice, LB-33 rated 1.56 compared with 1.93 and 5.95 ratings for LM-1 and Bengal, respectively (Table 6.7).

Yield was compared among LB-33 and its parents (LM-1 and Bengal) that were inoculated with *B. glumae* and *R. solani* and those non-inoculated. Both BPB and SB impacted yield more reduction on Bengal than LB-33 and LM-1. No difference in yield was detected between healthy and diseased plants for LB-33 (Figure 6.2).

Table 6.7. The diseases ratings of LM-1, Bengal with LB-33.

	Sheath blight			Bacterial panicle blight		
Year	LM-1	Bengal	LB-33	LM-1	Bengal	LB-33
2012	3.37	5.62	2	2.5	6.62	1.5
2013	2.12	6.33	1.33	1.37	5.25	1.62
Mean	2.74	5.97	1.66	1.93	5.93	1.56

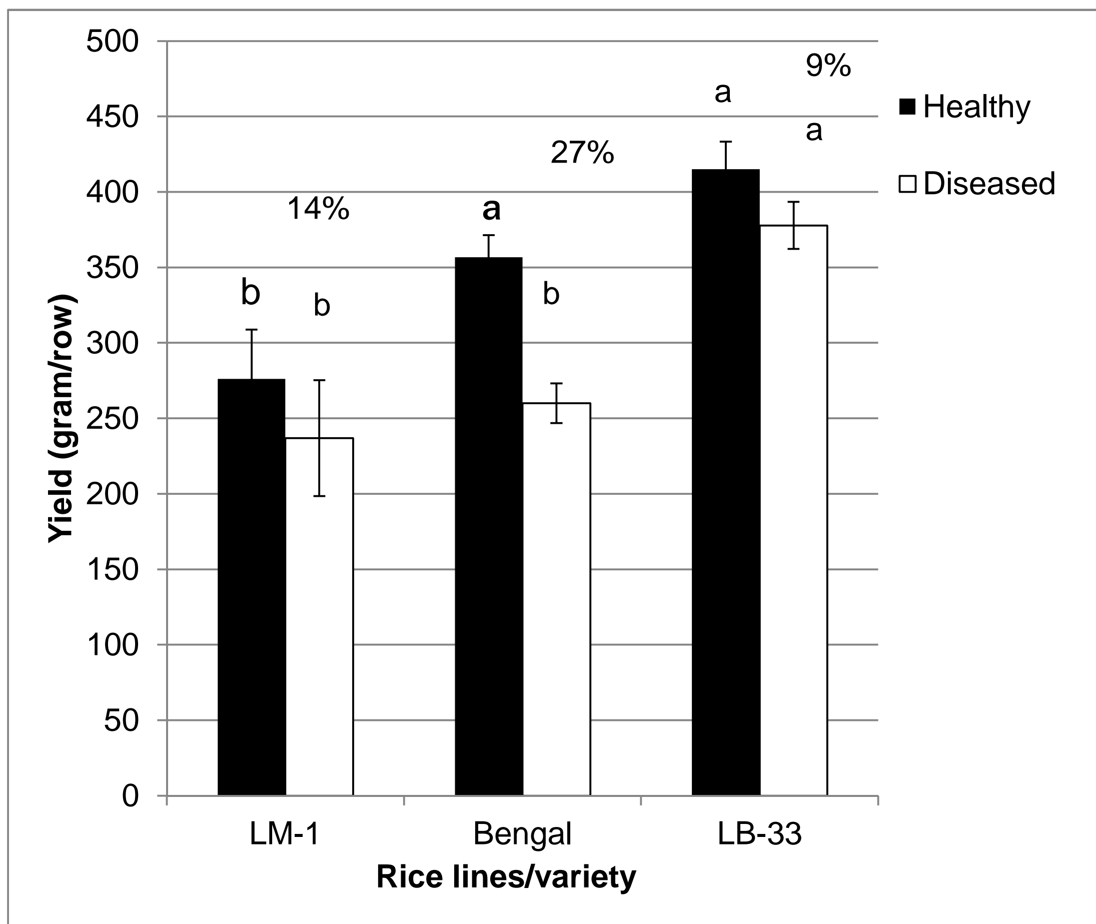


Figure 6.2. The effect of bacterial panicle blight and sheath blight infection on yield. Rows of LB-33, LM-1 and Bengal were inoculated with *B. glumae* and *R. solani*, harvested and percent yield reduction by BPB and SB was compared to non-inoculated control. Small case letters indicate statistically significant differences ($P > 0.01$) among treatments.

6.4. Discussion

This study characterized an inbred rice line, LB-33 with bacterial panicle blight and sheath blight resistance and high yield. To elucidate the superiority of LB-33 in terms of BPB and SB resistance as well as other important agronomic characters associated with high yield, a comparative study with its parents (Bengal and LM-1) was performed under two different environmental conditions. The important result of this study is that LB-33 achieved higher yield and showed resistant phenotypes to BPB and SB compared to parents, LM-1 and Bengal. The high yield of LB-33 may be due to a higher panicle length, number grains per panicle and larger flag leaf area.

LB-33 had significantly higher panicle length than both parents. Longer panicles have more secondary panicles and grains, and this is a contributing factor for high yield. LB-33 has the potential for producing high yield due to higher number grains per panicle. Also, LB-33 produced significantly higher yield per panicle than both parents under two different growing conditions.

Taller rice plants with a high degree of lodging resistance are important for forage and biofuel production. Semi-dwarf lines sustained more than double yield and milling qualities reduction than standard height plants (110-120 cm) due to the effect of SB (Marchetti, 1983). In addition, tall plants create better air transmission, and reduced relative humidity within plant canopy and this make the micro climate unfavorable for sheath blight development (Xing et al., 2003). LB-33 was taller than both of the parents which were considered as semi-dwarf rice. The average height of LB-33 was 108.6cm which might be suitable height for reduction of sheath blight infection in the field. During vegetative stage, Bengal was taller than LB-33, however, as the plants reached the

reproductive stage, Bengal was overtaken by LB-33 under all experimental conditions. LB-33 showed intermediate length in days to heading of the two parents under both growing conditions. Delayed heading might contribute to decreased BPB infection in the field by avoiding the favorable environment (high temperature and humidity) for pathogen growth especially; BPB has such a narrow window for infecting the flowering panicle.

Normally, plants grown under greenhouse are shorter and produce fewer yields as compared to filed grown plants. However, in our test, greenhouse grown plants are taller and produce more yields. An additional application of N_2 fertilizer at 1 week before heading and created soils with high organic matter than in the field might be the reason for superior agronomic traits of LB-33 in greenhouse. LB-33 showed more response to the extra dose of fertilizers, so LB-33 has more chance to increase yield by better crop management.

Most of the rice cultivars grown in Louisiana are susceptible to sheath blight and bacterial panicle blight, however, some rice cultivars shown partial resistance to bacterial panicle blight of rice (Blanche et al., 2012). A significant difference in sheath light and bacterial panicle blight was found among LM-1, Bengal and LB-33. Since LB-33 showed almost an immune response to the most important rice diseases of Louisiana, it might successfully incorporate resistance into the breeding program. BPB alone can reduce yield up to 75% in rice under the heavy infection in highly susceptible cultivars (Nandakumar et al., 2009). In case of severe infection, sheath blight can cause yield reduction by 12 to 49% (Wu et al., 2013). It was shown that LB-33 had the least and Bengal had the most yield reductions by BPB and SB. Interestingly there was no

significant different between healthy and diseased plant's yield of LB-33. Under field conditions of Louisiana inoculation with *R. solani* increases the incidence and severity of sheath blight and yield reduction ranged from 4% reduction in moderately susceptible cv. Francis to 21% in very susceptible cv. Cocodrie (Groth and Bond, 2007). For standard height rice plants, SB rating scales of 5 and 6 reduced the yield by 10 and 12% respectively (Marchetti, 1983). Bengal being a moderately SB susceptible cultivar, the 27% yield reduction might primarily due to the effect of BPB.

This study showed that there are significant improvements of LB-33 over its parents in terms of panicle length, number of grains per panicle, yield per panicle and larger flag leaf area. The high yield of LB-33 over its parents is due to the improvement of those yield attributing characters. Most importantly, LB-33 possesses high levels of resistance to bacterial panicle blight and sheath blight. So this rice line can be used in the future for the development of high yielding disease resistant rice cultivars.

CHAPTER 7
CONCLUSION

Random transposon mutagenesis has been widely used to identify the genetic elements underlying the virulence mechanism of different plant pathogenic bacteria. In this study we used mini-Tn*5gus* transposon to investigate the genetic basis of pigment production of *B. glumae* strain 411gr-6. We screened about 30,000 mutants and 50 mutants showing no, less and over production of pigment were selected for further study. Among identified genes related to pigment production, some genes are already known to be related with the pathogenesis of this pathogen. So it is interesting to know how the pigment production and pathogenesis is related in *B. glumae*. We demonstrated that a newly found TCRS, PidS/PidR, is an essential regulatory component of *B. glumae* for pigmentation in CPG medium, HR elicitation in tobacco, and full virulence in rice and onion. Remarkably, it was found that orthologs of PidS and PidR are highly conserved in many *Burkholderia* spp. *B. gladioli*, another bacterium causing BPB in rice (Ham et al., 2011), contains PidR and PidS orthologs showing highest homology with PidR and PidS. In this study we also provided the evidence that mutation in the genes involved in shikimate acid pathway (*aroA* and *aroB*) of *B. glumae* strain 411gr-6 resulted in lack of pigment production, fail to grow in M9 medium and almost non-pathogenic to rice. Also these mutants showed reduced tolerance to UV light. To the best of our knowledge it is the first report of involvement of shikimate acid pathway in virulence related phenotypes of plant pathogenic bacterium, *B. glumae*.

Mapping populations have several uses such as QTL identification, verifications and measurement of QTL effects, breeding values and introgression of interesting

characters to an adapted genetic background. The line partially resistant to BPB and SB, LM-1, was crossed to susceptible cultivar Bengal to produce a breeding and mapping population at the Rice Research Station, Crowley in 2008. 300 RILs derived from Bengal and LM-1 were evaluated for BPB and SB resistance and other important agronomic characters in the field from 2009. Several RILs showed enhanced BPB and SB resistance as well as other superior yield attributing characteristics. BPB and SB resistant line, LB-33 was characterized for resistance and also showed some promosing agronomic traits. To elucidate the superiority of LB-33 and in terms of BPB and SB resistance as well as other important agronomic characters associated with high yield, a comparative study with its parents (Bengal and LM-1) was performed in two different environmental conditions. A comprehensive study showed that there is significant improvement of LB-33 over its parents in terms of panicle length, number of grains per panicle, yield per panicle and larger flag leaf area. The high yield of LB-33 over its parents is due to the improvement of those yield attributing characters. Most importantly, LB-33 possesses high levels of resistance to bacterial panicle blight and sheath blight. So this rice line can be used for future breeding for the development of high yielding, disease resistance rice cultivars.

The role of non-pathogenic strains of *B. glumae* for the ability to suppress BPB and SB blight of rice was investigated. Since BPB and SB did not offer many viable options for their control, use of biological control agents can be alternative option. In a previous study, it was shown that some *B. glumae* strains isolated from the Southern United States were non-pathogenic to rice and did not cause any significant yield reduction when inoculated (Karki et al., 2012b). This study focuses on non-pathogenic

strains of *B. glumae* that were isolated from the same ecological niches and already verified that they cannot cause disease on rice. The naturally occurring non-pathogenic strains could suppress the disease caused by virulent strains. The ability of nine pathogenic and 11 non-pathogenic strains of *B. glumae* were tested for the ability to restrict the growth of *R. solani*. Some of the naturally avirulent strains showed antifungal activities *in vitro* that can be good candidates for biological control agents for controlling sheath blight of rice. Field efficacy tests were evaluated for some of the selected strains for the suppression of BPB and SB. All the tested strains reduced the severity of BPB and SB symptoms when they were applied as a pretreatment 1 day prior to pathogen inoculation. The results demonstrated that non-pathogenic *B. glumae* strains isolated form rice field, particularly 257sh-1 and 396gr-2 for BPB and 257sh-1 for SB, can suppress the diseases and prevent yield reduction.

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APPENDIX A. POLYMORPHIC MARKERS BETWEEN FOUR PARENTS USED IN RICE BREEDING

		Parents				
S.N	Polymorphic markers	Trenasse	Jupiter	Bengal	LM-1	Chromosome
1	RM 14133	L	U	U	L	2
2	RM12533	L	U	A	A	2
3	RM12977	L	U	A	A	2
4	RM 12853	U	L	L	L	2
5	RM 5607	L	L	L	U	2
6	RM 13633	U	U	L	U	2
7	RM 13433	U	U	U	L	2
8	RM 12172	U	L	L	L	2
9	RM 13893	U	U	U	L	2
10	RM 5472	L	L	U	L	2
11	RM13514	U	L	U	U	2
12	RM 13114	U	L	L	U	2
13	RM 12294	L	U	L	U	2
14	RM 13553	L	U	L	U	2
15	RM 7492	U	L	U	L	10
16	RM24865	L	U	L	U	10
17	RM 24944	L	U	L	U	10
18	RM 25761	U	L	L	U	10
19	RM 25898	U	L	L	U	10
20	RM 25688	L	U	L	U	10
21	RM 25626	L	U	U	U	10
22	RM 25510	U	L	U	L	10
23	RM 25292	U	L	L	U	10
24	RM 25212	L	U	U	L	10
25	RM 25164	U	L	L	U	10
26	RM 25147	L	U	U	L	10
27	RM 25330	L	U	L	U	10
28	RM 25439	L	U	L	U	10
29	RM 25535	L	U	U	L	10
30	RM 6868	U	L	L	L	10
31	RM 25571	U	L	U	U	10
32	RM 5756	U	L	L	L	10
33	RM 25658	L	U	L	U	10
34	RM 25912	U	L	L	U	10

35	RM 25299	L	U	L	U	10
36	RM 6824	L	U	L	U	10
37	RM 25940	U	L	U	L	10
38	RM 25824	U	L	L	L	10
39	RM 25102	U	A	A	L	10
40	RM 25147	U	U	U	L	10
41	RM 25164	U	L	L	U	10
42	RM7173	U	U	L	U	11
43	RM4B	L	U	L	U	11
44	RM26045	L	U	L	U	11
45	RM26203	L	U	L	U	11
46	RM441	L	U	L	U	11
47	RM26437	L	U	U	U	11
48	RM26288	L	L	L	U	11
49	RM26460	U	L	L	U	11
50	RM536	L	L	L	U	11
51	RM26487	U	L	U	U	11
52	RM26596	U	L	L	L	11
53	RM26646	U	L	L	U	11
54	RM27069	U	L	L	L	11
55	RM27080	U	L	U	L	11
56	RM27248	U	L	L	L	11
57	RM27273	U	L	U	L	11
58	RM27290	U	L	U	L	11
59	RM27206	U	U	U	L	11
60	RM27234	L	L	U	L	11
61	RM5961	L	L	U	L	11
62	RM26544	U	U	U	L	11
63	RM3323	L	U	U	U	12
64	RM27491	U	L	L	U	12
65	RM27508	U	L	L	U	12
66	RM27663	L	U	L	U	12
67	RM27792	U	L	U	L	12
68	RM27805	L	U	L	U	12
69	RM1261	L	U	L	U	12
70	RM28230	U	L	U	L	12
71	RM28261	L	U	L	U	12
72	RM309	U	L	L	U	12
73	RM28546	U	L	L	U	12
74	RM1227	U	L	U	L	12
75	RM28633	L	U	L	L	12

76	RM28828	L	U	U	U	12
77	RM28438	L	L	U	L	12
78	RM1103	U	U	L	U	12
79	RM28294	L	L	L	U	12

The abbreviation U, L and A refers to the PCR band size which is bigger, smaller and no PCR product respectively.

APPENDIX B. HEADING DATE OF RILS DURING 2013 AND 2012

		HD 2013			HD 2012		
S.N.	RILs	Rep I	Rep II	Mean	Rep I	Rep II	Mean
1	LB_1	108	107	107.5	90	90	90
2	LB_2	112	112	112	90	90	90
3	LB_3	112	112	112	88	88	88
4	LB_4	113	113	113	87	87	87
5	LB_5	106	106	106	89	89	89
6	LB_6	114	114	114	89	89	89
7	LB_7	114	114	114	88	88	88
8	LB_8	115	115	115	88	88	88
9	LB_9	117	115	116	99	96	97.5
10	LB_10	111	111	111	91	93	92
11	LB_11	115	115	115	88	88	88
12	LB_12	115	115	115	93	93	93
13	LB_13	114	114	114	92	91	91.5
14	LB_14	114	114	114	98	91	94.5
15	LB_15	117	117	117	99	100	99.5
16	LB_16	116	116	116	98	93	95.5
17	LB_17	117	115	116	98	97	97.5
18	LB_18	113	114	113.5	97	95	96
19	LB_19	113	113	113	91	88	89.5
20	LB_20	112	112	112	87	86	86.5
21	LB_21	118	120	119	98	98	98
22	LB_22	116	116	116	94	95	94.5
23	LB_23	115	115	115	99	98	98.5
24	LB_24	110	110	110	96	93	94.5
25	LB_25	115	116	115.5	99	96	97.5
26	LB_26	111	111	111	97	91	94
27	LB_27	109	109	109	96	93	94.5
28	LB_28	110	110	110	98	98	98
29	LB_29	109	109	109	89	91	90
30	LB_30	115	114	114.5	97	93	95
31	LB_31	118	118	118	100	96	98
32	LB_32	118	115	116.5	97	94	95.5
33	LB_33	118	118	118	97	95	96
34	LB_34	115	115	115	96	94	95
35	LB_35	115	115	115	90	93	91.5
36	LB_36	118	118	118	98	99	98.5

37	LB_37	108	108	108	91	92	91.5
38	LB_38	118	118	118	98	100	99
39	LB_39	112	113	112.5	97	97	97
40	LB_40	111	111	111	92	91	91.5
41	LB_41	112	112	112	93	91	92
42	LB_42	115	114	114.5	98	90	94
43	LB_43	120	118	119	86	86	86
44	LB_44	112	120	116	99	100	99.5
45	LB_45	113	113	113	92	97	94.5
46	LB_46	117	118	117.5	100	100	100
47	LB_47	114	114	114	100	100	100
48	LB_48	115	115	115	97	92	94.5
49	LB_49	113	113	113	98	95	96.5
50	LB_50	112	112	112	92	92	92
51	LB_51	112	112	112	93	92	92.5
52	LB_52	115	115	115	97	96	96.5
53	LB_53	116	115	115.5	93	93	93
54	LB_54	115	115	115	97	92	94.5
55	LB_55	117	118	117.5	99	98	98.5
56	LB_56	112	114	113	93	92	92.5
57	LB_57	114	114	114	92	96	94
58	LB_58	104	104	104	88	92	90
59	LB_59	113	113	113	97	93	95
60	LB_60	110	110	110	93	91	92
61	LB_61	110	110	110	93	93	93
62	LB_62	110	110	110	92	98	95
63	LB_63	109	109	109	91	89	90
64	LB_64	110	110	110	96	93	94.5
65	LB_65	112	112	112	96	93	94.5
66	LB_66	117	117	117	92	92	92
67	LB_67	110	110	110	97	92	94.5
68	LB_68	113	113	113	93	92	92.5
69	LB_69	115	115	115	100	100	100
70	LB_70	112	114	113	99	98	98.5
71	LB_71	110	110	110	97	93	95
72	LB_72	118	118	118	99	99	99
73	LB_73	109	109	109	86	86	86
74	LB_74	112	112	112	91	86	88.5
75	LB_75	109	109	109	91	91	91
76	LB_76	112	112	112	92	93	92.5
77	LB_77	114	114	114	92	91	91.5
78	LB_78	115	115	115	96	92	94
79	LB_79	112	112	112	93	93	93

80	LB_80	116	116	116	93	93	93
81	LB_81	116	116	116	98	93	95.5
82	LB_82	116	116	116	98	97	97.5
83	LB_83	109	109	109	97	96	96.5
84	LB_84	118	118	118	97	98	97.5
85	LB_85	118	118	118	97	96	96.5
86	LB_86	113	113	113	97	93	95
87	LB_87	115	115	115	92	89	90.5
88	LB_88	109	109	109	93	88	90.5
89	LB_89	114	114	114	97	96	96.5
90	LB_90	111	113	112	85	86	85.5
91	LB_91	113	113	113	96	93	94.5
92	LB_92	116	116	116	91	93	92
93	LB_93	115	115	115	91	92	91.5
94	LB_94	116	116	116	92	93	92.5
95	LB_95	117	117	117	97	97	97
96	LB_96	117	117	117	91	91	91
97	LB_97	115	115	115	92	91	91.5
98	LB_98	114	114	114	88	92	90
99	LB_99	115	115	115	86	86	86
100	LB_100	116	116	116	93	93	93
101	LB_101	115	115	115	93	96	94.5
102	LB_102	116	115	115.5	91	95	93
103	LB_103	117	117	117	93	93	93
104	LB_104	114	114	114	88	86	87
105	LB_105	109	108	108.5	82	82	82
106	LB_106	111	111	111	96	96	96
107	LB_107	113	113	113	93	93	93
108	LB_108	116	116	116	98	97	97.5
109	LB_109	114	114	114	93	93	93
110	LB_110	117	117	117	92	93	92.5
111	LB_111	116	116	116	98	96	97
112	LB_112	115	115	115	91	92	91.5
113	LB_113	115	115	115	100	99	99.5
114	LB_114	113	114	113.5	98	92	95
115	LB_115	117	117	117	95	100	97.5
116	LB_116	115	115	115	97	94	95.5
117	LB_117	114	114	114	91	90	90.5
118	LB_118	111	111	111	98	98	98
119	LB_119	114	114	114	95	95	95
120	LB_120	112	112	112	92	93	92.5
121	LB_121	111	111	111	88	88	88
122	LB_122	118	118	118	96	91	93.5

123	LB_123	115	115	115	96	91	93.5
124	LB_124	114	114	114	96	93	94.5
125	LB_125	113	113	113	97	93	95
126	LB_126	115	115	115	100	97	98.5
127	LB_127	118	118	118	88	93	90.5
128	LB_128	113	113	113	92	93	92.5
129	LB_129	109	109	109	92	89	90.5
130	LB_130	109	109	109	92	80	86
131	LB_131	115	115	115	92	92	92
132	LB_132	118	118	118	99	98	98.5
133	LB_133	116	116	116	97	96	96.5
134	LB_134	116	116	116	97	92	94.5
135	LB_135	111	111	111	93	98	95.5
136	LB_136	112	112	112	93	96	94.5
137	LB_137	115	115	115	93	93	93
138	LB_138	116	115	115.5	97	97	97
139	LB_139	116	116	116	97	97	97
140	LB_140	114	114	114	98	97	97.5
141	LB_141	115	114	114.5	88	96	92
142	LB_142	117	117	117	98	98	98
143	LB_143	109	109	109	88	90	89
144	LB_144	118	118	118	98	98	98
145	LB_145	116	116	116	98	98	98
146	LB_146	118	118	118	91	97	94
147	LB_147	110	110	110	94	90	92
148	LB_148	117	117	117	96	91	93.5
149	LB_149	111	111	111	93	93	93
150	LB_150	120	118	119	98	99	98.5
151	LB_151	112	112	112	93	95	94
152	LB_152	113	113	113	96	95	95.5
153	LB_153	109	109	109	93	93	93
154	LB_154	118	118	118	100	99	99.5
155	LB_155	115	115	115	92	92	92
156	LB_156	116	116	116	96	95	95.5
157	LB_157	118	117	117.5	96	93	94.5
158	LB_158	114	114	114	96	96	96
159	LB_159	114	114	114	96	92	94
160	LB_160	117	117	117	100	99	99.5
161	LB_161	114	114	114	96	91	93.5
162	LB_162	121	121	121	97	97	97
163	LB_163	118	118	118	96	93	94.5
164	LB_164	113	113	113	97	93	95
165	LB_165	118	118	118	100	100	100

166	LB_166	118	118	118	93	96	94.5
167	LB_167	113	113	113	93	92	92.5
168	LB_168	113	113	113	93	96	94.5
169	LB_169	113	113	113	93	98	95.5
170	LB_170	116	116	116	93	96	94.5
171	LB_171	115	115	115	96	96	96
172	LB_172	116	116	116	97	97	97
173	LB_173	118	118	118	97	98	97.5
174	LB_174	115	115	115	98	98	98
175	LB_175	120	120	120	99	99	99
176	LB_176	119	119	119	100	99	99.5
177	LB_177	118	117	117.5	86	87	86.5
178	LB_178	110	110	110	99	99	99
179	LB_179	122	122	122	98	99	98.5
180	LB_180	113	113	113	93	93	93
181	LB_181	115	115	115	93	93	93
182	LB_182	115	115	115	95	93	94
183	LB_183	110	110	110	88	93	90.5
184	LB_184	115	115	115	88	88	88
185	LB_185	113	113	113	92	93	92.5
186	LB_186	115	115	115	86	86	86
187	LB_187	115	114	114.5	86	93	89.5
188	LB_188	110	110	110	86	93	89.5
189	LB_189	115	115	115	91	92	91.5
190	LB_190	115	115	115	90	91	90.5
191	LB_191	116	115	115.5	87	87	87
192	LB_192	116	116	116	97	93	95
193	LB_193	115	114	114.5	93	91	92
194	LB_194	113	113	113	96	93	94.5
195	LB_195	112	112	112	88	88	88
196	LB_196	112	112	112	93	93	93
197	LB_197	109	109	109	85	85	85
198	LB_198	108	108	108	89	93	91
199	LB_199	110	110	110	96	96	96
200	LB_200	115	115	115	94	96	95
201	LB_201	110	110	110	96	96	96
202	LB_202	115	115	115	93	94	93.5
203	LB_203	113	113	113	88	95	91.5
204	LB_204	112	112	112	93	88	90.5
205	LB_205	111	111	111	88	93	90.5
206	LB_206	107	107	107	98	97	97.5
207	LB_207	115	115	115	88	91	89.5
208	LB_208	110	110	110	91	88	89.5

209	LB_209	111	111	111	86	91	88.5
210	LB_210	112	115	113.5	96	98	97
211	LB_211	116	116	116	97	95	96
212	LB_212	118	118	118	93	96	94.5
213	LB_213	118	118	118	98	98	98
214	LB_214	118	118	118	96	96	96
215	LB_215	112	112	112	96	98	97
216	LB_216	116	116	116	97	100	98.5
217	LB_217	112	112	112	93	93	93
218	LB_218	115	115	115	93	93	93
219	LB_219	111	111	111	91	91	91
220	LB_220	113	113	113	96	98	97
221	LB_221	120	120	120	100	100	100
222	LB_222	115	115	115	98	100	99
223	LB_223	115	115	115	93	96	94.5
224	LB_224	115	116	115.5	93	99	96
225	LB_225	115	115	115	94	97	95.5
226	LB_226	105	105	105	83	83	83
227	LB_227	110	110	110	96	98	97
228	LB_228	115	115	115	87	86	86.5
229	LB_229	111	111	111	94	98	96
230	LB_230	115	115	115	97	98	97.5
231	LB_231	117	117	117	93	100	96.5
232	LB_232	113	113	113	93	91	92
233	LB_233	118	118	118	97	100	98.5
234	LB_234	114	114	114	94	96	95
235	LB_235	110	110	110	85	85	85
236	LB_236	117	115	116	97	96	96.5
237	LB_237	114	114	114	93	93	93
238	LB_238	115	115	115	99	96	97.5
239	LB_239	115	115	115	97	97	97
240	LB_240	115	115	115	94	96	95
241	LB_241	118	118	118	84	84	84
242	LB_242	111	111	111	93	93	93
243	LB_243	115	115	115	96	96	96
244	LB_244	112	112	112	93	95	94
245	LB_245	114	114	114	94	96	95
246	LB_246	113	113	113	94	93	93.5
247	LB_247	115	115	115	96	95	95.5
248	LB_248	118	118	118	98	100	99
249	LB_249	116	116	116	91	93	92
250	LB_250	118	118	118	97	98	97.5
251	LB_251	110	110	110	93	98	95.5

252	LB_252	118	118	118	100	98	99
253	LB_253	115	115	115	91	91	91
254	LB_254	115	115	115	94	96	95
255	LB_255	116	115	115.5	93	93	93
256	LB_256	120	120	120	94	97	95.5
257	LB_257	115	115	115	94	93	93.5
258	LB_258	110	110	110	93	96	94.5
259	LB_259	111	111	111	88	91	89.5
260	LB_260	111	111	111	92	93	92.5
261	LB_261	111	111	111	87	87	87
262	LB_262	116	115	115.5	96	95	95.5
263	LB_263	118	118	118	96	96	96
264	LB_264	104	105	104.5	85	85	85
265	LB_265	114	115	114.5	93	94	93.5
266	LB_266	115	115	115	92	93	92.5
267	LB_267	115	116	115.5	98	96	97
268	LB_268	116	116	116	96	96	96
269	LB_269	114	114	114	93	96	94.5
270	LB_270	106	106	106	88	86	87
271	LB_271	107	107	107	86	86	86
272	LB_272	110	110	110	91	96	93.5
273	LB_273	118	115	116.5	93	95	94
274	LB_274	115	112	113.5	88	88	88
275	LB_275	117	115	116	98	96	97
276	LB_276	114	114	114	91	87	89
277	LB_277	117	115	116	93	93	93
278	LB_278	119	119	119	96	96	96
279	LB_279	117	115	116	96	97	96.5
280	LB_280	115	115	115	91	93	92
281	LB_281	113	113	113	89	93	91
282	LB_282	114	114	114	89	88	88.5
283	LB_283	108	108	108	90	88	89
284	LB_284	108	108	108	89	88	88.5
285	LB_285	109	109	109	91	93	92
286	LB_286	115	115	115	92	96	94
287	LB_287	109	109	109	90	91	90.5
288	LB_288	109	109	109	89	88	88.5
289	LB_289	114	114	114	88	91	89.5
290	LB_290	115	115	115	94	96	95
291	LB_291	116	114	115	93	96	94.5
292	LB_292	115	115	115	97	93	95
293	LB_293	106	106	106	91	93	92
294	LB_294	113	113	113	93	88	90.5

295	LB_295	112	112	112	91	95	93
296	LB_296	113	113	113	89	91	90
297	LB_297	114	114	114	93	93	93
298	LB_298	112	112	112	90	93	91.5
299	LB_299	115	115	115	95	95	95
300	LB_300	115	115	115	96	96	96
301	Bengal	115	115	115	93	93	93
302	LM-1	120	120	120	98	98	98

APPENDIX C. PLANT HEIGHT AND PANICLE LENGTH MEASURED IN RILS

S.N	RILs	Plant (H)	Plant (H)	Plant (H)	Mean (H)	SD	Panicle (L)	Panicle (L)	Panicle (L)	Mean (L)	SD
1	LB_1	82	100	91	91.00	9.00	18	15.5	15	16.1	1.61
2	LB_2	90	102	93	95.00	6.24	25	21	20	22.00	2.65
3	LB_3	91	100	96	95.6	4.51	21.5	18	17.5	19.00	2.18
4	LB_4	90	90	88	89.33	1.15	19	19.5	20	19.50	0.50
5	LB_5	110	101	109	106.6	4.93	18.5	22.5	25	22.00	3.28
6	LB_6	80	91	84	85.00	5.57	26	18	19	21.00	4.36
7	LB_7	108	93	97	99.33	7.77	21	21.5	18	20.17	1.89
8	LB_8	95	97	94	95.33	1.53	25.5	23	21	23.17	2.25
9	LB_9	96	98	89	94.33	4.73	19	19	19.5	19.17	0.29
10	LB_10	105	93	93	97.00	6.93	20.5	22	20	20.83	1.04
11	LB_11	98	99	102	99.67	2.08	20.5	22	20	20.83	1.04
12	LB_12	99	101	98	99.33	1.53	19	21	18	19.33	1.53
13	LB_13	95	96	102	97.67	3.79	18.5	20	19.5	19.33	0.76
14	LB_14	96	100	99	98.33	2.08	24	23	21.5	22.83	1.26
15	LB_15	80	83	95	86.00	7.94	18	20	19.5	19.17	1.04
16	LB_16	93	90	94	92.33	2.08	19.5	15.5	20	18.33	2.47
17	LB_17	95	86	88	89.67	4.73	21	18.5	21.5	20.33	1.61
18	LB_18	102	103	109	104.6	3.79	24	23	23	23.33	0.58
19	LB_19	95	106	102	101.0	5.57	25	20	22	22.33	2.52
20	LB_20	86	91	93	90.00	3.61	20	21	22	21.00	1.00
21	LB_21	91	97	91	93.00	3.46	21	19	21	20.33	1.15
22	LB_22	91	90	88	89.67	1.53	20	19	17	18.67	1.53
23	LB_23	95	96	103	98.00	4.36	19.5	18	17	18.17	1.26
24	LB_24	100	101	102	101.0	1.00	21	19	21	20.33	1.15
25	LB_25	93	97	98	96.00	2.65	19	17	17.5	17.83	1.04
26	LB_26	90	100	98	96.00	5.29	22.5	25	21	22.83	2.02
27	LB_27	100	103	106	103.0	3.00	19.5	25	26	23.50	3.50
28	LB_28	100	100	107	102.3	4.04	21	20	19	20.00	1.00
29	LB_29	103	98	101	100.6	2.52	15	18	17	16.67	1.53
30	LB_30	88	92	94	91.33	3.06	21	20	23	21.33	1.53
31	LB_31	88	82	80	83.33	4.16	22	20	22	21.33	1.15
32	LB_32	90	95	108	97.67	9.29	15.5	23	20	19.50	3.77
33	LB_33	105	105	107	105.6	1.15	21	26	24	23.67	2.52
34	LB_34	96	100	100	98.67	2.31	24	25	23	24.00	1.00
35	LB_35	112	110	109	110.3	1.53	22	20	21	21.00	1.00

36	LB_36	97	95	99	97.00	2.00	17	18	19	18.00	1.00
37	LB_37	83	84	91	86.00	4.36	18	17	15	16.67	1.53
38	LB_38	94	99	108	100.3	7.09	23	23	19	21.67	2.31
39	LB_39	82	92	90	88.00	5.29	20	21	20	20.33	0.58
40	LB_40	81	80	81	80.67	0.58	18	17	19.5	18.17	1.26
41	LB_41	85	96	100	93.67	7.77	21	19	24	21.33	2.52
42	LB_42	82	85	82	83.00	1.73	19	19	18.5	18.83	0.29
43	LB_43	80	86	81	82.33	3.21	17	15	18	16.67	1.53
44	LB_44	97	90	95	94.00	3.61	18	18.5	20	18.83	1.04
45	LB_45	102	96	96	98.00	3.46	22	19	21	20.67	1.53
46	LB_46	87	103	99	96.33	8.33	20	19	20	19.67	0.58
47	LB_47	96	100	98	98.00	2.00	20.5	20	18	19.50	1.32
48	LB_48	93	98	97	96.00	2.65	22.5	21	19.5	21.00	1.50
49	LB_49	102	96	96	98.00	3.46	22	21	19	20.67	1.53
50	LB_50	97	98	92	95.67	3.21	21	20	21	20.67	0.58
51	LB_51	95	102	96	97.67	3.79	23	23	21	22.33	1.15
52	LB_52	97	92	99	96.00	3.61	22	20	21	21.00	1.00
53	LB_53	94	109	102	101.6	7.51	20	23	23	22.00	1.73
54	LB_54	96	94	100	96.67	3.06	22	19.5	19	20.17	1.61
55	LB_55	81	82	83	82.00	1.00	16	15	16	15.67	0.58
56	LB_56	96	92	97	95.00	2.65	19	20	21	20.00	1.00
57	LB_57	98	105	110	104.3	6.03	22	22	19	21.00	1.73
58	LB_58	109	102	99	103.3	5.13	22.5	22.5	20	21.67	1.44
59	LB_59	88	94	93	91.67	3.21	17	17.5	20	18.17	1.61
60	LB_60	100	103	100	101.0	1.73	24	19.5	21	21.50	2.29
61	LB_61	92	94	94	93.33	1.15	16	15	16	15.67	0.58
62	LB_62	86	88	87	87.00	1.00	17	17	18.5	17.50	0.87
63	LB_63	95	103	99	99.00	4.00	20	23	22	21.67	1.53
64	LB_64	88	86	84	86.00	2.00	17	17.5	18	17.50	0.50
65	LB_65	84	87	82	84.33	2.52	19	17	19	18.33	1.15
66	LB_66	97	96	90	94.33	3.79	22	16.5	19	19.17	2.75
67	LB_67	79	75	80	78.00	2.65	21	18.5	15	18.17	3.01
68	LB_68	97	100	103	100.0	3.00	21	22	20	21.00	1.00
69	LB_69	96	93	96	95.00	1.73	22	21	21	21.33	0.58
70	LB_70	83	93	97	91.00	7.21	17	15	19	17.00	2.00
71	LB_71	89	10	100	66.33	49.1	17	18	19	18.00	1.00
72	LB_72	84	79	87	83.33	4.04	17	24	20	20.33	3.51
73	LB_73	89	93	90	90.67	2.08	19	18	17	18.00	1.00
74	LB_74	85	93	88	88.67	4.04	24	22	19.5	21.83	2.25

75	LB_75	99	95	91	95.00	4.00	20	21	18	19.67	1.53
76	LB_76	97	90	87	91.33	5.13	20	19	19	19.33	0.58
77	LB_77	91	87	88	88.67	2.08	20	21	21	20.67	0.58
78	LB_78	89	95	93	92.33	3.06	18	21	20	19.67	1.53
79	LB_79	78	89	82	83.00	5.57	19	16	17	17.33	1.53
80	LB_80	92	91	86	89.67	3.21	21	17	19	19.00	2.00
81	LB_81	90	92	90	90.67	1.15	20	20.5	20	20.17	0.29
82	LB_82	97	95	92	94.67	2.52	21	19	19	19.67	1.15
83	LB_83	98	90	97	95.00	4.36	19	19.5	22	20.17	1.61
84	LB_84	98	99	99	98.67	0.58	24	22	24	23.33	1.15
85	LB_85	97	83	79	86.33	9.45	16	18	15	16.33	1.53
86	LB_86	85	96	94	91.67	5.86	20	21	20	20.33	0.58
87	LB_87	93	95	97	95.00	2.00	21	21	20	20.67	0.58
88	LB_88	96	95	87	92.67	4.93	19	21	17.5	19.17	1.76
89	LB_89	97	88	97	94.00	5.20	23	17.5	20	20.17	2.75
90	LB_90	91	102	86	93.00	8.19	20	17	18	18.33	1.53
91	LB_91	80	87	91	86.00	5.57	18	22	18	19.33	2.31
92	LB_92	107	95	106	102.6	6.66	25	20	23	22.67	2.52
93	LB_93	87	101	93	93.67	7.02	19	31	15	21.67	8.33
94	LB_94	90	93	97	93.33	3.51	15	17	19	17.00	2.00
95	LB_95	90	92	96	92.67	3.06	20	16	20	18.67	2.31
96	LB_96	78	84	84	82.00	3.46	12	23	22	19.00	6.08
97	LB_97	105	107	106	106.0	1.00	19	21	20	20.00	1.00
98	LB_98	103	91	95	96.33	6.11	21	19	20	20.00	1.00
99	LB_99	101	109	106	105.3	4.04	23	19	21	21.00	2.00
100	LB_100	102	106	102	103.3	2.31	20	21	23	21.33	1.53
101	LB_101	109	103	112	108.0	4.58	19	18	20	19.00	1.00
102	LB_102	85	82	85	84.00	1.73	18	16	19	17.67	1.53
103	LB_103	95	94	95	94.67	0.58	19	18	17	18.00	1.00
104	LB_104	82	83	86	83.67	2.08	20	20	18	19.33	1.15
105	LB_105	85	85	85	85.00	0.00	18	17	15	16.67	1.53
106	LB_106	96	94	95	95.00	1.00	26	26	23	25.00	1.73
107	LB_107	101	105	97	101.0	4.00	21	20	20	20.33	0.58
108	LB_108	82	82	90	84.67	4.62	14	17	19	16.67	2.52
109	LB_109	74	71	77	74.00	3.00	16	16	15	15.67	0.58
110	LB_110	80	85	85	83.33	2.89	16	21	19	18.67	2.52
111	LB_111	94	99	97	96.67	2.52	16	22	18	18.67	3.06
112	LB_112	83	94	93	90.00	6.08	10	18	22	16.67	6.11
113	LB_113	94	85	100	93.00	7.55	20	18	23	20.33	2.52

114	LB_114	103	102	100	101.6	1.53	19	20	19	19.33	0.58
115	LB_115	89	85	83	85.67	3.06	21	12	17	16.67	4.51
116	LB_116	95	88	88	90.33	4.04	21	21	19	20.33	1.15
117	LB_117	96	101	97	98.00	2.65	21	20	20	20.33	0.58
118	LB_118	102	99	99	100.0	1.73	22	20	21	21.00	1.00
119	LB_119	98	95	98	97.00	1.73	20	20	21	20.33	0.58
120	LB_120	104	101	96	100.3	4.04	23	21	20	21.33	1.53
121	LB_121	98	96	97	97.00	1.00	22	19	17	19.33	2.52
122	LB_122	89	98	92	93.00	4.58	12	23	19	18.00	5.57
123	LB_123	91	97	100	96.00	4.58	23	22	20	21.67	1.53
124	LB_124	81	88	91	86.67	5.13	21	19	19	19.67	1.15
125	LB_125	82	86	88	85.33	3.06	18	18	18	18.00	0.00
126	LB_126	103	96	91	96.67	6.03	25	24	18	22.33	3.79
127	LB_127	102	81	78	87.00	13.08	24	21	17	20.67	3.51
128	LB_128	97	94	103	98.00	4.58	20	33	19	24.00	7.81
129	LB_129	83	89	92	88.00	4.58	22	21	19	20.67	1.53
130	LB_130	108	90	95	97.67	9.29	22	24	22	22.67	1.15
131	LB_131	89	87	83	86.33	3.06	18	22	20	20.00	2.00
132	LB_132	98	84	91	91.00	7.00	22	19	18	19.67	2.08
133	LB_133	98	100	90	96.00	5.29	22	17	20	19.67	2.52
134	LB_134	95	89	89	91.00	3.46	26	21	19	22.00	3.61
135	LB_135	95	93	99	95.67	3.06	22	17	29	22.67	6.03
136	LB_136	90	89	92	90.33	1.53	17	17	22	18.67	2.89
137	LB_137	95	108	100	101.0	6.56	18	27	21	22.00	4.58
138	LB_138	101	104	101	102.0	1.73	23	23	19	21.67	2.31
139	LB_139	93	91	93	92.33	1.15	21	17	19	19.00	2.00
140	LB_140	101	97	103	100.3	3.06	22	20	20	20.67	1.15
141	LB_141	96	98	93	95.67	2.52	22	19	21	20.67	1.53
142	LB_142	95	108	100	101.0	6.56	20	21	18	19.67	1.53
143	LB_143	111	107	96	104.6	7.77	25	26	20	23.67	3.21
144	LB_144	86	91	92	89.67	3.21	15	18	21	18.00	3.00
145	LB_145	95	98	103	98.67	4.04	20	22	22	21.33	1.15
146	LB_146	98	95	96	96.33	1.53	24	19	21	21.33	2.52
147	LB_147	87	79	80	82.00	4.36	17	16	17	16.67	0.58
148	LB_148	96	103	94	97.67	4.73	23	22	24	23.00	1.00
149	LB_149	85	88	81	84.67	3.51	19	22	17	19.33	2.52
150	LB_150	94	89	87	90.00	3.61	20	19	17	18.67	1.33
151	LB_151	89	86	95	90.00	4.58	22	17	19	19.33	2.52
152	LB_152	86	96	90	90.67	5.03	22	21	20	21.00	1.00

153	LB_153	83	79	86	82.67	3.51	21	17	21	19.67	2.31
154	LB_154	92	87	87	88.67	2.89	18	18	18	18.00	0.00
155	LB_155	97	91	79	89.00	9.17	22	20	19	20.33	1.53
156	LB_156	98	92	92	94.00	3.46	24	22	22	22.67	1.15
157	LB_157	92	84	88	88.00	4.00	19	17	19	18.33	1.15
158	LB_158	88	82	88	86.00	3.46	21	19	21	20.33	1.15
159	LB_159	80	81	84	81.67	2.08	21	18	22	20.33	2.08
160	LB_160	105	92	94	97.00	7.00	22	18	23	21.00	2.65
161	LB_161	96	84	95	91.67	6.66	17	23	22	20.67	3.21
162	LB_162	87	87	88	87.33	0.58	21	19	18	19.33	1.53
163	LB_163	95	97	94	95.33	1.53	21	21	19	20.33	1.15
164	LB_164	98	88	89	91.67	5.51	16	18	21	18.33	2.52
165	LB_165	84	92	82	86.00	5.29	16	17	16	16.33	0.58
166	LB_166	95	101	99	98.33	3.06	20	19	22	20.33	1.53
167	LB_167	93	87	85	88.33	4.16	19	21	18	19.33	1.53
168	LB_168	94	87	93	91.33	3.79	19	19	20	19.33	0.58
169	LB_169	88	81	82	83.67	3.79	18	21	22	20.33	2.08
170	LB_170	94	87	89	90.00	3.61	19	16	22	19.00	3.00
171	LB_171	77	69	72	72.67	4.04	20	18	17	18.33	1.53
172	LB_172	96	92	101	96.33	4.51	20	15	17	17.33	2.52
173	LB_173	95	98	99	97.33	2.08	19	18	21	19.33	1.53
174	LB_174	85	88	90	87.67	2.52	24	23	20	22.33	2.08
175	LB_175	87	99	91	92.33	6.11	20	19	18	19.00	1.00
176	LB_176	100	100	95	98.33	2.89	21	20	19	20.00	1.00
177	LB_177	99	93	98	96.67	3.21	20	18	18	18.67	1.15
178	LB_178	101	99	98	99.33	1.53	18	21	18	19.00	1.73
179	LB_179	71	83	78	77.33	6.03	15	17	19	17.00	2.00
180	LB_180	81	87	95	87.67	7.02	19	26	20	21.67	3.79
181	LB_181	96	98	97	97.00	1.00	17	22	15	18.00	3.61
182	LB_182	101	109	106	105.3	4.04	20	20	21	20.33	0.58
183	LB_183	97	106	99	100.6	4.73	23	27	21	23.67	3.06
184	LB_184	104	108	113	108.3	4.51	25	27	25	25.67	1.15
185	LB_185	94	95	98	95.67	2.08	19	20	20	19.67	0.58
186	LB_186	107	110	111	109.3	2.08	27	23	25	25.00	2.00
187	LB_187	108	107	105	106.6	1.53	18	19	18	18.33	0.58
188	LB_188	98	97	96	97.00	1.00	21	19	19	19.67	1.15
189	LB_189	95	101	94	96.67	3.79	20	21	21	20.67	0.58
190	LB_190	99	101	106	102.0	3.61	20	16	21	19.00	2.65
191	LB_191	107	109	103	106.3	3.06	24	24	20	22.67	2.31

192	LB_192	110	99	101	103.3	5.86	23	23	27	24.33	2.31
193	LB_193	104	104	97	101.6	4.04	20	22	19	20.33	1.53
194	LB_194	95	105	100	100.0	5.00	19	17	21	19.00	2.00
195	LB_195	94	92	91	92.33	1.53	19	24	21	21.33	2.52
196	LB_196	100	88	87	91.67	7.23	19	19	19	19.00	0.00
197	LB_197	102	100	101	101.0	1.00	23	21	21	21.67	1.15
198	LB_198	95	102	92	96.33	5.13	20	19	17	18.67	1.53
199	LB_199	105	103	110	106.0	3.61	22	22	21	21.67	0.58
200	LB_200	99	99	100	99.33	0.58	20	23	20	21.00	1.73
201	LB_201	84	86	84	84.67	1.15	16	14	18	16.00	2.00
202	LB_202	95	103	96	98.00	4.36	22	24	21	22.33	1.53
203	LB_203	109	112	109	110.0	1.73	21	23	19	21.00	2.00
204	LB_204	100	102	89	97.00	7.00	18	23	20	20.33	2.52
205	LB_205	94	95	95	94.67	0.58	19	20	21	20.00	1.00
206	LB_206	89	93	106	96.00	8.89	22	22	24	22.67	1.15
207	LB_207	97	99	104	100.0	3.61	18	22	23	21.00	2.65
208	LB_208	91	101	100	97.33	5.51	18	24	21	21.00	3.00
209	LB_209	93	95	89	92.33	3.06	18	19	18	18.33	0.58
210	LB_210	98	96	89	94.33	4.73	23	23	19	21.67	2.31
211	LB_211	89	97	97	94.33	4.62	21	22	22	21.67	0.58
212	LB_212	88	84	89	87.00	2.65	17	21	20	19.33	2.08
213	LB_213	89	96	92	92.33	3.51	17	24	18	19.67	3.79
214	LB_214	108	106	99	104.3	4.73	19	23	22	21.33	2.08
215	LB_215	94	92	97	94.33	2.52	21	22	24	22.33	1.53
216	LB_216	103	95	101	99.67	4.16	25	20	23	22.67	2.52
217	LB_217	94	90	87	90.33	3.51	19	18	17	18.00	1.00
218	LB_218	90	100	94	94.67	5.03	20	18	21	19.67	1.53
219	LB_219	94	94	96	94.67	1.15	21	18	19	19.33	1.53
220	LB_220	105	98	96	99.67	4.73	25	22	21	22.67	2.08
221	LB_221	84	78	78	80.00	3.46	15	15	18	16.00	1.73
222	LB_222	94	96	89	93.00	3.61	21	21	16	19.33	2.89
223	LB_223	84	103	103	96.67	10.97	11	24	22	19.00	7.00
224	LB_224	78	81	79	79.33	1.53	18	20	18	18.67	1.15
225	LB_225	107	110	111	109.3	2.08	22	22	20	21.33	1.15
226	LB_226	90	93	92	91.67	1.53	19	24	18	20.33	3.21
227	LB_227	101	98	101	100.0	1.73	23	22	23	22.67	0.58
228	LB_228	90	97	92	93.00	3.61	19	23	22	21.33	2.08
229	LB_229	97	104	96	99.00	4.36	23	23	20	22.00	1.73
230	LB_230	96	88	91	91.67	4.04	18	17	14	16.33	2.08

231	LB_231	99	98	97	98.00	1.00	21	22	22	21.67	0.58
232	LB_232	94	91	91	92.00	1.73	22	22	22	22.00	0.00
233	LB_233	80	84	91	85.00	5.57	19	18	22	19.67	2.08
234	LB_234	99	104	102	101.6	2.52	22	21	23	22.00	1.00
235	LB_235	120	114	114	116.0	3.46	24	27	25	25.33	1.53
236	LB_236	98	101	101	100.0	1.73	22	23	22	22.33	0.58
237	LB_237	94	88	84	88.67	5.03	19	19	21	19.67	1.15
238	LB_238	95	106	95	98.67	6.35	20	20	19	19.67	0.58
239	LB_239	84	94	92	90.00	5.29	18	21	21	20.00	1.73
240	LB_240	76	79	84	79.67	4.04	25	28	18	23.67	5.13
241	LB_241	87	87	84	86.00	1.73	17	17	15	16.33	1.15
242	LB_242	78	83	75	78.67	4.04	12	20	16	16.00	4.00
243	LB_243	79	81	97	85.67	9.87	20	13	22	18.33	4.73
244	LB_244	88	92	85	88.33	3.51	18	17	19	18.00	1.00
245	LB_245	83	85	81	83.00	2.00	17	16	16	16.33	0.58
246	LB_246	104	85	89	92.67	10.02	21	16	22	19.67	3.21
247	LB_247	96	96	95	95.67	0.58	20	21	23	21.33	1.53
248	LB_248	87	92	98	92.33	5.51	18	18	21	19.00	1.73
249	LB_249	86	91	86	87.67	2.89	20	20	19	19.67	0.58
250	LB_250	85	89	91	88.33	3.06	17	20	20	19.00	1.73
251	LB_251	93	92	90	91.67	1.53	21	18	20	19.67	1.53
252	LB_252	90	95	96	93.67	3.21	21	24	20	21.67	2.08
253	LB_253	96	95	96	95.67	0.58	19	19	18	18.67	0.58
254	LB_254	82	76	83	80.33	3.79	17	16	20	17.67	2.08
255	LB_255	89	83	84	85.33	3.21	21	20	21	20.67	0.58
256	LB_256	85	88	94	89.00	4.58	18	22	20	20.00	2.00
257	LB_257	96	89	92	92.33	3.51	20	18	20	19.33	1.15
258	LB_258	95	100	89	94.67	5.51	19	18	19	18.67	0.58
259	LB_259	79	80	80	79.67	0.58	21	14	17	17.33	3.51
260	LB_260	82	88	78	82.67	5.03	20	23	17	20.00	3.00
261	LB_261	95	93	95	94.33	1.15	19	21	19	19.67	1.15
262	LB_262	95	92	91	92.67	2.08	18	17	14	16.33	2.08
263	LB_263	90	91	93	91.33	1.53	22	22	19	21.00	1.73
264	LB_264	89	84	87	86.67	2.52	16	17	20	17.67	2.08
265	LB_265	94	96	98	96.00	2.00	20	19	19	19.33	0.58
266	LB_266	87	84	101	90.67	9.07	17	19	20	18.67	1.53
267	LB_267	91	95	98	94.67	3.51	20	20	21	20.33	0.58
268	LB_268	92	96	91	93.00	2.65	24	23	23	23.33	0.58
269	LB_269	89	98	97	94.67	4.93	20	19	21	20.00	1.00

270	LB_270	91	92	100	94.33	4.93	17	16	21	18.00	2.65
271	LB_271	105	101	95	100.3	5.03	19	18	17	18.00	1.00
272	LB_272	94	100	102	98.67	4.16	18	19	22	19.67	2.08
273	LB_273	93	98	99	96.67	3.21	20	20	21	20.33	0.58
274	LB_274	105	104	106	105.0	1.00	20	20	21	20.33	0.58
275	LB_275	99	100	105	101.3	3.21	25	23	20	22.67	2.52
276	LB_276	101	97	98	98.67	2.08	16	22	19	19.00	3.00
277	LB_277	92	92	91	91.67	0.58	17	15	16	16.00	1.00
278	LB_278	104	94	96	98.00	5.29	22	18	20	20.00	2.00
279	LB_279	107	105	102	104.6	2.52	19	19	21	19.67	1.15
280	LB_280	96	93	86	91.67	5.13	20	19	25	21.33	3.21
281	LB_281	90	89	88	89.00	1.00	19	17	29	21.67	6.43
282	LB_282	78	72	75	75.00	3.00	20	14	18	17.33	3.06
283	LB_283	102	103	104	103.0	1.00	21	20	22	21.00	1.00
284	LB_284	105	96	113	104.6	8.50	21	18	21	20.00	1.73
285	LB_285	85	96	91	90.67	5.51	22	18	20	20.00	2.00
286	LB_286	97	104	97	99.33	4.04	26	21	17	21.33	4.51
287	LB_287	85	91	94	90.00	4.58	19	17	28	21.33	5.86
288	LB_288	96	91	91	92.67	2.89	20	20	19	19.67	0.58
289	LB_289	81	95	91	89.00	7.21	19	22	16	19.00	3.00
290	LB_290	92	85	89	88.67	3.51	19	20	20	19.67	0.58
291	LB_291	91	89	98	92.67	4.73	21	20	24	21.67	2.08
292	LB_292	97	93	96	95.33	2.08	17	21	18	18.67	2.08
293	LB_293	105	99	112	105.3	6.51	23	22	24	23.00	1.00
294	LB_294	106	102	96	101.3	5.03	20	24	23	22.33	2.08
295	LB_295	102	102	105	103.0	1.73	22	23	20	21.67	1.53
296	LB_296	95	82	92	89.67	6.81	19	21	19	19.67	1.15
297	LB_297	99	92	94	95.00	3.61	22	19	16	19.00	3.00
298	LB_298	89	85	91	88.33	3.06	20	20	21	20.33	0.58
299	LB_299	82	86	95	87.67	6.66	16	19	24	19.67	4.04
300	LB_300	81	82	73	78.67	4.93	19	19	22	20.00	1.73
301	Bengal	92	87	90	89.67	2.52	17	20	17	18.00	1.73
302	LM-1	82	80	82	81.33	1.15	21	19	18	19.33	1.53

APPENDIX D. FLAG LEAF AREA MEASURED IN RILS

	Replication I							Replication II					
S.N	RILs	L	B	B	B	M (B)	A	L	B	B	B	M (B)	A
1	LB_1	23.50	1.00	1.50	1.20	1.23	28.98	28.00	1.30	1.70	1.60	1.53	42.93
2	LB_2	23.00	1.00	1.20	1.00	1.07	24.53	28.50	1.00	1.30	1.00	1.10	31.35
3	LB_3	30.00	1.00	1.80	1.50	1.43	43.00	33.00	1.50	2.00	1.50	1.67	55.00
4	LB_4	24.50	1.00	1.10	0.90	1.00	24.50	25.00	1.00	1.20	0.90	1.03	25.83
5	LB_5	38.00	1.00	1.30	1.30	1.20	45.60	36.00	1.50	1.30	1.00	1.27	45.60
6	LB_6	27.50	1.20	1.50	1.20	1.30	35.75	31.50	1.60	1.40	1.50	1.50	47.25
7	LB_7	30.50	1.30	1.40	1.00	1.23	37.62	32.00	1.20	1.30	1.20	1.23	39.47
8	LB_8	29.00	1.40	1.60	1.50	1.50	43.50	23.00	1.40	1.50	1.30	1.40	32.20
9	LB_9	27.00	1.30	1.50	1.40	1.40	37.80	26.00	1.00	1.40	1.00	1.13	29.47
10	LB_10	23.00	1.40	1.40	1.50	1.43	32.97	25.00	1.50	1.60	1.50	1.53	38.33
11	LB_11	27.00	1.50	1.60	1.70	1.60	43.20	28.00	1.60	1.70	1.40	1.57	43.87
12	LB_12	28.00	1.30	1.50	1.20	1.33	37.33	25.50	1.10	1.50	1.40	1.33	34.00
13	LB_13	20.50	1.30	1.40	1.20	1.30	26.65	21.50	1.50	1.60	1.50	1.53	32.97
14	LB_14	34.00	1.50	1.80	1.60	1.63	55.53	32.50	1.70	1.70	1.50	1.63	53.08
15	LB_15	28.00	1.20	1.30	1.00	1.17	32.67	32.00	1.60	1.50	1.20	1.43	45.87
16	LB_16	28.50	1.00	1.50	1.30	1.27	36.10	24.00	1.20	1.60	1.70	1.50	36.00
17	LB_17	28.00	1.20	1.50	1.20	1.30	36.40	29.00	1.20	1.50	1.40	1.37	39.63
18	LB_18	36.00	1.00	1.50	1.60	1.37	49.20	37.00	1.20	1.60	1.50	1.43	53.03
19	LB_19	22.00	1.90	1.50	1.50	1.63	35.93	26.00	1.50	2.00	1.60	1.70	44.20
20	LB_20	29.00	1.40	1.50	1.50	1.47	42.53	29.00	1.50	1.50	1.30	1.43	41.57
21	LB_21	30.00	1.50	1.60	1.50	1.53	46.00	25.00	1.10	1.40	1.30	1.27	31.67
22	LB_22	27.00	1.30	1.50	1.20	1.33	36.00	29.00	1.30	1.50	1.20	1.33	38.67
23	LB_23	23.00	1.50	1.60	1.60	1.57	36.03	24.00	1.60	1.40	1.20	1.40	33.60
24	LB_24	24.00	1.50	1.70	1.50	1.57	37.60	30.00	1.50	1.70	1.50	1.57	47.00
25	LB_25	29.00	1.50	1.60	1.50	1.53	44.47	29.00	1.20	1.50	1.30	1.33	38.67
26	LB_26	25.00	1.50	1.70	1.20	1.47	36.67	31.50	1.50	1.60	1.50	1.53	48.30
27	LB_27	25.00	1.50	1.50	1.30	1.43	35.83	33.50	1.20	1.50	1.30	1.33	44.67
28	LB_28	39.00	1.30	1.60	1.60	1.50	58.50	36.00	1.20	1.50	1.60	1.43	51.60
29	LB_29	28.50	1.10	1.30	1.30	1.23	35.15	25.00	1.00	1.20	1.30	1.17	29.17
30	LB_30	31.50	1.10	1.20	1.40	1.23	38.85	20.00	1.00	1.20	1.25	1.15	23.00
31	LB_31	30.00	1.25	1.50	1.60	1.45	43.50	33.50	1.30	1.50	1.65	1.48	49.69
32	LB_32	28.50	1.20	1.40	1.60	1.40	39.90	26.50	1.50	1.70	1.70	1.63	43.28
33	LB_33	35.00	1.70	1.70	1.80	1.73	60.67	27.50	1.10	1.30	1.40	1.27	34.83
34	LB_34	37.00	1.15	1.40	1.50	1.35	49.95	39.00	1.50	1.55	1.50	1.52	59.15
35	LB_35	35.00	1.50	1.75	1.80	1.68	58.92	28.50	1.65	1.80	1.65	1.70	48.45
36	LB_36	34.00	1.30	1.40	1.45	1.38	47.03	31.00	1.30	1.50	1.75	1.52	47.02
37	LB_37	27.00	0.95	1.10	1.15	1.07	28.80	22.50	0.85	1.00	1.50	1.12	25.13

38	LB_38	38.50	1.40	1.70	1.75	1.62	62.24	37.00	1.30	1.50	1.65	1.48	54.88
39	LB_39	29.00	1.20	1.30	1.50	1.33	38.67	28.50	1.10	1.25	1.30	1.22	34.68
40	LB_40	25.00	1.20	1.25	1.40	1.28	32.08	31.00	1.40	1.50	1.60	1.50	46.50
41	LB_41	25.50	1.40	1.60	1.50	1.50	38.25	29.50	1.20	1.35	1.40	1.32	38.84
42	LB_42	27.50	1.00	1.20	1.30	1.17	32.08	21.50	1.10	1.35	1.40	1.28	27.59
43	LB_43	21.00	1.20	1.45	1.60	1.42	29.75	26.00	1.40	1.50	1.65	1.52	39.43
44	LB_44	25.00	1.20	1.50	1.60	1.43	35.83	27.00	1.30	1.60	1.50	1.47	39.60
45	LB_45	32.50	1.40	1.40	1.40	1.40	45.50	30.00	1.15	1.40	1.45	4.78	40
46	LB_46	25.00	1.20	1.30	1.40	1.30	32.50	24.50	1.40	1.50	1.65	1.52	37.16
47	LB_47	26.50	1.25	1.40	1.50	1.38	36.66	31.50	1.20	1.50	1.60	1.43	45.15
48	LB_48	34.50	1.40	1.60	1.70	1.57	54.05	34.00	1.40	1.40	1.50	1.43	48.73
49	LB_49	25.50	1.50	1.50	1.60	1.53	39.10	24.00	1.20	1.30	1.60	1.37	32.80
50	LB_50	35.00	1.30	1.40	1.50	1.40	49.00	24.00	1.35	1.50	1.60	1.48	35.60
51	LB_51	35.00	1.40	1.60	1.65	1.55	54.25	30.50	1.35	1.45	1.30	1.37	41.68
52	LB_52	22.50	1.35	1.60	1.30	1.42	31.88	22.50	1.40	1.45	1.40	1.42	31.88
53	LB_53	33.00	1.35	1.50	1.60	1.48	48.95	26.00	1.10	1.30	1.35	1.25	32.50
54	LB_54	34.00	1.30	1.60	1.60	1.50	51.00	28.50	1.20	1.40	1.50	1.37	38.95
55	LB_55	20.50	1.20	1.30	1.50	1.33	27.33	24.50	1.30	1.40	1.45	1.38	33.89
56	LB_56	31.00	1.20	1.30	1.50	1.33	41.33	32.50	1.30	1.30	1.60	1.40	45.50
57	LB_57	32.50	1.20	1.50	1.50	1.40	45.50	27.00	1.15	1.45	1.60	1.40	37.80
58	LB_58	33.50	1.60	1.90	2.05	1.85	61.98	30.00	1.30	1.50	1.65	1.48	44.50
59	LB_59	27.00	1.30	1.40	1.50	1.40	37.80	22.00	1.20	1.20	1.20	1.20	26.40
60	LB_60	29.50	1.20	1.30	1.30	1.27	37.37	29.00	1.25	1.35	1.50	1.37	39.63
61	LB_61	24.00	1.20	1.40	1.50	1.37	32.80	22.50	1.20	1.30	1.40	1.30	29.25
62	LB_62	29.50	1.50	1.55	1.70	1.58	46.71	29.00	1.25	1.40	1.50	1.38	40.12
63	LB_63	30.00	1.30	1.60	1.65	1.52	45.50	36.00	1.40	1.60	1.90	1.63	58.80
64	LB_64	21.50	1.20	1.50	1.45	1.38	29.74	25.50	1.30	1.60	1.40	1.43	36.55
65	LB_65	34.50	1.40	1.30	1.40	1.37	47.15	25.50	1.30	1.50	1.50	1.43	36.55
66	LB_66	31.50	1.30	1.50	1.60	1.47	46.20	27.00	1.05	1.30	1.30	1.22	32.85
67	LB_67	28.50	1.30	1.45	1.50	1.42	40.38	26.00	1.20	1.40	1.35	1.32	34.23
68	LB_68	27.50	1.00	1.20	1.25	1.15	31.63	29.00	1.20	1.30	1.30	1.27	36.73
69	LB_69	29.00	1.40	1.50	1.50	1.47	42.53	32.00	1.30	1.50	1.40	1.40	44.80
70	LB_70	30.00	1.40	1.65	1.00	1.35	40.50	30.50	1.50	1.50	1.55	1.52	46.26
71	LB_71	32.00	1.50	1.60	1.70	1.60	51.20	25.00	1.60	1.90	1.50	1.67	41.67
72	LB_72	24.00	1.45	1.60	1.60	1.55	37.20	28.00	1.10	1.40	1.40	1.30	36.40
73	LB_73	30.50	1.20	1.40	1.40	1.33	40.67	32.00	1.00	1.30	1.30	1.20	38.40
74	LB_74	32.50	1.50	1.50	1.60	1.53	49.83	26.50	1.10	1.30	1.40	1.27	33.57
75	LB_75	32.00	1.50	1.70	1.70	1.63	52.27	26.50	1.15	1.30	1.40	1.28	34.01
76	LB_76	26.50	1.60	1.60	1.50	1.57	41.52	27.50	1.30	1.40	1.60	1.43	39.42
77	LB_77	28.50	1.00	1.20	1.20	1.13	32.30	26.50	1.20	1.40	1.50	1.37	36.22
78	LB_78	27.00	1.40	1.35	1.40	1.38	37.35	28.00	1.30	1.30	1.50	1.37	38.27

79	LB_79	27.00	1.45	1.50	1.60	1.52	40.95	22.00	1.25	1.40	1.40	1.35	29.70
80	LB_80	26.00	1.20	1.40	1.40	1.33	34.67	25.00	1.20	1.40	1.30	1.30	32.50
81	LB_81	20.50	1.20	1.40	1.50	1.37	28.02	17.00	1.30	1.45	1.50	1.42	24.08
82	LB_82	28.00	1.30	1.55	1.65	1.50	42.00	28.00	1.60	1.70	1.65	1.65	46.20
83	LB_83	26.00	1.45	1.50	1.70	1.55	40.30	24.00	1.00	1.10	1.30	1.13	27.20
84	LB_84	32.50	1.40	1.50	1.70	1.53	49.83	24.00	1.50	1.70	1.80	1.67	40.00
85	LB_85	22.50	1.20	1.25	1.30	1.25	28.13	21.50	1.00	1.00	2.00	1.33	28.67
86	LB_86	32.00	1.40	1.50	1.60	1.50	48.00	30.00	1.20	1.30	1.60	1.37	41.00
87	LB_87	32.00	1.30	1.60	1.60	1.50	48.00	27.00	1.50	1.70	1.70	1.63	44.10
88	LB_88	20.50	1.10	1.40	1.45	1.32	26.99	24.50	1.10	1.30	1.40	1.27	31.03
89	LB_89	32.00	1.50	1.70	1.70	1.63	52.27	30.50	1.30	1.50	1.60	1.47	44.73
90	LB_90	31.50	1.30	1.40	1.50	1.40	44.10	26.00	1.20	1.30	1.40	1.30	33.80
91	LB_91	24.50	1.10	1.40	1.40	1.30	31.85	31.50	1.20	1.55	1.60	1.45	45.68
92	LB_92	23.00	1.30	1.70	1.60	1.53	35.27	24.50	1.10	1.50	1.40	1.33	32.67
93	LB_93	37.00	1.20	1.40	1.30	1.30	48.10	34.00	1.20	1.20	1.10	1.17	39.67
94	LB_94	27.00	1.50	1.60	1.40	1.50	40.50	34.00	1.10	1.50	1.40	1.33	45.33
95	LB_95	29.50	0.90	1.50	1.60	1.33	39.33	28.50	1.00	1.30	1.40	1.23	35.15
96	LB_96	33.00	1.10	1.40	1.40	1.30	42.90	32.00	1.10	1.40	1.40	1.30	41.60
97	LB_97	33.00	1.00	1.20	1.70	1.30	42.90	33.70	1.20	1.60	1.50	1.43	48.30
98	LB_98	20.50	0.90	1.20	1.30	1.13	23.23	30.00	1.10	1.50	1.20	1.27	38.00
99	LB_99	18.00	1.00	1.30	1.25	1.18	21.30	21.50	0.90	1.25	1.40	1.18	25.44
100	LB_100	31.50	1.00	1.70	1.65	1.45	45.68	30.00	1.30	1.70	1.90	1.63	49.00
101	LB_101	23.50	1.00	1.30	1.30	1.20	28.20	24.00	1.00	1.40	1.50	1.30	31.20
102	LB_102	22.50	1.20	1.50	1.40	1.37	30.75	27.50	1.00	1.40	1.40	1.27	34.83
103	LB_103	29.50	1.05	1.50	1.55	1.37	40.32	28.00	1.20	1.50	1.50	1.40	39.20
104	LB_104	30.00	1.10	1.50	1.50	1.37	41.00	28.00	1.30	1.50	1.50	1.43	40.13
105	LB_105	21.50	0.90	1.20	1.20	1.10	23.65	21.00	1.05	1.30	1.30	1.22	25.55
106	LB_106	33.00	0.90	1.65	1.65	1.40	46.20	26.50	1.25	1.50	1.50	1.42	37.54
107	LB_107	22.50	1.20	1.80	1.70	1.57	35.25	24.50	1.20	1.50	1.40	1.37	33.48
108	LB_108	24.50	1.10	1.30	1.30	1.23	30.22	29.50	1.00	1.30	1.20	1.17	34.42
109	LB_109	22.00	0.70	1.20	1.20	1.03	22.73	19.50	0.90	1.00	1.15	1.02	19.83
110	LB_110	32.00	1.10	1.40	1.40	1.30	41.60	30.00	1.00	1.40	1.40	1.27	38.00
111	LB_111	25.50	1.20	1.60	1.70	1.50	38.25	25.50	1.30	1.55	1.40	1.42	36.13
112	LB_112	31.00	0.80	1.55	1.60	1.32	40.82	31.00	1.00	1.40	1.40	1.27	39.27
113	LB_113	28.50	0.80	1.50	1.50	1.27	36.10	26.00	1.00	1.60	1.60	1.40	36.40
114	LB_114	32.50	0.90	1.45	1.50	1.28	41.71	24.00	1.00	1.20	1.20	1.13	27.20
115	LB_115	27.50	0.90	1.20	1.20	1.10	30.25	29.00	0.95	1.30	1.20	1.15	33.35
116	LB_116	28.50	1.00	1.30	1.25	1.18	33.73	29.50	0.90	1.30	1.20	1.13	33.43
117	LB_117	29.50	1.10	1.50	1.40	1.33	39.33	32.00	0.90	1.40	1.40	1.23	39.47
118	LB_118	26.50	1.30	1.65	1.50	1.48	39.31	29.00	1.10	1.40	1.50	1.33	38.67
119	LB_119	25.00	1.00	1.30	1.20	1.17	29.17	24.00	0.90	1.20	1.30	1.13	27.20

120	LB_120	30.50	0.90	1.60	1.50	1.33	40.67	24.50	0.90	1.50	1.70	1.37	33.48
121	LB_121	29.50	0.90	1.30	1.40	1.20	35.40	26.00	0.90	1.30	1.50	1.23	32.07
122	LB_122	23.50	1.00	1.50	1.50	1.33	31.33	29.00	0.90	1.25	1.40	1.18	34.32
123	LB_123	25.00	0.95	1.20	1.30	1.15	28.75	30.00	0.90	1.50	1.60	1.33	40.00
124	LB_124	33.00	1.20	1.50	1.50	1.40	46.20	31.50	1.00	1.50	1.50	1.33	42.00
125	LB_125	23.00	0.95	1.40	1.40	1.25	28.75	25.00	1.10	1.50	1.60	1.40	35.00
126	LB_126	34.50	1.80	1.60	1.55	1.65	56.93	29.00	0.90	1.60	1.75	1.42	41.08
127	LB_127	27.00	1.00	1.30	1.45	1.25	33.75	28.00	0.90	1.40	1.30	1.20	33.60
128	LB_128	30.00	1.60	1.50	1.40	1.50	45.00	32.00	1.80	1.60	1.40	1.60	51.20
129	LB_129	34.00	1.50	1.60	1.20	1.43	48.73	31.00	1.30	1.60	1.20	1.37	42.37
130	LB_130	23.00	1.30	1.20	1.00	1.17	26.83	31.50	1.40	1.30	1.20	1.30	40.95
131	LB_131	26.00	1.30	1.50	1.20	1.33	34.67	28.50	1.20	1.50	1.40	1.37	38.95
132	LB_132	23.00	1.50	1.70	1.60	1.60	36.80	24.00	1.50	1.70	1.40	1.53	36.80
133	LB_133	29.00	1.90	1.70	1.40	1.67	48.33	21.50	1.70	2.00	1.50	1.73	37.27
134	LB_134	36.00	2.00	1.80	1.50	1.77	63.60	28.00	1.50	1.60	1.40	1.50	42.00
135	LB_135	36.00	1.50	1.20	1.20	1.30	46.80	36.00	1.50	1.70	1.40	1.53	55.20
136	LB_136	35.00	1.70	1.60	1.40	1.57	54.83	33.00	1.50	1.30	1.30	1.37	45.10
137	LB_137	44.00	2.20	2.00	1.80	2.00	88.00	39.00	1.80	1.70	1.50	1.67	65.00
138	LB_138	24.00	1.70	1.70	1.20	1.53	36.80	31.00	1.70	1.60	1.40	1.57	48.57
139	LB_139	33.00	1.50	1.40	1.40	1.43	47.30	24.00	1.70	1.50	1.30	1.50	36.00
140	LB_140	31.00	1.70	1.90	2.00	1.87	57.87	32.00	1.60	1.80	1.90	1.77	56.53
141	LB_141	32.00	1.60	1.60	1.60	1.60	51.20	33.00	1.60	1.60	1.40	1.53	50.60
142	LB_142	27.50	1.70	1.60	1.50	1.60	44.00	26.00	1.70	1.90	1.60	1.73	45.07
143	LB_143	31.00	2.00	1.80	1.50	1.77	54.77	29.00	1.70	1.70	1.40	1.60	46.40
144	LB_144	25.00	1.30	1.40	1.00	1.23	30.83	22.50	1.60	1.50	1.50	1.53	34.50
145	LB_145	34.00	1.80	1.90	1.80	1.83	62.33	30.00	1.70	1.80	1.70	1.73	52.00
146	LB_146	24.00	1.50	1.40	1.20	1.37	32.80	35.00	1.80	1.20	1.60	1.53	53.67
147	LB_147	29.00	1.40	1.30	1.20	1.30	37.70	31.00	1.60	1.50	2.20	1.77	54.77
148	LB_148	30.00	1.50	1.70	1.60	1.60	48.00	34.00	1.80	1.70	1.20	1.57	53.27
149	LB_149	23.00	1.60	1.60	1.30	1.50	34.50	32.00	1.60	1.80	1.70	1.70	54.40
150	LB_150	28.00	1.60	1.30	1.20	1.37	38.27	32.00	1.00	1.50	1.50	1.33	42.67
151	LB_151	27.00	1.60	1.60	1.30	1.50	40.50	22.50	1.70	1.50	1.20	1.47	33.00
152	LB_152	25.50	1.50	1.50	1.10	1.37	34.85	31.00	1.70	1.60	1.50	1.60	49.60
153	LB_153	19.50	1.30	1.20	1.00	1.17	22.75	20.00	1.20	1.00	1.50	1.23	24.67
154	LB_154	26.00	1.60	1.70	1.40	1.57	40.73	24.00	1.60	1.70	1.40	1.57	37.60
155	LB_155	27.00	1.50	1.40	1.40	1.43	38.70	26.00	1.50	1.50	1.60	1.53	39.87
156	LB_156	23.00	1.40	1.30	1.20	1.30	29.90	32.50	1.60	1.55	1.40	1.52	49.29
157	LB_157	23.00	1.40	1.40	1.20	1.33	30.67	30.00	1.40	1.50	1.40	1.43	43.00
158	LB_158	28.00	1.50	1.40	1.30	1.40	39.20	29.00	1.60	1.50	1.30	1.47	42.53
159	LB_159	25.00	1.60	1.50	1.70	1.60	40.00	24.50	1.40	1.50	1.20	1.37	33.48
160	LB_160	28.50	1.60	1.60	1.50	1.57	44.65	29.00	1.60	1.50	1.40	1.50	43.50

161	LB_161	29.00	1.00	1.60	1.70	1.43	41.57	22.00	1.40	1.60	1.60	1.53	33.73
162	LB_162	26.50	1.10	1.30	1.40	1.27	33.57	26.00	1.10	1.30	1.40	1.27	32.93
163	LB_163	29.50	1.00	1.55	1.70	1.42	41.79	28.00	1.00	1.40	1.45	1.28	35.93
164	LB_164	30.50	0.95	1.40	1.40	1.25	38.13	29.00	1.20	1.40	1.30	1.30	37.70
165	LB_165	31.50	0.90	1.40	1.50	1.27	39.90	26.50	0.80	1.15	1.20	1.05	27.83
166	LB_166	24.50	1.10	1.50	1.60	1.40	34.30	23.00	1.20	1.40	1.40	1.33	30.67
167	LB_167	24.00	1.00	1.40	1.40	1.27	30.40	26.50	1.00	1.14	1.40	1.18	31.27
168	LB_168	27.00	1.00	1.40	1.35	1.25	33.75	28.50	1.00	1.30	1.20	1.17	33.25
169	LB_169	33.00	1.10	1.70	1.80	1.53	50.60	30.50	1.10	1.30	1.50	1.30	39.65
170	LB_170	25.00	0.95	1.30	1.30	1.18	29.58	31.00	1.80	1.30	1.30	1.47	45.47
171	LB_171	24.00	1.00	1.40	1.40	1.27	30.40	28.00	1.20	1.20	1.20	1.20	33.60
172	LB_172	21.50	1.00	1.25	1.35	1.20	25.80	25.50	1.00	1.30	1.30	1.20	30.60
173	LB_173	29.50	1.30	1.50	1.60	1.47	43.27	23.50	1.20	1.60	1.50	1.43	33.68
174	LB_174	31.00	1.30	1.60	1.55	1.48	45.98	27.00	1.00	1.30	1.40	1.23	33.30
175	LB_175	32.50	1.40	1.60	1.60	1.53	49.83	30.50	1.00	1.30	1.40	1.23	37.62
176	LB_176	27.50	1.10	1.40	1.40	1.30	35.75	26.50	0.90	1.20	1.30	1.13	30.03
177	LB_177	25.00	1.10	1.45	1.50	1.35	33.75	31.50	1.10	1.50	1.65	1.42	44.63
178	LB_178	27.00	0.90	1.20	1.20	1.10	29.70	23.00	0.80	1.10	1.25	1.05	24.15
179	LB_179	24.00	1.10	1.40	1.35	1.28	30.80	24.,5	0.90	1.25	1.30	1.15	35.42
180	LB_180	23.50	1.00	1.35	1.30	1.22	28.59	21.00	1.00	1.20	1.20	1.13	23.80
181	LB_181	27.00	1.40	1.50	1.20	1.37	36.90	26.50	1.50	1.80	1.60	1.63	43.28
182	LB_182	26.00	1.90	1.90	1.60	1.80	46.80	27.00	1.50	1.80	1.60	1.63	44.10
183	LB_183	30.00	1.40	1.40	1.20	1.33	40.00	29.00	1.60	1.50	1.40	1.50	43.50
184	LB_184	28.00	1.40	1.40	1.50	1.43	40.13	28.50	1.50	1.50	1.30	1.43	40.85
185	LB_185	27.50	1.60	1.70	1.40	1.57	43.08	28.00	1.50	1.60	1.30	1.47	41.07
186	LB_186	23.00	1.80	1.70	1.50	1.67	38.33	27.00	1.50	1.60	1.30	1.47	39.60
187	LB_187	27.50	1.80	1.80	1.50	1.70	46.75	31.00	1.80	1.50	2.00	1.77	54.77
188	LB_188	33.00	1.40	1.50	1.50	1.47	48.40	30.00	1.20	1.20	1.40	1.27	38.00
189	LB_189	35.00	1.60	1.90	1.70	1.73	60.67	39.00	1.40	1.60	1.60	1.53	59.80
190	LB_190	34.00	1.50	1.60	1.60	1.57	53.27	35.00	1.50	1.50	1.40	1.47	51.33
191	LB_191	31.00	1.40	1.30	1.10	1.27	39.27	31.00	1.40	1.40	1.40	1.40	43.40
192	LB_192	33.00	1.50	1.60	1.20	1.43	47.30	30.00	1.20	1.20	1.40	1.27	38.00
193	LB_193	37.00	1.20	1.40	1.40	1.33	49.33	32.00	1.60	1.50	1.40	1.50	48.00
194	LB_194	37.00	1.60	1.30	1.10	1.33	49.33	35.00	1.20	1.20	1.30	1.23	43.17
195	LB_195	29.00	1.60	1.50	1.40	1.50	43.50	32.00	1.40	1.40	1.30	1.37	43.73
196	LB_196	27.00	1.50	1.50	1.20	1.40	37.80	29.00	1.80	1.60	1.20	1.53	44.47
197	LB_197	28.00	1.70	1.50	1.20	1.47	41.07	34.00	1.60	1.40	1.40	1.47	49.87
198	LB_198	27.00	1.60	1.50	1.40	1.50	40.50	28.00	1.50	1.40	1.30	1.40	39.20
199	LB_199	37.00	1.70	1.50	1.50	1.57	57.97	34.00	1.60	1.40	1.20	1.40	47.60
200	LB_200	28.00	1.60	1.50	1.20	1.43	40.13	29.00	1.60	1.60	1.90	1.70	49.30
201	LB_201	22.00	1.40	1.20	1.00	1.20	26.40	21.00	1.40	1.20	1.20	1.27	26.60

202	LB_202	23.00	1.40	1.30	1.20	1.30	29.90	25.00	1.40	1.40	1.20	1.33	33.33
203	LB_203	25.00	1.60	1.50	1.20	1.43	35.83	27.00	1.50	1.50	1.30	1.43	38.70
204	LB_204	31.00	1.50	1.50	1.20	1.40	43.40	30.00	1.40	1.00	1.40	1.27	38.00
205	LB_205	24.00	1.50	1.50	1.30	1.43	34.40	24.00	1.30	1.00	1.20	1.17	28.00
206	LB_206	35.00	1.30	1.50	1.40	1.40	49.00	32.00	1.70	1.50	1.40	1.53	49.07
207	LB_207	39.00	1.50	1.40	1.20	1.37	53.30	40.00	1.70	1.60	1.50	1.60	64.00
208	LB_208	29.50	1.30	1.20	1.20	1.23	36.38	26.00	1.50	1.20	1.20	1.30	33.80
209	LB_209	30.00	1.50	1.40	1.40	1.43	43.00	26.00	1.40	1.40	1.20	1.33	34.67
210	LB_210	31.00	1.70	1.50	1.20	1.47	45.47	32.50	1.60	1.60	1.20	1.47	47.67
211	LB_211	29.00	1.50	1.30	1.30	1.37	39.63	28.00	1.50	1.50	1.50	1.50	42.00
212	LB_212	34.00	1.70	1.60	1.50	1.60	54.40	32.00	1.60	1.50	1.30	1.47	46.93
213	LB_213	37.00	1.50	1.50	1.60	1.53	56.73	36.00	1.70	1.40	1.30	1.47	52.80
214	LB_214	39.00	2.00	1.70	1.20	1.63	63.70	34.50	1.70	1.60	1.30	1.53	52.90
215	LB_215	39.00	1.20	1.60	1.50	1.43	55.90	40.00	2.00	2.10	1.60	1.90	76.00
216	LB_216	34.00	1.50	1.70	1.65	1.62	54.97	41.00	1.30	1.50	1.40	1.40	57.40
217	LB_217	25.50	1.10	1.50	1.50	1.37	34.85	27.00	1.30	1.60	1.60	1.50	40.50
218	LB_218	29.00	1.60	1.50	1.30	1.47	42.53	37.50	1.30	1.60	1.60	1.50	56.25
219	LB_219	31.50	1.30	1.60	1.55	1.48	46.73	29.50	1.30	1.70	1.60	1.53	45.23
220	LB_220	35.00	1.50	1.80	1.80	1.70	59.50	36.50	1.20	1.60	1.60	1.47	53.53
221	LB_221	28.50	1.40	1.70	1.50	1.53	43.70	32.00	1.20	1.40	1.40	1.33	42.67
222	LB_222	31.50	1.40	1.50	1.40	1.43	45.15	29.00	1.40	1.60	1.50	1.50	43.50
223	LB_223	28.50	1.50	1.90	1.75	1.72	48.93	27.00	1.45	1.60	1.60	1.55	41.85
224	LB_224	32.00	1.20	1.30	1.30	1.27	40.53	30.50	1.20	1.30	1.30	1.27	38.63
225	LB_225	31.50	1.20	1.60	1.50	1.43	45.15	37.00	1.20	1.60	1.60	1.47	54.27
226	LB_226	34.00	1.00	1.20	1.10	1.10	37.40	24.50	1.20	1.20	1.20	1.20	29.40
227	LB_227	30.00	1.20	1.70	1.70	1.53	46.00	27.00	1.80	1.50	1.30	1.53	41.40
228	LB_228	27.50	1.00	1.50	1.60	1.37	37.58	26.50	1.20	1.30	1.35	1.28	34.01
229	LB_229	30.00	1.10	1.50	1.50	1.37	41.00	31.00	1.40	1.60	1.45	1.48	45.98
230	LB_230	26.00	1.10	1.40	1.50	1.33	34.67	32.50	1.40	1.60	1.60	1.53	49.83
231	LB_231	30.50	1.30	1.60	1.50	1.47	44.73	29.50	1.15	1.40	1.50	1.35	39.83
232	LB_232	30.00	1.20	1.50	1.40	1.37	41.00	28.00	1.30	1.50	1.55	1.45	40.60
233	LB_233	24.00	1.40	1.60	1.50	1.50	36.00	25.50	1.50	1.50	1.50	1.50	38.25
234	LB_234	30.50	1.10	1.50	1.50	1.37	41.68	32.00	0.80	1.00	1.20	1.00	32.00
235	LB_235	37.50	0.90	1.20	1.30	1.13	42.50	35.50	1.10	1.40	1.50	1.33	47.33
236	LB_236	38.00	1.20	1.60	1.50	1.43	54.47	28.50	1.00	1.50	1.60	1.37	38.95
237	LB_237	34.50	1.30	1.60	1.70	1.53	52.90	32.00	1.40	1.60	1.60	1.53	49.07
238	LB_238	36.50	1.20	1.60	1.50	1.43	52.32	31.00	1.00	1.50	1.70	1.40	43.40
239	LB_239	27.00	1.10	1.40	1.35	1.28	34.65	29.00	1.30	1.50	1.40	1.40	40.60
240	LB_240	29.50	1.40	1.60	1.60	1.53	45.23	25.00	1.40	1.50	1.40	1.43	35.83
241	LB_241	31.00	1.50	1.70	1.60	1.60	49.60	29.50	1.50	1.80	1.70	1.67	49.17
242	LB_242	30.00	1.00	1.50	1.50	1.33	40.00	34.50	1.00	1.50	1.60	1.37	47.15

243	LB_243	25.00	1.40	1.70	1.60	1.57	39.17	25.50	1.50	1.70	1.60	1.60	40.80
244	LB_244	24.00	1.10	1.30	1.40	1.27	30.40	31.50	1.20	1.30	1.20	1.23	38.85
245	LB_245	21.50	1.10	1.50	1.50	1.37	29.38	37.50	1.30	1.50	1.65	1.48	55.63
246	LB_246	26.50	1.10	1.50	1.60	1.40	37.10	21.50	1.10	1.50	1.60	1.40	30.10
247	LB_247	27.50	1.10	1.30	1.30	1.23	33.92	27.50	1.10	1.30	1.35	1.25	34.38
248	LB_248	31.00	1.40	1.50	1.40	1.43	44.43	31.50	1.10	1.50	1.50	1.37	43.05
249	LB_249	25.00	1.30	1.50	1.50	1.43	35.83	25.00	1.30	1.60	1.50	1.47	36.67
250	LB_250	28.00	1.10	1.50	1.40	1.33	37.33	34.50	1.30	1.40	1.30	1.33	46.00
251	LB_251	31.00	1.20	1.40	1.30	1.30	40.30	28.50	1.10	1.30	1.20	1.20	34.20
252	LB_252	33.00	1.50	1.50	1.40	1.47	48.40	31.50	1.00	1.80	1.80	1.53	48.30
253	LB_253	28.50	1.20	1.40	1.30	1.30	37.05	29.50	1.20	1.50	1.50	1.40	41.30
254	LB_254	26.00	1.10	1.30	1.30	1.23	32.07	28.00	1.20	1.70	1.70	1.53	42.93
255	LB_255	28.50	1.10	1.40	1.50	1.33	38.00	26.50	1.00	1.40	1.50	1.30	34.45
256	LB_256	19.00	1.10	1.50	1.30	1.30	24.70	22.50	1.40	1.60	1.50	1.50	33.75
257	LB_257	34.00	1.00	1.50	1.50	1.33	45.33	26.00	1.10	1.50	1.60	1.40	36.40
258	LB_258	24.50	0.90	1.10	1.10	1.03	25.32	20.50	0.90	1.10	1.10	1.03	21.18
259	LB_259	30.50	1.20	1.30	1.50	1.33	40.67	32.00	1.20	1.50	1.50	1.40	44.80
260	LB_260	32.00	1.10	1.20	1.20	1.17	37.33	29.50	1.50	1.50	1.50	1.50	44.25
261	LB_261	37.50	1.20	1.60	1.70	1.50	56.25	32.50	1.40	1.50	1.60	1.50	48.75
262	LB_262	22.00	1.30	1.40	1.60	1.43	31.53	27.50	1.20	1.50	1.50	1.40	38.50
263	LB_263	25.50	1.20	1.30	1.40	1.30	33.15	31.00	1.00	1.30	1.40	1.23	38.23
264	LB_264	31.00	1.30	1.50	1.60	1.47	45.47	37.00	1.30	1.50	1.50	1.43	53.03
265	LB_265	25.50	1.50	1.70	1.80	1.67	42.50	28.00	1.40	1.60	1.10	1.37	38.27
266	LB_266	29.50	1.20	1.20	1.20	1.20	35.40	28.00	1.20	1.50	1.60	1.43	40.13
267	LB_267	26.50	1.20	1.40	1.50	1.37	36.22	27.00	1.10	1.40	1.60	1.37	36.90
268	LB_268	36.00	1.50	1.70	1.70	1.63	58.80	36.00	1.30	1.60	1.60	1.50	54.00
269	LB_269	27.00	1.20	1.60	1.50	1.43	38.70	33.00	1.30	1.50	1.50	1.43	47.30
270	LB_270	24.50	1.00	1.10	1.20	1.10	26.95	20.50	0.80	1.00	1.20	1.00	20.50
271	LB_271	31.00	1.00	1.30	1.30	1.20	37.20	32.00	1.00	1.20	1.30	1.17	37.33
272	LB_272	26.50	1.30	1.30	1.30	1.30	34.45	27.00	1.20	1.50	1.60	1.43	38.70
273	LB_273	27.00	1.10	1.50	1.50	1.37	36.90	28.50	1.10	1.50	1.60	1.40	39.90
274	LB_274	31.50	1.30	1.50	1.60	1.47	46.20	35.00	1.10	1.50	1.60	1.40	49.00
275	LB_275	36.00	1.40	1.50	1.40	1.43	51.60	31.00	1.40	1.60	1.60	1.53	47.53
276	LB_276	29.00	1.30	1.50	1.60	1.47	42.53	25.00	1.20	1.40	1.50	1.37	34.17
277	LB_277	26.50	1.20	1.35	1.45	1.33	35.33	31.00	1.30	1.60	1.50	1.47	45.47
278	LB_278	26.00	1.00	1.30	1.40	1.23	32.07	32.50	1.20	1.40	1.50	1.37	44.42
279	LB_279	28.00	1.10	1.30	1.50	1.30	36.40	28.00	1.10	1.50	1.60	1.40	39.20
280	LB_280	25.50	1.20	1.50	1.70	1.47	37.40	29.00	1.30	1.50	1.60	1.47	42.53
281	LB_281	29.50	1.40	1.50	1.60	1.50	44.25	26.00	1.30	1.50	1.60	1.47	38.13
282	LB_282	28.00	1.10	1.40	1.50	1.33	37.33	31.00	1.00	1.40	1.40	1.27	39.27
283	LB_283	24.00	1.00	1.20	1.20	1.13	27.20	26.00	1.00	1.10	1.20	1.10	28.60

284	LB_284	25.50	1.00	1.20	1.30	1.17	29.75	30.00	0.90	1.10	1.30	1.10	33.00
285	LB_285	27.00	1.10	1.30	1.30	1.23	33.30	30.50	1.10	1.30	1.30	1.23	37.62
286	LB_286	29.00	1.30	1.40	1.40	1.37	39.63	26.00	1.20	1.50	1.60	1.43	37.27
287	LB_287	24.00	0.90	1.20	1.20	1.10	26.40	26.00	0.90	1.20	1.40	1.17	30.33
288	LB_288	22.00	1.10	1.30	1.40	1.27	27.87	25.50	1.00	1.20	1.20	1.13	28.90
289	LB_289	24.50	1.30	1.50	1.70	1.50	36.75	27.50	1.40	1.50	1.60	1.50	41.25
290	LB_290	26.00	1.20	1.40	1.40	1.33	34.67	27.00	1.00	1.20	1.40	1.20	32.40
291	LB_291	24.50	1.10	1.50	1.60	1.40	34.30	34.00	1.10	1.40	1.50	1.33	45.33
292	LB_292	24.00	1.15	1.40	1.20	1.25	30.00	30.00	1.10	1.40	1.60	1.37	41.00
293	LB_293	24.50	1.20	1.50	1.40	1.37	33.48	28.50	1.20	1.50	1.60	1.43	40.85
294	LB_294	30.00	1.20	1.50	1.40	1.37	41.00	31.00	1.00	1.40	1.50	1.30	40.30
295	LB_295	27.50	1.10	1.30	1.50	1.30	35.75	32.00	1.30	1.60	1.70	1.53	49.07
296	LB_296	29.00	0.95	1.30	1.40	1.22	35.28	23.00	0.90	1.30	1.40	1.20	27.60
297	LB_297	38.00	1.40	1.70	1.80	1.63	62.07	36.50	1.40	1.70	1.70	1.60	58.40
298	LB_298	29.00	1.40	1.60	1.70	1.57	45.43	30.00	1.20	1.45	1.40	1.35	40.50
299	LB_299	27.00	1.15	1.30	1.40	1.28	34.65	31.50	1.10	1.30	1.40	1.27	39.90
300	LB_300	24.00	1.20	1.35	1.50	1.35	32.40	21.50	1.10	1.30	1.40	1.27	27.23
301	Bengal	31.00	1.20	1.30	1.40	1.30	40.30	34.50	1.10	1.30	1.40	1.27	43.70
302	LM-1	30.50	1.30	1.50	1.70	1.50	45.75	28.50	1.10	1.30	1.30	1.23	35.15

		Replication III							
S.N	RILs	L	B	B	B	Mean (B)	A	Mean (A)	SD
1	LB_1	24.00	1.40	1.60	1.40	1.47	35.20	35.71	6.99
2	LB_2	26.00	1.00	1.20	1.00	1.07	27.73	27.87	3.41
3	LB_3	29.50	1.60	1.80	1.50	1.63	48.18	48.73	6.02
4	LB_4	27.00	1.40	1.20	1.00	1.20	32.40	27.58	4.23
5	LB_5	43.00	1.20	1.20	1.00	1.13	48.73	46.64	1.81
6	LB_6	28.00	1.00	1.50	1.30	1.27	35.47	39.49	6.72
7	LB_7	28.00	1.50	1.60	1.50	1.53	42.93	40.01	2.70
8	LB_8	23.50	1.40	1.50	1.35	1.42	33.29	36.33	6.23
9	LB_9	25.00	1.20	1.30	1.20	1.23	30.83	32.70	4.47
10	LB_10	27.50	1.40	1.60	1.40	1.47	40.33	37.21	3.81
11	LB_11	24.50	1.20	1.25	1.40	1.28	31.44	39.50	6.99
12	LB_12	25.50	1.60	1.40	1.20	1.40	35.70	35.68	1.67
13	LB_13	26.00	1.70	1.70	1.50	1.63	42.47	34.03	7.96
14	LB_14	32.00	1.60	1.40	1.50	1.50	48.00	52.21	3.84
15	LB_15	35.00	1.40	1.50	1.40	1.43	50.17	42.90	9.12
16	LB_16	29.00	1.60	1.50	1.20	1.43	41.57	37.89	3.19
17	LB_17	25.00	1.50	1.20	1.20	1.30	32.50	36.18	3.57
18	LB_18	37.00	1.40	1.50	1.50	1.47	54.27	52.17	2.64
19	LB_19	25.00	1.30	1.60	1.50	1.47	36.67	38.93	4.58
20	LB_20	32.50	1.00	1.50	1.30	1.27	41.17	41.76	0.70
21	LB_21	22.00	1.00	1.50	1.30	1.27	27.87	35.18	9.56
22	LB_22	25.00	1.50	1.30	1.40	1.40	35.00	36.56	1.90
23	LB_23	22.00	1.10	1.50	1.30	1.30	28.60	32.74	3.79
24	LB_24	25.50	1.30	1.40	1.40	1.37	34.85	39.82	6.37
25	LB_25	28.00	1.20	1.50	1.20	1.30	36.40	39.84	4.16
26	LB_26	30.00	1.30	1.50	1.30	1.37	41.00	41.99	5.88
27	LB_27	28.00	1.50	1.40	1.30	1.40	39.20	39.90	4.46
28	LB_28	29.00	1.60	1.70	1.70	1.67	48.33	52.81	5.19
29	LB_29	27.00	1.20	1.30	1.40	1.30	35.10	33.14	3.44
30	LB_30	29.50	1.30	1.40	1.30	1.33	39.33	33.73	9.29
31	LB_31	23.50	1.20	1.40	1.50	1.37	32.12	41.77	8.91
32	LB_32	29.00	1.30	1.50	1.60	1.47	42.53	41.91	1.78
33	LB_33	31.50	1.30	1.70	1.80	1.60	50.40	48.63	13.01
34	LB_34	3.70	1.40	1.45	1.50	1.45	5.37	38.16	28.77
35	LB_35	29.00	1.40	1.60	1.20	1.40	40.60	49.32	9.19
36	LB_36	27.00	1.20	1.30	1.40	1.30	35.10	43.05	6.88
37	LB_37	27.00	1.00	1.10	1.20	1.10	29.70	27.88	2.42
38	LB_38	29.50	1.25	1.50	1.60	1.45	42.78	53.30	9.83

39	LB_39	32.00	1.20	1.45	1.45	1.37	43.73	39.03	4.54
40	LB_40	37.00	1.40	1.40	1.50	1.43	53.03	43.87	10.72
41	LB_41	26.50	1.20	1.40	1.45	1.35	35.78	37.62	1.63
42	LB_42	22.50	1.25	1.30	1.40	1.32	29.63	29.77	2.25
43	LB_43	25.00	1.50	1.70	1.70	1.63	40.83	36.67	6.04
44	LB_44	36.00	1.40	1.60	1.90	1.63	58.80	44.74	12.32
45	LB_45	29.00	1.10	1.20	1.20	1.17	39.78	74.28	5.84
46	LB_46	31.50	1.50	1.70	1.65	1.62	50.93	40.19	9.58
47	LB_47	24.00	1.10	1.30	1.40	1.27	30.40	37.40	7.40
48	LB_48	29.50	1.55	1.55	1.50	1.53	45.23	49.34	4.44
49	LB_49	21.00	1.00	1.30	1.50	1.27	26.60	32.83	6.25
50	LB_50	34.50	1.40	1.60	1.55	1.52	52.33	45.64	8.85
51	LB_51	30.50	1.40	1.60	1.60	1.53	46.77	47.57	6.32
52	LB_52	29.00	1.10	1.20	1.30	1.20	34.80	32.85	1.69
53	LB_53	28.00	1.20	1.30	1.30	1.27	35.47	38.97	8.77
54	LB_54	37.00	1.30	1.50	1.50	1.43	53.03	47.66	7.61
55	LB_55	22.00	1.20	1.35	1.40	1.32	28.97	30.06	3.41
56	LB_56	34.00	1.40	1.50	1.65	1.52	51.57	46.13	5.15
57	LB_57	25.00	1.40	1.50	1.60	1.50	37.50	40.27	4.53
58	LB_58	36.00	1.50	1.80	1.80	1.70	61.20	55.89	9.87
59	LB_59	24.00	1.45	1.45	2.00	1.63	39.20	34.47	7.02
60	LB_60	25.00	1.20	1.35	1.50	1.35	33.75	36.92	2.97
61	LB_61	22.50	1.50	1.50	1.50	1.50	33.75	31.93	2.37
62	LB_62	31.50	1.50	1.65	1.30	1.48	46.73	44.52	3.81
63	LB_63	35.00	1.00	1.40	1.45	1.28	44.92	49.74	7.85
64	LB_64	32.00	1.50	1.60	1.60	1.57	50.13	38.81	10.38
65	LB_65	28.00	1.25	1.30	1.40	1.32	36.87	40.19	6.03
66	LB_66	32.00	1.20	1.35	1.45	1.33	42.67	40.57	6.92
67	LB_67	30.50	1.35	1.50	1.60	1.48	45.24	39.95	5.52
68	LB_68	32.00	1.10	1.30	1.40	1.27	40.53	36.30	4.47
69	LB_69	32.00	1.20	1.40	1.40	1.33	42.67	43.33	1.27
70	LB_70	26.00	1.30	1.30	1.35	1.32	34.23	40.33	6.01
71	LB_71	25.50	1.40	1.50	1.50	1.47	37.40	43.42	7.07
72	LB_72	30.50	1.25	1.40	1.60	1.42	43.21	38.94	3.72
73	LB_73	27.50	1.10	1.15	1.30	1.18	32.54	37.20	4.19
74	LB_74	23.50	1.20	1.50	1.50	1.40	32.90	38.77	9.59
75	LB_75	32.00	1.25	1.40	1.50	1.38	44.27	43.51	9.15
76	LB_76	26.00	1.50	1.70	1.70	1.63	42.47	41.13	1.56
77	LB_77	30.50	1.30	1.50	1.60	1.47	44.73	37.75	6.36
78	LB_78	26.50	1.40	1.60	1.50	1.50	39.75	38.46	1.21
79	LB_79	28.50	1.30	1.50	1.50	1.43	40.85	37.17	6.47

80	LB_80	23.50	1.20	1.30	1.40	1.30	30.55	32.57	2.06
81	LB_81	30.00	1.40	1.70	1.75	1.62	48.50	33.53	13.11
82	LB_82	23.00	1.20	1.50	1.70	1.47	33.73	40.64	6.34
83	LB_83	28.50	1.20	1.40	1.30	1.30	37.05	34.85	6.82
84	LB_84	36.00	1.50	1.70	1.90	1.70	61.20	50.34	10.61
85	LB_85	25.50	1.10	1.20	1.30	1.20	30.60	29.13	1.30
86	LB_86	29.00	1.20	1.40	1.40	1.33	38.67	42.56	4.86
87	LB_87	28.00	1.50	1.50	1.60	1.53	42.93	45.01	2.65
88	LB_88	35.00	1.50	1.65	1.60	1.58	55.42	37.81	15.38
89	LB_89	35.00	1.50	1.70	1.80	1.67	58.33	51.78	6.81
90	LB_90	29.00	1.30	1.60	1.80	1.57	45.43	41.11	6.37
91	LB_91	22.00	1.10	1.30	1.40	1.27	27.87	35.13	9.35
92	LB_92	19.50	1.60	1.40	1.20	1.40	27.30	31.74	4.06
93	LB_93	38.00	1.15	1.20	1.10	1.15	43.70	43.82	4.22
94	LB_94	29.00	1.30	1.45	1.50	1.42	41.08	42.31	2.64
95	LB_95	31.00	0.90	1.40	1.30	1.20	37.20	37.23	2.09
96	LB_96	29.00	1.10	1.40	1.40	1.30	37.70	40.73	2.71
97	LB_97	31.50	1.30	1.80	1.80	1.63	51.45	47.55	4.32
98	LB_98	30.50	1.00	1.35	1.00	1.12	34.06	31.76	7.65
99	LB_99	20.50	1.00	1.50	1.40	1.30	26.65	24.46	2.81
100	LB_100	29.00	1.40	1.70	1.60	1.57	45.43	46.70	1.99
101	LB_101	23.50	0.90	1.30	1.40	1.20	28.20	29.20	1.73
102	LB_102	26.00	1.10	1.40	1.40	1.30	33.80	33.13	2.12
103	LB_103	32.50	1.30	1.60	1.60	1.50	48.75	42.76	5.22
104	LB_104	29.00	1.20	1.40	1.60	1.40	40.60	40.58	0.43
105	LB_105	25.50	1.00	1.40	1.35	1.25	31.88	27.03	4.31
106	LB_106	28.50	1.10	1.40	1.40	1.30	37.05	40.26	5.15
107	LB_107	29.00	1.20	1.70	1.60	1.50	43.50	37.41	5.35
108	LB_108	26.50	1.05	1.30	1.20	1.18	31.36	32.00	2.17
109	LB_109	20.00	0.80	1.10	1.15	1.02	20.33	20.96	1.55
110	LB_110	29.00	1.00	1.30	1.30	1.20	34.80	38.13	3.40
111	LB_111	25.50	1.20	1.50	1.40	1.37	34.85	36.41	1.72
112	LB_112	29.00	0.90	1.40	1.50	1.27	36.73	38.94	2.06
113	LB_113	30.50	0.80	1.40	1.40	1.20	36.60	36.37	0.25
114	LB_114	27.50	1.20	1.40	1.60	1.40	38.50	35.80	7.62
115	LB_115	30.50	0.90	1.50	1.30	1.23	37.62	33.74	3.70
116	LB_116	30.50	0.95	1.20	1.40	1.18	36.09	34.42	1.46
117	LB_117	28.30	1.30	1.30	1.40	1.33	37.73	38.84	0.96
118	LB_118	29.00	1.10	1.30	1.30	1.23	35.77	37.91	1.89
119	LB_119	25.00	0.90	1.20	1.20	1.10	27.50	27.96	1.06
120	LB_120	30.00	1.10	1.50	1.60	1.40	42.00	38.72	4.58

121	LB_121	22.50	1.10	1.50	1.40	1.33	30.00	32.49	2.72
122	LB_122	28.00	1.10	1.30	1.40	1.27	35.47	33.71	2.13
123	LB_123	32.00	0.90	1.30	1.20	1.13	36.27	35.01	5.73
124	LB_124	27.00	1.05	1.40	1.40	1.28	34.65	40.95	5.85
125	LB_125	23.50	1.00	1.40	1.50	1.30	30.55	31.43	3.22
126	LB_126	34.00	1.20	1.40	1.45	1.35	45.90	47.97	8.12
127	LB_127	29.00	1.00	1.20	1.30	1.17	33.83	33.73	0.12
128	LB_128	29.00	1.30	1.60	1.50	1.47	42.53	46.24	4.47
129	LB_129	30.50	1.50	1.40	1.00	1.30	39.65	43.58	4.66
130	LB_130	28.00	1.20	1.30	1.30	1.27	35.47	34.42	7.12
131	LB_131	27.00	1.20	1.40	1.20	1.27	34.20	35.94	2.62
132	LB_132	23.00	1.40	1.50	1.30	1.40	32.20	35.27	2.66
133	LB_133	27.00	1.70	1.60	1.40	1.57	42.30	42.63	5.54
134	LB_134	26.00	1.20	1.40	1.20	1.27	32.93	46.18	15.75
135	LB_135	36.00	1.50	1.50	1.00	1.33	48.00	50.00	4.54
136	LB_136	26.50	1.50	1.00	1.40	1.30	34.45	44.79	10.20
137	LB_137	40.00	1.80	2.00	1.50	1.77	70.67	74.56	11.98
138	LB_138	28.00	1.70	1.70	1.40	1.60	44.80	43.39	6.01
139	LB_139	24.00	1.50	1.50	1.30	1.43	34.40	39.23	7.03
140	LB_140	23.00	1.90	1.80	1.50	1.73	39.87	51.42	10.03
141	LB_141	30.50	1.50	1.60	1.40	1.50	45.75	49.18	2.99
142	LB_142	26.00	1.70	1.40	1.40	1.50	39.00	42.69	3.24
143	LB_143	31.00	1.70	1.60	1.40	1.57	48.57	49.91	4.34
144	LB_144	28.50	1.50	1.50	1.30	1.43	40.85	35.39	5.07
145	LB_145	30.00	1.60	1.80	1.80	1.73	52.00	55.44	5.97
146	LB_146	32.00	1.60	1.50	1.40	1.50	48.00	44.82	10.79
147	LB_147	32.00	1.50	1.60	1.50	1.53	49.07	47.18	8.69
148	LB_148	29.00	1.60	1.50	1.40	1.50	43.50	48.26	4.89
149	LB_149	28.00	1.80	1.50	1.00	1.43	40.13	43.01	10.26
150	LB_150	29.00	1.60	1.50	1.40	1.50	43.50	41.48	2.81
151	LB_151	27.00	1.60	1.50	1.40	1.50	40.50	38.00	4.33
152	LB_152	27.00	1.70	1.70	1.20	1.53	41.40	41.95	7.39
153	LB_153	18.00	1.40	1.20	1.20	1.27	22.80	23.41	1.09
154	LB_154	27.00	1.60	1.50	1.30	1.47	39.60	39.31	1.59
155	LB_155	30.50	1.60	1.40	1.20	1.40	42.70	40.42	2.06
156	LB_156	28.50	1.60	1.60	1.40	1.53	43.70	40.96	9.98
157	LB_157	31.50	1.00	1.20	1.30	1.17	36.75	36.81	6.17
158	LB_158	24.00	1.40	1.40	1.30	1.37	32.80	38.18	4.95
159	LB_159	30.50	1.40	1.40	1.50	1.43	43.72	39.07	5.18
160	LB_160	21.50	1.60	1.50	1.40	1.50	32.25	40.13	6.85
161	LB_161	25.00	1.30	1.50	1.40	1.40	35.00	36.77	4.20

162	LB_162	24.50	1.10	1.30	1.30	1.23	30.22	32.24	1.78
163	LB_163	26.00	1.10	1.60	1.60	1.43	37.27	38.33	3.07
164	LB_164	35.00	1.20	1.40	1.50	1.37	47.83	41.22	5.73
165	LB_165	34.50	1.10	1.60	1.60	1.43	49.45	39.06	10.84
166	LB_166	23.50	1.10	1.40	1.50	1.33	31.33	32.10	1.93
167	LB_167	35.50	0.90	1.40	1.60	1.30	46.15	35.94	8.85
168	LB_168	32.50	1.20	1.30	1.25	1.25	40.63	35.88	4.12
169	LB_169	25.00	1.10	1.50	1.40	1.33	33.33	41.19	8.74
170	LB_170	33.50	0.90	1.30	1.35	1.18	39.64	38.23	8.04
171	LB_171	26.00	1.50	1.35	1.30	1.38	35.97	33.32	2.79
172	LB_172	25.00	1.50	1.40	1.50	1.47	36.67	31.02	5.45
173	LB_173	22.00	1.25	1.70	1.80	1.58	34.83	37.26	5.23
174	LB_174	29.00	1.05	1.30	1.40	1.25	36.25	38.51	6.64
175	LB_175	38.00	1.20	1.60	1.50	1.43	54.47	47.31	8.70
176	LB_176	24.00	1.10	1.35	1.30	1.25	30.00	31.93	3.31
177	LB_177	26.50	1.00	1.30	1.30	1.20	31.80	36.73	6.91
178	LB_178	26.50	0.80	1.00	1.05	0.95	25.18	26.34	2.95
179	LB_179	25.50	1.00	1.30	1.20	1.17	29.75	31.99	3.02
180	LB_180	18.00	1.00	1.20	1.25	1.15	20.70	24.36	3.98
181	LB_181	24.00	1.60	1.60	1.50	1.57	37.60	39.26	3.50
182	LB_182	27.00	1.60	1.80	1.50	1.63	44.10	45.00	1.56
183	LB_183	27.00	1.60	1.50	1.40	1.50	40.50	41.33	1.89
184	LB_184	32.00	1.60	1.80	1.60	1.67	53.33	44.77	7.42
185	LB_185	29.00	1.50	1.50	1.30	1.43	41.57	41.91	1.05
186	LB_186	29.00	1.50	1.70	1.40	1.53	44.47	40.80	3.24
187	LB_187	32.00	1.90	1.80	1.60	1.77	56.53	52.68	5.21
188	LB_188	24.00	1.40	1.50	1.20	1.37	32.80	39.73	7.94
189	LB_189	28.00	1.40	1.40	1.20	1.33	37.33	52.60	13.23
190	LB_190	34.00	0.90	1.20	1.10	1.07	36.27	46.96	9.31
191	LB_191	28.00	1.20	1.20	1.40	1.27	35.47	39.38	3.97
192	LB_192	30.00	1.40	1.40	1.50	1.43	43.00	42.77	4.65
193	LB_193	29.50	1.60	1.50	1.30	1.47	43.27	46.87	3.19
194	LB_194	35.00	1.70	1.60	1.60	1.63	57.17	49.89	7.02
195	LB_195	31.00	1.60	1.40	1.30	1.43	44.43	43.89	0.49
196	LB_196	31.00	1.80	1.70	1.40	1.63	50.63	44.30	6.42
197	LB_197	31.00	1.90	1.70	1.60	1.73	53.73	48.22	6.49
198	LB_198	30.00	1.60	1.50	1.40	1.50	45.00	41.57	3.04
199	LB_199	38.00	1.60	1.50	1.40	1.50	57.00	54.19	5.73
200	LB_200	29.00	1.50	1.50	1.60	1.53	44.47	44.63	4.59
201	LB_201	20.00	1.40	1.20	1.20	1.27	25.33	26.11	0.68
202	LB_202	21.00	1.20	1.20	1.50	1.30	27.30	30.18	3.03

203	LB_203	31.00	1.30	1.30	1.20	1.27	39.27	37.93	1.84
204	LB_204	32.00	1.50	1.40	1.20	1.37	43.73	41.71	3.22
205	LB_205	28.00	1.50	1.40	1.30	1.40	39.20	33.87	5.62
206	LB_206	30.00	1.50	1.40	1.20	1.37	41.00	46.36	4.64
207	LB_207	34.00	1.50	1.50	1.40	1.47	49.87	55.72	7.37
208	LB_208	35.00	1.50	1.40	1.30	1.40	49.00	39.73	8.13
209	LB_209	27.00	1.40	1.40	1.20	1.33	36.00	37.89	4.48
210	LB_210	31.00	1.60	1.50	1.20	1.43	44.43	45.86	1.65
211	LB_211	29.00	2.20	1.40	1.20	1.60	46.40	42.68	3.43
212	LB_212	33.00	1.70	1.50	1.40	1.53	50.60	50.64	3.73
213	LB_213	32.00	1.40	1.30	1.30	1.33	42.67	50.73	7.26
214	LB_214	34.00	1.70	1.60	1.20	1.50	51.00	55.87	6.85
215	LB_215	37.00	1.10	1.50	1.50	1.37	50.57	60.82	13.41
216	LB_216	39.00	1.80	1.90	1.50	1.73	67.60	59.99	6.70
217	LB_217	34.50	1.50	1.60	1.65	1.58	54.63	43.33	10.19
218	LB_218	30.50	1.50	1.90	1.80	1.73	52.87	50.55	7.15
219	LB_219	35.50	1.20	1.50	1.40	1.37	48.52	46.83	1.64
220	LB_220	41.00	1.50	1.95	1.70	1.72	70.38	61.14	8.54
221	LB_221	32.00	1.40	1.60	1.50	1.50	48.00	44.79	2.83
222	LB_222	35.00	1.50	1.70	1.55	1.58	55.42	48.02	6.46
223	LB_223	33.00	1.40	1.70	1.70	1.60	52.80	47.86	5.55
224	LB_224	32.00	1.20	1.30	1.50	1.33	42.67	40.61	2.02
225	LB_225	30.00	1.10	1.40	1.60	1.37	41.00	46.81	6.79
226	LB_226	20.00	0.90	1.10	1.10	1.03	20.67	29.16	8.37
227	LB_227	22.00	1.30	1.60	1.70	1.53	33.73	40.38	6.20
228	LB_228	28.00	1.40	1.60	1.55	1.52	42.47	38.02	4.25
229	LB_229	32.00	1.25	1.50	1.55	1.43	45.87	44.28	2.84
230	LB_230	30.00	1.20	1.30	1.30	1.27	38.00	40.83	7.97
231	LB_231	26.50	1.30	1.60	1.50	1.47	38.87	41.14	3.15
232	LB_232	37.00	1.20	1.45	1.50	1.38	51.18	44.26	6.00
233	LB_233	24.00	1.10	1.20	1.30	1.20	28.80	34.35	4.94
234	LB_234	27.00	1.30	1.50	1.50	1.43	38.70	37.46	4.96
235	LB_235	37.50	1.10	1.30	1.40	1.27	47.50	45.78	2.84
236	LB_236	32.00	1.20	1.50	1.60	1.43	45.87	46.43	7.77
237	LB_237	35.50	1.20	1.70	1.60	1.50	53.25	51.74	2.32
238	LB_238	30.50	1.30	1.50	1.60	1.47	44.73	46.82	4.81
239	LB_239	30.00	1.10	1.50	1.40	1.33	40.00	38.42	3.28
240	LB_240	25.00	1.30	1.40	1.50	1.40	35.00	38.69	5.68
241	LB_241	35.00	1.50	1.90	1.80	1.73	60.67	53.14	6.52
242	LB_242	27.50	1.00	1.50	1.50	1.33	36.67	41.27	5.36
243	LB_243	26.00	1.20	1.60	1.60	1.47	38.13	39.37	1.34

244	LB_244	24.50	1.00	1.30	1.30	1.20	29.40	32.88	5.19
245	LB_245	24.00	1.10	1.50	1.40	1.33	32.00	39.00	14.45
246	LB_246	22.00	1.10	1.50	1.50	1.37	30.07	32.42	4.05
247	LB_247	22.00	1.20	1.40	1.50	1.37	30.07	32.79	2.37
248	LB_248	30.50	1.20	1.40	1.20	1.27	38.63	42.04	3.03
249	LB_249	32.00	1.30	1.50	1.50	1.43	45.87	39.46	5.57
250	LB_250	27.50	1.20	1.50	1.45	1.38	38.04	40.46	4.81
251	LB_251	33.50	1.40	1.50	1.40	1.43	48.02	40.84	6.92
252	LB_252	32.00	1.60	1.70	1.70	1.67	53.33	50.01	2.88
253	LB_253	23.50	1.10	1.30	1.40	1.27	29.77	36.04	5.83
254	LB_254	24.00	1.20	1.60	1.60	1.47	35.20	36.73	5.59
255	LB_255	28.00	1.10	1.50	1.30	1.30	36.40	36.28	1.78
256	LB_256	25.00	1.10	1.50	1.50	1.37	34.17	30.87	5.35
257	LB_257	23.00	1.50	1.60	1.30	1.47	33.73	38.49	6.08
258	LB_258	24.50	1.00	1.20	1.10	1.10	26.95	24.48	2.97
259	LB_259	29.00	1.30	1.20	1.60	1.37	39.63	41.70	2.73
260	LB_260	29.50	1.20	1.50	1.40	1.37	40.32	40.63	3.47
261	LB_261	38.00	1.50	1.50	1.40	1.47	55.73	53.58	4.19
262	LB_262	26.50	1.10	1.40	1.40	1.30	34.45	34.83	3.50
263	LB_263	28.00	1.00	1.30	1.30	1.20	33.60	34.99	2.81
264	LB_264	35.50	1.20	1.90	1.70	1.60	56.80	51.77	5.77
265	LB_265	25.50	1.40	1.50	1.50	1.47	37.40	39.39	2.73
266	LB_266	29.00	1.00	1.40	1.50	1.30	37.70	37.74	2.37
267	LB_267	30.00	1.40	1.50	1.50	1.47	44.00	39.04	4.31
268	LB_268	29.50	1.30	1.60	1.60	1.50	44.25	52.35	7.41
269	LB_269	25.00	1.40	1.40	1.30	1.37	34.17	40.06	6.67
270	LB_270	24.50	0.80	1.10	1.30	1.07	26.13	24.53	3.51
271	LB_271	29.00	1.30	1.40	1.50	1.40	40.60	38.38	1.93
272	LB_272	27.50	1.30	1.30	1.50	1.37	37.58	36.91	2.20
273	LB_273	24.50	1.20	1.30	1.50	1.33	32.67	36.49	3.63
274	LB_274	34.00	1.50	1.60	1.60	1.57	53.27	49.49	3.56
275	LB_275	37.50	1.30	1.50	1.50	1.43	53.75	50.96	3.16
276	LB_276	29.50	1.40	1.40	1.50	1.43	42.28	39.66	4.76
277	LB_277	30.50	1.50	1.80	1.90	1.73	52.87	44.56	8.80
278	LB_278	26.50	1.30	1.60	1.60	1.50	39.75	38.74	6.24
279	LB_279	25.00	1.00	1.50	1.30	1.27	31.67	35.76	3.81
280	LB_280	25.00	1.10	1.40	1.40	1.30	32.50	37.48	5.02
281	LB_281	24.50	1.40	1.50	1.60	1.50	36.75	39.71	3.99
282	LB_282	29.00	1.20	1.50	1.50	1.40	40.60	39.07	1.64
283	LB_283	28.50	1.00	1.20	1.30	1.17	33.25	29.68	3.17
284	LB_284	31.00	1.00	1.00	1.20	1.07	33.07	31.94	1.90

285	LB_285	37.50	1.20	1.40	1.50	1.37	51.25	40.72	9.37
286	LB_286	31.50	1.20	1.40	1.60	1.40	44.10	40.33	3.47
287	LB_287	23.50	1.00	1.30	1.10	1.13	26.63	27.79	2.21
288	LB_288	38.20	1.10	1.30	1.40	1.27	48.39	35.05	11.56
289	LB_289	28.00	1.30	1.50	1.60	1.47	41.07	39.69	2.55
290	LB_290	28.50	1.30	1.50	1.60	1.47	41.80	36.29	4.91
291	LB_291	30.50	1.20	1.50	1.60	1.43	43.72	41.12	5.96
292	LB_292	31.20	1.20	1.40	1.50	1.37	42.64	37.88	6.87
293	LB_293	20.50	1.30	1.50	1.50	1.43	29.38	34.57	5.81
294	LB_294	28.50	1.20	1.50	1.60	1.43	40.85	40.72	0.37
295	LB_295	28.00	1.70	1.80	1.90	1.80	50.40	45.07	8.10
296	LB_296	24.00	1.00	1.20	1.30	1.17	28.00	30.29	4.33
297	LB_297	27.00	1.50	1.70	1.70	1.63	44.10	54.86	9.49
298	LB_298	30.00	1.30	1.50	1.60	1.47	44.00	43.31	2.54
299	LB_299	28.00	1.30	1.40	1.40	1.37	38.27	37.61	2.69
300	LB_300	24.00	1.10	1.35	1.40	1.28	30.80	30.14	2.64
301	Bengal	33.00	1.60	1.30	1.40	1.43	47.30	43.77	3.50
302	LM-1	26.50	1.20	1.30	1.40	1.30	34.45	38.45	6.33

APPENDIX E. BACTERIAL PANICLE BLIGHT AND SHEATH BLIGHT RATING IN RILS

		BPB 2012			BPB 2013			SB 2012		
S.N	RILs	RepI	RepII	Mean	RepI	RepII	Mean	RepI	RepII	Mean
1	LB_1	7	7	7	3	3	3	4	3	3.5
2	LB_2	7	7	7	4	4	4	6	7	6.5
3	LB_3	2	2	2	5	5	5	3	4	3.5
4	LB_4	5	5	5	7	6	6.5	5	6	5.5
5	LB_5	4	5	4.5	6	7	6.5	5	5	5
6	LB_6	8	9	8.5	4	3	3.5	8	8	8
7	LB_7	3	3	3	4	3	3.5	4	3	3.5
8	LB_8	5	5	5	4	4	4	7	7	7
9	LB_9	4	4	4	4	5	4.5	3	2	2.5
10	LB_10	6	6	6	5	4	4.5	3	6	4.5
11	LB_11	3	4	3.5	3	3	3	4	4	4
12	LB_12	2	2	2	5	4	4.5	4	3	3.5
13	LB_13	3	3	3	7	5	6	3	5	4
14	LB_14	3	4	3.5	3	2	2.5	5	3	4
15	LB_15	2	2	2	4	3	3.5	3	3	3
16	LB_16	4	5	4.5	2	2	2	7	7	7
17	LB_17	3	3	3	4	5	4.5	6	6	6
18	LB_18	2	2	2	3	2	2.5	5	4	4.5
19	LB_19	2	3	2.5	5	4	4.5	5	4	4.5
20	LB_20	6	5	5.5	3	3	3	8	8	8
21	LB_21	3	3	3	1	1	1	6	5	5.5
22	LB_22	5	5	5	6	6	6	7	5	6
23	LB_23	4	4	4	2	3	2.5	8	7	7.5
24	LB_24	3	3	3	4	2	3	4	8	6
25	LB_25	3	2	2.5	4	5	4.5	2	2	2
26	LB_26	3	3	3	2	2	2	4	4	4
27	LB_27	2	3	2.5	5	4	4.5	3	4	3.5
28	LB_28	4	6	5	4	2	3	7	7	7
29	LB_29	5	4	4.5	5	4	4.5	5	6	5.5
30	LB_30	5	5	5	4	6	5	4	6	5
31	LB_31	5	5	5	1	1	1	8	7	7.5
32	LB_32	4	5	4.5	6	6	6	7	7	7
33	LB_33	2	1	1.5	2	2	2	2	2	2
34	LB_34	3	3	3	3	2	2.5	2	3	2.5
35	LB_35	2	2	2	3	2	2.5	2	2	2
36	LB_36	3	3	3		7	3.5	3	4	3.5
37	LB_37	7	5	6	5	5	5	9	9	9

38	LB_38	5	3	4	2	4	3	4	4	4
39	LB_39	3	3	3	1	2	1.5	8	8	8
40	LB_40	6	5	5.5	3	4	3.5	9	9	9
41	LB_41	5	4	4.5	3	3	3	5	5	5
42	LB_42	5	7	6	7	7	7	4	7	5.5
43	LB_43	3	2	2.5	3	3	3	7	4	5.5
44	LB_44	6	6	6	5	5	5	9	4	6.5
45	LB_45	4	4	4	4	4	4	6	3	4.5
46	LB_46	5	5	5	4	6	5	6	6	6
47	LB_47	3	3	3	3	4	3.5	4	4	4
48	LB_48	2	2	2	3	2	2.5	6	4	5
49	LB_49	6	4	5	2	2	2	5	6	5.5
50	LB_50	3	4	3.5	6	5	5.5	7	5	6
51	LB_51	3	3	3	4	4	4	3	5	4
52	LB_52	2	2	2	3	2	2.5	2	2	2
53	LB_53	2	2	2	3	3	3	4	8	6
54	LB_54	4	3	3.5	5	4	4.5	8	6	7
55	LB_55	3	4	3.5	3	4	3.5	7	5	6
56	LB_56	4	6	5	4	4	4	8	8	8
57	LB_57	3	3	3	3	3	3	3	3	3
58	LB_58	8	5	6.5	3	4	3.5	2	8	5
59	LB_59	3	3	3	2	4	3	2	3	2.5
60	LB_60	2	5	3.5	6	7	6.5	3	6	4.5
61	LB_61	5	4	4.5	3	3	3	1	3	2
62	LB_62	6	5	5.5	4	5	4.5	3	2	2.5
63	LB_63	3	5	4	2	1	1.5	2	2	2
64	LB_64	5	5	5	2	2	2	3	4	3.5
65	LB_65	4	4	4	4	4	4	5	7	6
66	LB_66	6	6	6	2	3	2.5	2	2	2
67	LB_67	3	3	3	2	3	2.5	4	5	4.5
68	LB_68	2	2	2	2	2	2	8	7	7.5
69	LB_69	2	2	2	3	2	2.5	1	3	2
70	LB_70	6	6	6	3	3	3	4	1	2.5
71	LB_71	3	3	3	1	1	1	5	5	5
72	LB_72	5	5	5	2	2	2	5	5	5
73	LB_73	4	4	4	4	4	4	6	4	5
74	LB_74	3	3	3	3	2	2.5	4	6	5
75	LB_75	4	4	4	2	2	2	3	5	4
76	LB_76	1	2	1.5	2	2	2	4	3	3.5
77	LB_77	2	2	2	2	2	2	2	2	2
78	LB_78	2	3	2.5	4	3	3.5	9	6	7.5
79	LB_79	3	5	4	5	4	4.5	6	7	6.5
80	LB_80	6	5	5.5	4	4	4	3	4	3.5

81	LB_81	3	3	3	2	3	2.5	1	3	2
82	LB_82	5	5	5	1	2	1.5	3	2	2.5
83	LB_83	3	2	2.5	4	4	4	2	2	2
84	LB_84	2	2	2	3	3	3	6	3	4.5
85	LB_85	7	6	6.5	4	4	4	3	6	4.5
86	LB_86	3	5	4	5	5	5	6	3	4.5
87	LB_87	4	3	3.5	4	3	3.5	8	5	6.5
88	LB_88	3	3	3	3	2	2.5	8	7	7.5
89	LB_89	3	4	3.5	2	5	3.5	6	5	5.5
90	LB_90	6	6	6	2	2	2	8	8	8
91	LB_91	5	5	5	4	4	4	5	7	6
92	LB_92	3	3	3	1	1	1	7	6	6.5
93	LB_93	3	5	4	4	4	4	5	5	5
94	LB_94	6	5	5.5	3	3	3	8	8	8
95	LB_95	5	6	5.5	5	6	5.5	8	7	7.5
96	LB_96	6	6	6	6	5	5.5	8	8	8
97	LB_97	3	3	3	3	2	2.5	4	6	5
98	LB_98	3	3	3	4	3	3.5	4	5	4.5
99	LB_99	2	3	2.5	5	4	4.5	3	4	3.5
100	LB_100	5	4	4.5	3	2	2.5	4	3	3.5
101	LB_101	2	2	2	1	1	1	3	3	3
102	LB_102	4	4	4	5	5	5	7	7	7
103	LB_103	3	3	3	2	2	2	4	4	4
104	LB_104	2	3	2.5	7	7	7	8	5	6.5
105	LB_105	8	8	8	9	9	9	9	9	9
106	LB_106	3	3	3	3	2	2.5	7	6	6.5
107	LB_107	1	2	1.5	1	2	1.5	3	2	2.5
108	LB_108	3	4	3.5	2	1	1.5	4	4	4
109	LB_109	6	6	6	6	6	6	8	6	7
110	LB_110	3	3	3	4	3	3.5	4	4	4
111	LB_111	4	3	3.5	2	1	1.5	2	2	2
112	LB_112	5	4	4.5	4	3	3.5	7	5	6
113	LB_113	3	3	3	2	1	1.5	3	4	3.5
114	LB_114	7	8	7.5	4	4	4	7	3	5
115	LB_115	2	4	3	1	1	1	4	5	4.5
116	LB_116	3	5	4	1	1	1	5	4	4.5
117	LB_117	2	3	2.5	3	2	2.5	4	6	5
118	LB_118	5	5	5	3	3	3	3	3	3
119	LB_119	1	2	1.5	3	2	2.5	5	4	4.5
120	LB_120	6	7	6.5	5	3	4	2	5	3.5
121	LB_121	4	5	4.5	6	5	5.5	4	5	4.5
122	LB_122	5	4	4.5	6	3	4.5	5	4	4.5
123	LB_123	6	6	6	5	4	4.5	5	7	6

124	LB_124	4	4	4	4	3	3.5	7	7	7
125	LB_125	3	3	3	3	2	2.5	5	7	6
126	LB_126	2	2	2	2	1	1.5	1	1	1
127	LB_127	3	3	3	5	5	5	4	4	4
128	LB_128	4	3	3.5	3	3	3	2	3	2.5
129	LB_129	3	4	3.5	4	3	3.5	6	6	6
130	LB_130	3	4	3.5	4	4	4	2	5	3.5
131	LB_131	7	7	7	3	4	3.5	6	5	5.5
132	LB_132	2	2	2	4	2	3	2	2	2
133	LB_133	2	2	2	4	2	3	4	3	3.5
134	LB_134	4	4	4	5	3	4	5	4	4.5
135	LB_135	3	3	3	3	4	3.5	4	5	4.5
136	LB_136	2	3	2.5	3	4	3.5	7	7	7
137	LB_137	5	4	4.5	4	2	3	2	2	2
138	LB_138	3	4	3.5	3	2	2.5	3	3	3
139	LB_139	5	5	5	3	3	3	2	4	3
140	LB_140	3	3	3	3	1	2	5	3	4
141	LB_141	5	5	5	7	5	6	7	6	6.5
142	LB_142	2	2	2	3	1	2	3	2	2.5
143	LB_143	7	7	7	8	8	8	9	8	8.5
144	LB_144	3	3	3	2	1	1.5	6	5	5.5
145	LB_145	2	2	2	3	2	2.5	4	3	3.5
146	LB_146	3	4	3.5	2	2	2	5	5	5
147	LB_147	6	5	5.5	6	6	6	4	6	5
148	LB_148	4	3	3.5	2	2	2	6	6	6
149	LB_149	5	5	5	5	4	4.5	8	8	8
150	LB_150	2	2	2	4	4	4	3	4	3.5
151	LB_151	2	3	2.5	4	3	3.5	6	3	4.5
152	LB_152	7	7	7	4	5	4.5	6	4	5
153	LB_153	9	8	8.5	4	4	4	7	8	7.5
154	LB_154	3	3	3	7	6	6.5	2	2	2
155	LB_155	3	3	3	5	4	4.5	5	5	5
156	LB_156	2	3	2.5	4	5	4.5	5	5	5
157	LB_157	6	6	6	2	2	2	6	6	6
158	LB_158	2	4	3	3	3	3	3	4	3.5
159	LB_159	4	4	4	4	4	4	4	3	3.5
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161	LB_161	2	3	2.5	3	3	3	5	3	4
162	LB_162	3	4	3.5	7	4	5.5	3	5	4
163	LB_163	3	5	4	4	3	3.5	2	3	2.5
164	LB_164	4	5	4.5	6	4	5	5	4	4.5
165	LB_165	5	3	4	6	4	5	6	4	5
166	LB_166	3	2	2.5	3	3	3	4	3	3.5

167	LB_167	4	6	5	4	3	3.5	3	3	3
168	LB_168	6	6	6	3	2	2.5	7	8	7.5
169	LB_169	7	6	6.5	2	3	2.5	5	7	6
170	LB_170	4	5	4.5	4	4	4	3	4	3.5
171	LB_171	4	4	4	3	2	2.5	7	5	6
172	LB_172	4	4	4	5	4	4.5	2	2	2
173	LB_173	2	2	2	5	3	4	5	4	4.5
174	LB_174	2	3	2.5	4	4	4	6	3	4.5
175	LB_175	2	2	2	4	3	3.5	4	2	3
176	LB_176	3	3	3	6	6	6	2	4	3
177	LB_177	4	4	4	3	2	2.5	6	4	5
178	LB_178	5	5	5	5	5	5	4	7	5.5
179	LB_179	2	2	2	1	1	1	3	3	3
180	LB_180	6	7	6.5	3	4	3.5	2	6	4
181	LB_181	4	4	4	2	3	2.5	5	5	5
182	LB_182	6	6	6	4	3	3.5	4	6	5
183	LB_183	3	2	2.5	3	3	3	5	4	4.5
184	LB_184	2	2	2	2	2	2	4	3	3.5
185	LB_185	5	5	5	6	6	6	6	6	6
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187	LB_187	3	5	4	4	3	3.5	5	5	5
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189	LB_189	3	3	3	3	3	3	6	4	5
190	LB_190	3	3	3	4	4	4	3	6	4.5
191	LB_191	2	2	2	3	3	3	2	3	2.5
192	LB_192	4	4	4	3	4	3.5	3	3	3
193	LB_193	1	2	1.5	2	2	2	3	3	3
194	LB_194	2	3	2.5	4	4	4	3	3	3
195	LB_195	3	3	3	2	2	2	4	4	4
196	LB_196	6	6	6	3	2	2.5	6	4	5
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202	LB_202	3	4	3.5	7	7	7	7	6	6.5
203	LB_203	4	3	3.5	2	2	2	5	6	5.5
204	LB_204	6	7	6.5	3	4	3.5	7	7	7
205	LB_205	7	6	6.5	5	5	5	9	8	8.5
206	LB_206	2	2	2	2	2	2	4	4	4
207	LB_207	7	7	7	6	6	6	7	7	7
208	LB_208	2	5	3.5	5	6	5.5	3	3	3
209	LB_209	3	3	3	5	4	4.5	9	5	7

210	LB_210	2	2	2	1	2	1.5	3	3	3
211	LB_211	2	3	2.5	1	3	2	6	5	5.5
212	LB_212	5	4	4.5	4	4	4	6	7	6.5
213	LB_213	5	5	5	3	5	4	7	4	5.5
214	LB_214	2	2	2	1	1	1	3	3	3
215	LB_215	3	5	4	3	3	3	5	5	5
216	LB_216	2	2	2	2	3	2.5	4	3	3.5
217	LB_217	3	1	2	4	4	4	6	4	5
218	LB_218	3	4	3.5	3	1	2	3	5	4
219	LB_219	3	4	3.5	3	3	3	5	5	5
220	LB_220	2	2	2	3	3	3	3	3	3
221	LB_221	6	7	6.5	2	3	2.5	3	3	3
222	LB_222	5	5	5	2	3	2.5	6	3	4.5
223	LB_223	2	2	2	4	5	4.5	3	4	3.5
224	LB_224	4	4	4	3	4	3.5	5	4	4.5
225	LB_225	2	2	2	2	2	2	3	2	2.5
226	LB_226	7	7	7	8	8	8	10	10	10
227	LB_227	2	2	2	3	3	3	4	3	3.5
228	LB_228	2	2	2	4	4	4	3	4	3.5
229	LB_229	2	2	2	3	4	3.5	2	3	2.5
230	LB_230	4	5	4.5	4	3	3.5	3	5	4
231	LB_231	2	3	2.5	2	5	3.5	5	3	4
232	LB_232	4	5	4.5	1	1	1	5	6	5.5
233	LB_233	3	3	3	3	2	2.5	7	7	7
234	LB_234	3	2	2.5	5	5	5	3	3	3
235	LB_235	4	5	4.5	1	2	1.5	5	5	5
236	LB_236	2	2	2	2	2	2	4	3	3.5
237	LB_237	4	5	4.5	4	5	4.5	3	5	4
238	LB_238	3	2	2.5	2	2	2	0	1	0.5
239	LB_239	3	3	3	3	2	2.5	6	4	5
240	LB_240	2	3	2.5	4	4	4	7	6	6.5
241	LB_241	4	5	4.5	4	4	4	4	5	4.5
242	LB_242	5	5	5	5	4	4.5	4	5	4.5
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244	LB_244	3	2	2.5	3	3	3	4	4	4
245	LB_245	7	6	6.5	6	5	5.5	2	4	3
246	LB_246	3	4	3.5	4	4	4	3	2	2.5
247	LB_247	3	3	3	2	3	2.5	4	4	4
248	LB_248	4	3	3.5	3	4	3.5	3	3	3
249	LB_249	4	4	4	4	4	4	5	5	5
250	LB_250	3	2	2.5	3	4	3.5	2	4	3
251	LB_251	4	4	4	5	3	4	2	2	2
252	LB_252	3	3	3	4	4	4	0	0	0

253	LB_253	6	6	6	5	5	5	7	7	7
254	LB_254	3	2	2.5	7	7	7	2	2	2
255	LB_255	6	6	6	4	4	4	6	7	6.5
256	LB_256	4	5	4.5	2	2	2	5	6	5.5
257	LB_257	2	2	2	3	3	3	2	3	2.5
258	LB_258	2	2	2	3	3	3	1	2	1.5
259	LB_259	7	7	7	2	2	2	7	7	7
260	LB_260	3	3	3	2	1	1.5	4	5	4.5
261	LB_261	5	4	4.5	5	4	4.5	4	7	5.5
262	LB_262	4	4	4	4	4	4	3	7	5
263	LB_263	3	3	3	3	4	3.5	5	5	5
264	LB_264	8	8	8	7	7	7	3	3	3
265	LB_265	5	4	4.5	4	2	3	6	6	6
266	LB_266	3	3	3	5	5	5	3	3	3
267	LB_267	2	2	2	4	4	4	3	4	3.5
268	LB_268	4	4	4	2	2	2	6	5	5.5
269	LB_269	4	4	4	2	2	2	7	7	7
270	LB_270	4	3	3.5	5	5	5	5	5	5
271	LB_271	4	3	3.5	3	4	3.5	7	6	6.5
272	LB_272	4	4	4	6	6	6	8	7	7.5
273	LB_273	5	5	5	4	2	3	8	8	8
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275	LB_275	2	2	2	4	4	4	3	5	4
276	LB_276	2	2	2	3	3	3	5	5	5
277	LB_277	4	4	4	4	3	3.5	3	5	4
278	LB_278	3	3	3	3	2	2.5	5	4	4.5
279	LB_279	3	3	3	6	6	6	5	3	4
280	LB_280	5	5	5	6	5	5.5	4	4	4
281	LB_281	4	3	3.5	4	6	5	4	3	3.5
282	LB_282	6	6	6	8	6	7	8	8	8
283	LB_283	6	6	6	4	4	4	8	6	7
284	LB_284	3	3	3	5	5	5	4	3	3.5
285	LB_285	2	3	2.5	5	5	5	3	4	3.5
286	LB_286	2	2	2	5	3	4	2	2	2
287	LB_287	2	3	2.5	4	4	4	5	5	5
288	LB_288	5	6	5.5	5	5	5	7	7	7
289	LB_289	3	3	3	2	2	2	4	2	3
290	LB_290	4	4	4	2	3	2.5	5	4	4.5
291	LB_291	3	3	3	3	3	3	4	4	4
292	LB_292	2	2	2	2	2	2	3	4	3.5
293	LB_293	9	7	8	4	4	4	5	7	6
294	LB_294	3	3	3	5	4	4.5	4	3	3.5
295	LB_295	3	3	3	4	5	4.5	5	4	4.5

296	LB_296	5	4	4.5	5	6	5.5	6	6	6
297	LB_297	4	4	4	6	6	6	3	3	3
298	LB_298	8	8	8	4	2	3	5	5	5
299	LB_299	3	2	2.5	5	5	5	4	3	3.5
300	LB_300	3	3	3	3	2	2.5	4	4	4
301	LM-1	2	3	2.5	2	2.2	2.1	3	3.2	3.1
302	Bengal	3.7	3.5	6.6	2	3	5	3	2.5	5.6

APPENDIX F. BACTERIAL PANICLE BLIGHT AND SHEATH BLIGHT SYMPTOMS IN LB-33 AND ITS PARENTS

Bacterial panicle blight symptoms

Sheath blight symptoms

LM-1 LB-33 Bengal

APPENDIX G. PHENOTYPIC VARIATIONS AMONG LB-33 AND PARENTS

LB-33 Bengal LM-1

APPENDIX H. BIOLOGICAL CONTROL OF BACTERIAL PANICLE BLIGHT AND SHEATH BLIGHT OF RICE

Bacterial panicle blight (BPB) of rice, caused by *Burkholderia glumae*, is the major disease of rice in the Southern United States. This disease may cause up-to 70% loss when climatic conditions are optimal for disease development, particularly high night temperature and relative humidity. Despite its huge economic importance in rice producing areas around the world, not many studies have been conducted to develop efficient control measures. Oxolinic acid is used as a chemical for controlling seedling rot and grain rot of rice caused by *B. glumae* in Japan, however, this chemical is not registered in US (Nandakumar et al., 2009). Pretreatment of seeds before sowing and spraying during the heading stage of rice with oxolinic acid (OA) significantly reduced the population of *B. glumae* and showed a high efficacy for the control of seedling rot and grain rot of rice (Hikichi and Egami, 1995).

Sheath blight of rice caused by *Rhizoctonia solani* is another most important rice disease of Louisiana that greatly reduces yield and grain quality. No rice cultivars grown in the world are immune to this disease. Application of fungicides is always associated with financial cost, environmental hazards and development of resistance by the pathogen. Biological control of sheath blight is considered as an promising alternative to chemical control (Wiwattanapatapee et al., 2007). Several species of *Bacillus* have been widely studied for the control of sheath blight because of their broad range of anti-microbial activity (Peng et al., 2013).

Bacteria belonging to the *Burkholderia cepacia* complex (Bcc), a group of remarkably versatile bacteria, have been used frequently as antagonistic

microorganisms to control diseases of agricultural importance (Parke and Gurian-Sherman, 2001). Bcc complex has the capacity to produce volatile and non-volatile compounds, such as antibiotics, alkaloids and siderophores, which are involved in bio-control activity (Roitman et al., 1990). Different strains of *B. glumae* (formerly *Pseudomonas glumae*) collected from various locations in Japan were considerably different in pathogenicity level, i.e. some strains maintained strong pathogenicity while others are non-pathogenic (Furaya, 1991). The seedling rot caused by *B. glumae* was suppressed by pre-treatment of non-pathogenic strains and the suppression of disease was only observed with living avirulent cells, suggesting the mechanism of disease suppression by non-pathogenic strains could be due to competition for infection sites and nutrients (Furaya, 1991).

Previously, we reported genotypic and phenotypic diversity among *B. glumae* strains. The objectives of this study were to evaluate the effect of non-pathogenic strains of *B. glumae* for the in-vitro suppression of pathogenic *B. glumae* and *R. solani* as well as suppression of bacterial panicle blight and sheath blight under field conditions.

APPENDIX I. MATERIAL AND METHODS FOR BIOLOGICAL CONTROL

Antifungal activity against *R. solani*

Antifungal activity of *B. glumae* strains against *R. solani* was determined following a previously reported method (O'Grady et al., 2011) with some modifications. Briefly, each *B. glumae* strain was grown overnight at 37°C. For inoculating potato dextrose agar (PDA) plates, 1 ml of overnight culture was centrifuged, washed with Luria Bertani (LB) broth twice and re-suspended in 100 µl of LB broth. 10 µl of aliquots of a cell suspension was dropped in three corners of each plate and incubated at 37°C overnight. Fungal inocula, 5 mm in diameter were cut from 1-week old culture plates and placed in the center of the plate containing the bacterial colonies. Plates were incubated at 25°C for 2 days, and the inhibition zone between *R. solani* and bacteria colonies was measured.

Antibacterial activity against *B. glumae*

A bacterial suspension was prepared as described above. First, 50 µl aliquots of a cell suspension of 336gr-1, a reference virulent strain of *B. glumae*, was uniformly spread on LB agar plates then10 µl aliquots of cell suspension of the tested strains were dropped in the center of each plate. The plates were incubated overnight at 37°C and the inhibition zone was measured.

Pretreatments of non-pathogenic strains of *B. glumae* against BPB and SB

Highly susceptible rice cultivar Trenasse was grown in a rice field at the Rice Research Station, Crowley, LA, in a row containing 25-30 plants with standard agronomic practices. For sheath blight, plants at late tillering stage were inoculated with four selected non-pathogenic strains of *B. glumae* at a concentration of OD_{600}= 2

($20 X 10^8$ cfu/ml) and water as a control, one day prior to inoculation with *R. solani*. Visual sheath blight symptoms were evaluated about 1 month after the inoculation of *R. solani*. For the bacterial panicle blight study, five non-pathogenic strains at a concentration of OD_{600}= 2 and water as a control were sprayed at the flowering stage. Following the next day a reference virulent strain 336gr-1 was inoculated at a concentration of OD_{600}= 0.1 ($1X10^8$ cfu/ml). After 10 days, visual symptoms of BPB were evaluated. In both, inoculated rice plants experiments plants were harvested at the maturity and yield was compared with a non-inoculated water control. The experiment was conducted in a row having about twenty five plants and each treatment was repeated four times. Data were analyzed using SAS software, version 9.2 (SAS Institute). Analysis of variance was performed at 95% confidence interval.

APPENDIX J. RESULTS FOR BIOLOGICAL CONTROL

B. glumae strains antifungal activity against _R. solani_

Nine pathogenic and eleven non-pathogenic strains of *B. glumae* varied in the ability to restrict the growth of *R. solani in vitro* (Figure 7.1 [A] and [B]). The results obtained by measuring the inhibition zone of *R. solani* grown on PDA plates showed that highly virulent, toxoflavin and pigment producing strain, 411gr-6, showed maximum antifungal activity which was significantly different at 95% confidence interval. Another toxoflavin producing but dark pigment lacking strain, 117g1-7-a, also showed strong antifungal activity. All the dark pigment producing but toxoflavin lacking, non-pathogenic strains (237gr-5, 961149-4-4 and 257sh-1) showed distinct levels of inhibition to *R. solani* (Figure 7.1[B]). All pigment producing (191sh-1, 201sh-1, 411gr-6, 11sh2-2-a, 189gr-8 and 261gr-9) and non-producing (336gr-1 and 117g1-7-a) virulent (toxoflavin producing) strains showed some level of inhibition. All the pathogenic strains and seven of the eleven non-pathogenic strains tested showed various levels of antifungal activities against *R. solani* in the laboratory

B. glumae strains antibacterial activity against a pathogenic _B. glumae_ strain

None of seven pathogenic and 11 non-pathogenic *B. glumae* strains caused a zone of inhibition against virulent *B. glumae* strain, 336gr-1. These results indicate lack of antibacterial activity against a pathogenic strain.

Suppression of the development of BPB and SB

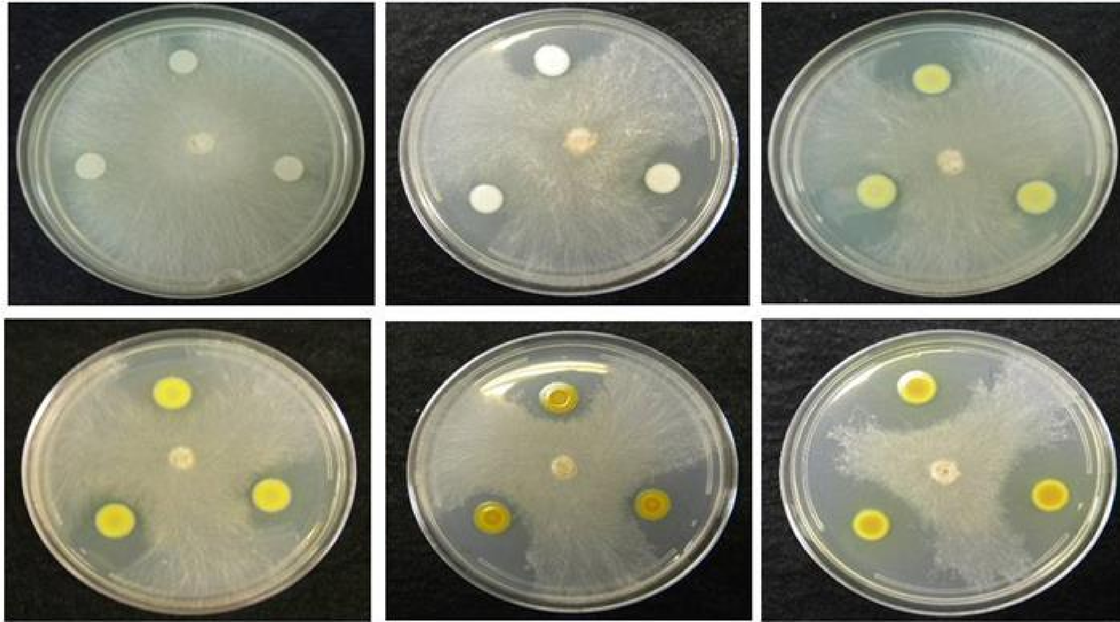
Application of non-pathogenic strains of *B. glumae* that showed antifungal activity against *R. solani in vitro* exhibited variable suppression of disease when applied to the susceptible cultivar, Trenasse, 1 day prior to inoculation with *R. solani* and *B. glumae*.

As consistent with the *in vitro* test of *R. solani*'s growth inhibition, all the tested strains significantly reduced SB infection when plants were pretreated with those strains prior to the pathogen inoculation. Plants pretreated with 257sh-1 and 396gr-2 were the best strains for reducing BPB symptoms and were statistically similar to the non-inoculated treatment. However, other treatments pretreated with 366gr-2 and 98gr-1 produced more BPB symptoms which are statistically similar to the water pretreatment control. For SB experiment, except pre-treatment with 961149-4-4, all the treatments reduced SB symptoms in comparison to water control. Pretreatment with 257sh-1 and 396gr-1 produced considerably less disease symptoms which is statistically similar to the water pretreatment control

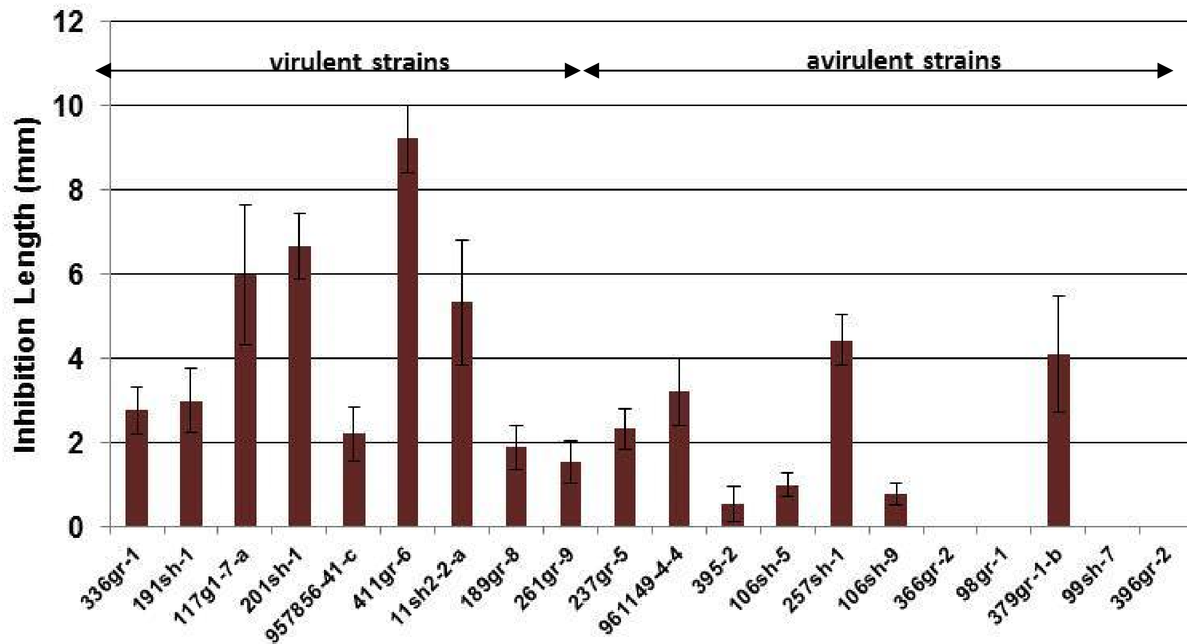
The effect of pretreatment of non-pathogenic strains of *B. glumae* on rice yield

The effect on rice yield resulting from pretreatment of plants with non-pathogenic strains of *B. glumae* for both BPB and SB diseases were determined on susceptible cultivar Trenasse. BPB yield effects were consistent with disease rating, pre-treatments of all the strains, had higher yield than the water pretreatment control. However, pretreatment with 396gr-2, produced the higher yield that was significantly different than the water pretreatment control Similarly, Pretreatment with 257sh-1 produced yield similar to the non-inoculated control that was significantly different than the water pretreatment control For SB, all the strains pretreatments produced higher yield than the water pretreatment control but were not significantly different than the non-inoculated water control However, yields obtained from inoculated and water pretreatment controls were significantly different with each other

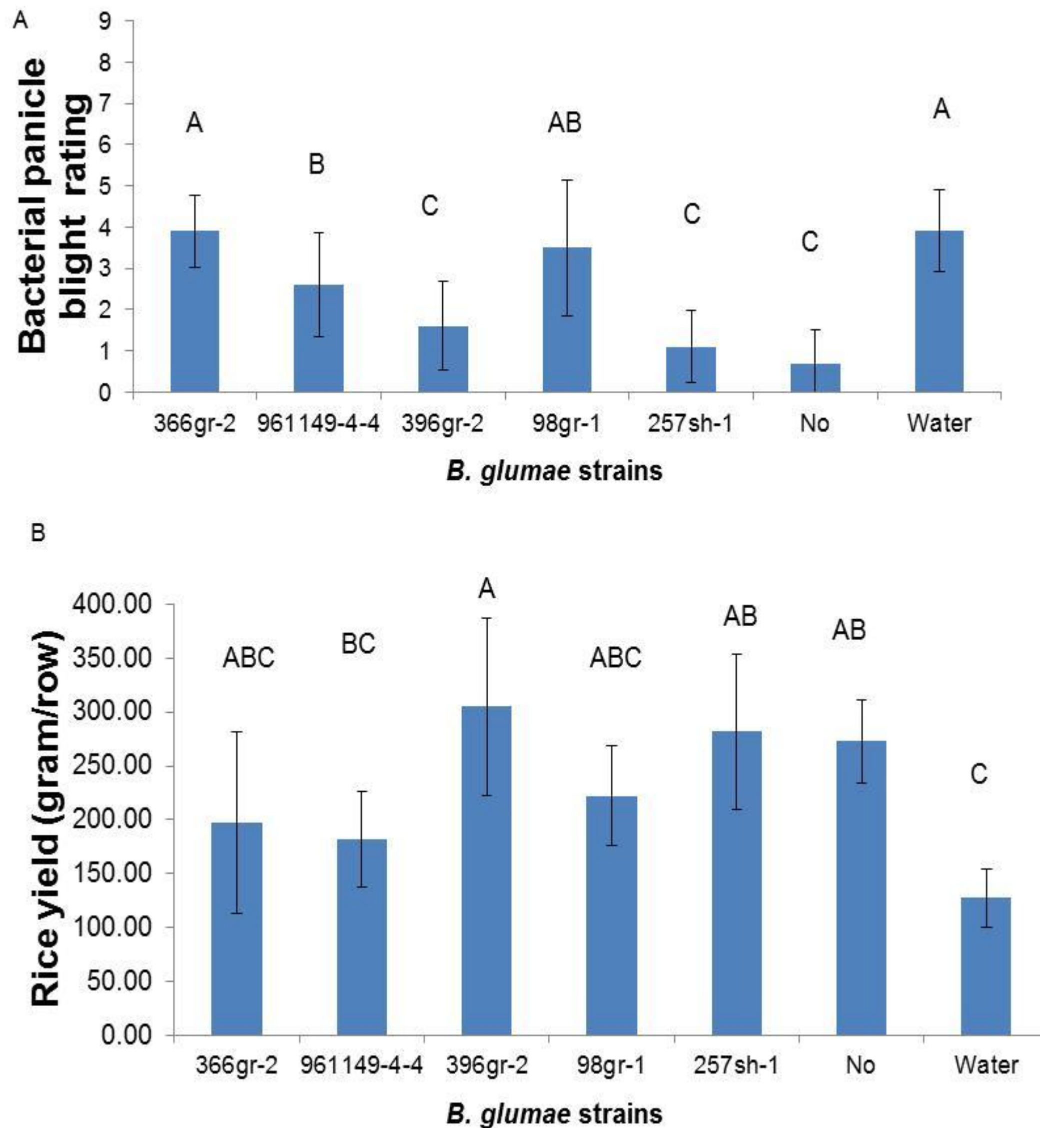
A



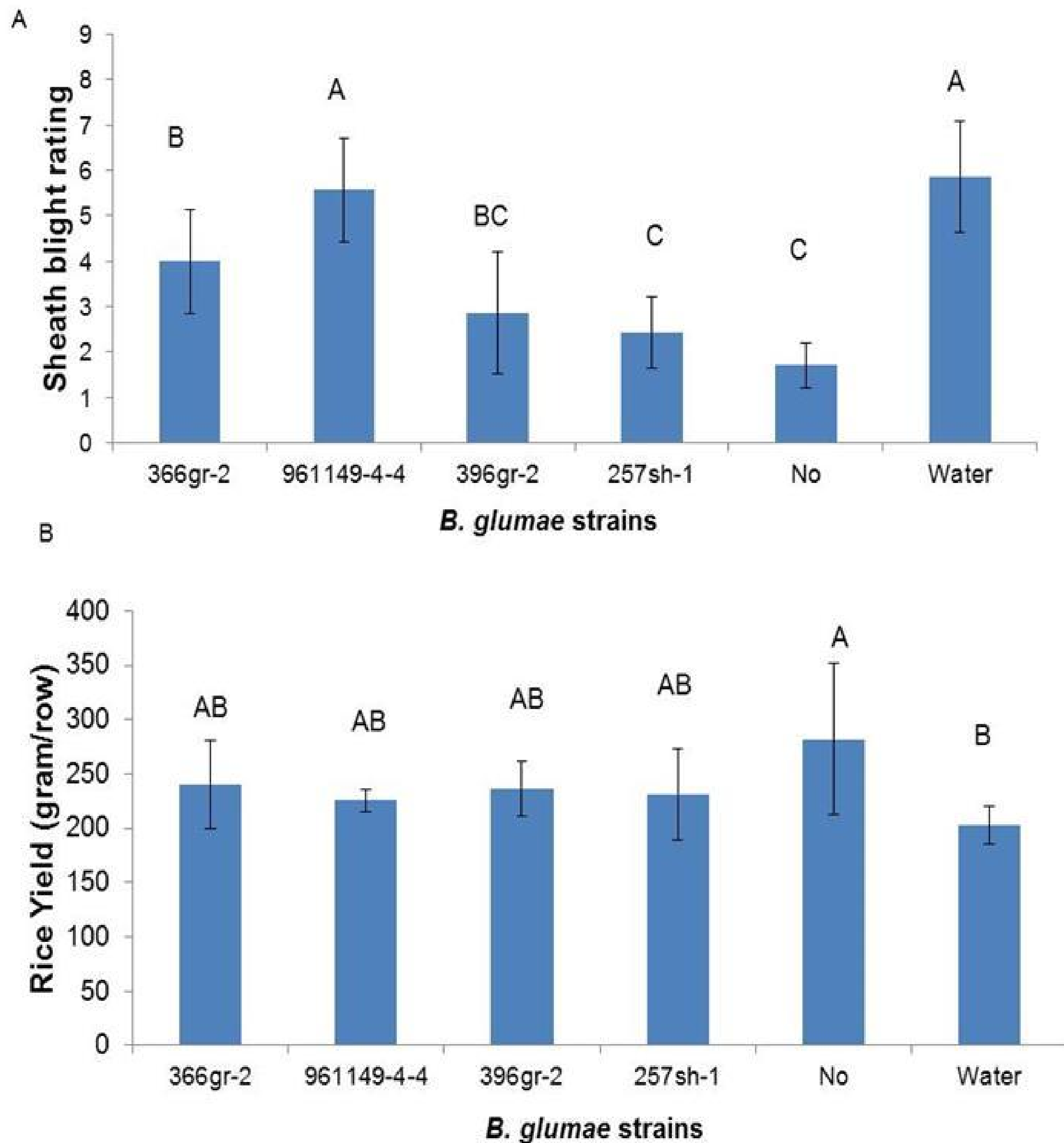
B



The antifungal activities of *Burkholderia glumae* strains against *Rhizoctonia solani*. (A) Images of antifungal activities on potato dextrose agar (PDA) plates. (B) Quantitative data of antifungal activities shown by individual strains of *B. glumae*.



The effect of pretreatment of with *Burkholderia glumae* strains on bacterial panicle blight of rice. A) Suppression of BPB symptom development by pretreatment with *B. glumae* strains. (B) Yields following subsequent inoculation of pathogenic strain of *B. glumae*. The various treatments indicated on X-axis are: 366gr-1, 961149-4-4, 396gr-2, 98gr-1 and 237sh-1 are non-pathogenic *B. glumae* strains, No (nothing was inoculated) and Water (Pretreatment with only followed by virulent *B. glumae*). Error bars indicates standard deviations. Yield indicates the dry weight of rice seeds at 13% moisture level after



The effects of pretreatments with *Burkholderia glumae* strains on sheath blight of rice. A) Suppression of SB symptom development resulting from the pretreatment with *B. glumae* strains. (B) Yields following subsequent inoculation with *R. solani*. The various treatments indicated on X-axis are: 366gr-1, 961149-4-4, 396gr-2, 98gr-1 and 237sh-1 are non-pathogenic *B. glumae* strains, No (nothing was inoculated) and water (Pretreatment with only followed by virulent *B. glumae*). Error bars indicates standard deviations. Yield indicates the dry weight of rice seeds at 13% moisture level after harvest from a single row. The letter above the column indicate significantly differences among the treatments (P<0.01).

APPENDIX K. DISCUSSION FOR BIOLOGICAL CONTROL

This study focuses on non-pathogenic strains of *B. glumae* isolated from the same ecological niches and already verified that they cannot cause disease on rice. These naturally occurring, non-pathogenic strains might suppress disease caused by virulent strains.

The ability of nine pathogenic and 11 non-pathogenic strains of *B. glumae* to restrict the growth of *R. solani in vitro* and compared their antifungal activity The results obtained by measuring the inhibition zone of *R. solani* showed that all the tested pathogenic strains had some level of antifungal activity. Since all the tested pathogenic strains produce toxoflavin (Karki et al., 2012b) and toxoflavin has antibacterial, antifungal and herbicidal effects (Tomohisa, 2002). It is probable that the observed antifungal activity by virulent strains is due to toxoflavin. The non-toxoflavin-producing avirulent strains (237gr-5, 961149-4-4 and 257sh-1) showed distinct level of antifungal activity, however, these strains produce other dark pigments different from toxoflavin, and these pigments inhibit the growth of *Collectotrichum orbiculare* (Karki et al., 2012b). From these observations, it is likely that toxoflavin and dark pigments are the some of the compounds responsible for the anti-fungal activity against *R. solani*. However, one avirulent strain, 379gr-1-b lacking toxoflavin and dark pigments production showed inhibition that could be interesting for further study. These naturally occuring avirulent strains showing antifungal activities can be good candidates biological control agents for sheath blight of rice. Our results also showed none of the tested *B. glumae* strains were capable of inhibiting *B. glumae* strains, *in vitro*, which indicates that these strains lack

antibacterial activity. It might be possible that *B. glumae* has a well-adapted system to protect cells from the toxic effect of toxoflavin and pigments.

Field efficacy tests were evaluated for some of the selected strains for the suppression of BPB and SB. All the tested strains reduced BPB and SB symptoms to some extent when they were applied as a pretreatment 1 day prior to pathogen inoculation. For BPB, pretreatment with 257sh-1 and 396gr-2 produced the least BPB symptoms which were statistically similar to non-inoculated treatment. In a previous study, it was shown that seedling rot disease caused by pathogenic strains of *B. glumae* was only suppressed by the living cells of non-pathogenic strains. The mechanism of disease suppression by avirulent strains may be due to competition for infection sites and nutrients. For SB except for 961149-4-4, pretreatment with, all the other strains resulted less severe SB symptoms than the water control. Pretreatment with 257sh-1 and 396gr-2 produced considerably less disease symptoms which were statistically similar with non-inoculated treatment. Contrary with the *in vitro* assay of growth inhibition of *R. solani*, it would be interesting to understand the reason behind suppression of SB by the *B. glumae* strain 396gr-1. SB suppression could be either experimental error or induction of plant defense system by 396gr-2.

BPB and SB can both significantly reduce rice yield. This study determined that pretreatment with selected non-pathogenic strains of *B. glumae* can reduce yield loss associated with both BPB and SB. For BPB, as consistent with disease rating all the strains pre-treatments had higher yield than the water pretreatment control. Pretreatment with 396gr-2 and 257sh-1, produced the highest yield which was significantly different than the water control treatment For SB, all the strains

pretreatment treatments produced a higher yield than the water control but was not significantly different from non-inoculated control. In conclusion, this study demonstrated that non-pathogenic *B. glumae* strains isolated from rice particularly 257sh-1 and 396gr-2 for BPB and 257sh-1 for SB, can suppress disease and prevent the yield reduction caused by these diseases. Because these *B. glumae* strains isolated from rice plants are naturally non-pathogenic and defective in multiple virulence factors and key regulatory systems including the quorum-sensing system (Karki, 2010), it would be safe to use these strains in the environment for sustainable agriculture production.

APPENDIX L. LETTER OF PERMISSION

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THE VITA

Hari Sharan Karki was born in Sathighar-2, Kavre, Nepal. His primary and secondary schooling was done in different schools of Kathmandu and Kavre districts. He moved away from his home after school leaving certificate to study Intermediate of Science in Agriculture in 1995. He then moved to Institute of Agriculture and Animal Science, Rampur, to pursue Bachelor of Science in agriculture in 1998. He graduated in early 2003 with Bachelor of Science in Agriculture with majoring in Ecology. He started working as a volunteer in Nepal-Australia Community Resource Management and Livelihood Project, where he learnt some aspects of natural resources and economics for the ultra-poor people. Three months later he got job offer in Male's International (sister company of Vossen & Co, Belgium based multinational company) as an agriculture officer, where he able to develop several economically viable cultivation practices package of essential oil bearing plants in community forests of sub-tropical region of Nepal. During his job period, he introduced some wild species of essential oils bearing plants in the community forests for cultivation in commercial scale and developed himself as an expert of essential oil bearing plants in sub-tropical region of Nepal. In 2007, he joined Chaudary Biosys Nepal, enterprises of Chaudary Group (one of the largest conglomerates of Nepal) as a local advisor for B2B program (Program funded by Government of Denmark). Since 2008, he has been working as a graduate assistant in Dr. Ham's Lab.