UNDERSTANDING DURABLE DISEASE RESISTANCE IN RICE

by

SEWEON LEE

B.S., Kyonggi University, Suwon, Korea, 1993
M.S., Dankook University, Cheonan, Korea, 1996

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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Abstract

Both qualitative and quantitative resistance mechanisms are important contributors to disease resistance in rice. To identify useful sources of durable resistance for Korean breeding programs, the distribution of rice blast isolates compatible to widely used resistance (*R*) genes was analyzed. Interactions of 3,747 Korean rice *Magnaporthe oryzae* isolates with eight monogenic lines, each harboring a major blast *R* gene, were tested. Lines with *R* gene *Pi-9* and *Pi-5* were susceptible to the fewest *M. oryzae* isolates, and therefore, this gene might be applied for blast resistance in breeding programs in Korea. Six major blast resistance genes were susceptible to more than 60 % of the population, suggesting limited utility of these genes in breeding programs. Quantitative trait loci (QTL)-based resistance is predicted to provide durable and broad spectrum resistance to rice diseases. A candidate gene approach was applied to a population of 164 recombinant inbred lines to identify sources of quantitative reisistance. Resistance gene analogs and defense response genes were mapped on the rice chromosomes, and analyzed for their association with blast and bacterial blight resistance QTL. A total of 21 putative QTL for blast resistance were identified on chromosomes 1, 4, 5, 6, 8, 9 and 12. Four putative QTL for bacterial blight resistance were identified on chromosome 3, 5 and 10. Thirteen RGA markers were associated with 11 different QTL on chromosome 1, 5, 8, and 9. The role of one disease resistance QTL associated gene, *Os02g39330*, encoding a chitinase was investigated for contributing to basal defense responses. RNAi silencing was used to evaluate contributions of the gene for the resistance to *Rhizoctonia solani* and *M. oryzae.* Five transgenic lines harboring the silencing construct and which differed in the level of expression of *Os02g39330* were screened for responses to *R. solani* and *M. oryzae.* The chitinase gene expression levels were inversely correlated with sheath blight disease severity, suggesting a role for this defense gene in resistance to *R. solani.* Rice blast disease was not affected by silencing *Os02g39330.* Both qualitative and QTL-based resistances provide valuable sources of disease resistance, and a combination of *R* gene *Pi-9* and QTL harboring the *Os02g39330* chitinase may help to stabilize resistance.

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CHAPTER 1 - Literature Review : Resistance To Rice Diseases by Quantitative Trait Loci

1. General Aspects of Plant Pathogen Interaction

Due to the innate nature of plants as a fixed living creature and their high carbohydrate content, they are easy targets for attack by multiple parasites, such as nematodes, insects, and microorganisms including viruses, bacteria and fungi. To protect themselves against the parasites, plants have evolved two different strategies, constitutive and inducible defense mechanisms (Odjakova and Hadjiivanova, 2001). Examples of constitutive defense mechanisms are the waxy exterior plant surface and the constant production of antimicrobial compounds (Agrios, 2005; Odjakova and Hadjiivanova, 2001). Inducible defense mechanisms are triggered by infection of pathogens, and rely on detection of pathogen attack, delivery of signals to the nucleus by various signal molecules, up- or down-regulation of defense genes, and inactivation of the pathogens with the gene product or the compounds produced by action of the gene product (Agrios, 2005; Odjakova and Hadjiivanova, 2001).

Plant-pathogen interactions can be classified as non-host interactions, compatible, and incompatible interactions. In a non-host interaction, a plant species is resistant to all strains of a given pathogen, therefore, the putative pathogenic microbes are unable to reproduce and colonize it (Heath, 1991; Mysore and Ryu, 2004). Incompatible interactions between a resistant host and an avirulent pathogen result in a hypersensitive response (HR, Hammond-Kosack and Jones, 1996). The HR is characterized by localized cell death at the site of infection, thus confining the spread of the infected microbes (Van Loon, 1997; Hammond-Kosack and Jones, 1996; Baker et

al., 1997; Fritig et al., 1998). Compatible interactions occur when a virulent pathogen infects a susceptible or moderately resistant host, resulting in severe damage or death due to colonization of the plants (Agrios, 2005).

Following pathogen infection, the initial plant response is a change in plasma membrane permeability, influx of calcium and protons, and efflux of chloride (McDowell and Dangl, 2000). Subsequently, ion fluxes in plant-pathogen interactions lead to production of reactive oxygen species (ROS) in the apoplast. Superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl free radical ($OH^{\bullet}$) are designated ROS due to their highly oxidizing potentials. High production of ROS is harmful to plants, however, regulated production of ROS in biotic and abiotic stresses can trigger plant defense responses. In a plant-pathogen interaction, the production of ROS is catalyzed by a plasma membrane-located NADPH oxidase and/or apoplastic-localized peroxidases (Somssich and Hahlbrock, 1998). The initial transient production of ROS, an oxidative burst, can trigger a complex but highly organized signaling pathway for defense responses, including hypersensitive response induction and defense gene expression (Piffanelli et al., 1999; Lamb and Dixon, 1997; Alvarez et al., 1998). The burst of ROS, especially H_2O_2, at the plant cell surface induces the cross-linking of the structural proteins in the cell wall, which fortifies the cell surface against pathogen infiltration (Scheel, 1998). The oxidative burst also triggers plant defense responses by inducing signal molecules, including salicyclic acid (SA), jasmonate (JA), or ethylene (ET), which may then move into the nucleus (Van Wees et al., 2000; Devadas et al., 2002).

SA plays a central role in plant defense against pathogens (Kunkel and Brooks, 2002). The accumulation of SA is observed in the immediate vicinity of incompatible interactions and exogenous application of SA induces higher levels of resistance to a broad range of pathogens.

SA is derived from the phenylpropanoid pathway, and can lead to disease resistance by encoding pathogenesis-related (PR) proteins. JA is produced from linolenic acid, and is released from membrane unsaturated fatty acids by the action of membrane-located phospholipases; the phospholipases are induced following pathogen attack. Linolenic acid is catalyzed to JA and related molecules via a series of enzymatic steps. Ethylene (ET), the only gaseous plant hormone, is produced at high levels during compatible and incompatible interactions. ET preferentially induces basic pathogenesis-related proteins. These signal molecules affect each other's signaling potential positively or negatively, therefore, the cross talk among signaling molecules allows plants to effectively regulate defense responses. The delivered signals control up-/or down-regulated transcription factors, resulting in reprogramming of cellular metabolic pathway for pathogen defense.

A large number of genes involved in pathogen defense have been cloned. Two major groups of genes may be induced in pathogen defense response; resistance or R-genes, which are involved in the recognition of pathogen avirulence effector genes (Bent, 1996) and plant defense response (DR) genes. Many different R genes have been cloned; those in common families share sequences motifs (Martin et al., 1993; Jones et al., 1994; Staskawicz et al., 1995). Commonly found motifs include gene products with a leucine rich repeats (LRR) and nucleotide binding sites (NB) (Hulbert et al., 2001). Conserved motifs in the same family reflect similar functions in the signal transduction in pathogen defense (Hulbert et al., 2001). These R-genes are usually associated with qualitative resistance; however, R-genes, particularly defeated R-genes, may be associated with quantitative resistance (Wang et al., 1994). DR genes are up- or down-regulated after pathogen attack, and frequently, the altered enzymatic activities are directly related to pathogen suppression. The gene products of DR genes are as follows: (a) structural proteins such

as peroxidases, oxidases participating in the confinement of the pathogen at the penetration sites, (b) enzymes involved in secondary metabolism for anti-pathogenic chemicals, e.g., enzymes in the phenylpropanoid pathway, and (c) enzymes directly involved in the defense responses including PR1, chitinases, and glucanases (Stintzi et al., 1993). Overexpression of these R-genes and DR genes in plants results in increased resistance to pathogens (Wu et al., 1995; Kachroo et al., 2003; Livingstone et al., 2005; Hu et al., 2003). Furthermore, suppression of these genes increases the susceptibility to disease (Pallas et al., 1996; Zimmermann et al., 2006; Christensen et al., 2004; Manosalva et al., 2009). However, studies using overexpression or suppression lines for the R-genes or DR-genes do not always correlate to increased resistance or augmented susceptibility, respectively. Possible explanations for these results are redundancy of the genes or gene functions, or effects of the host genetic background.

2. Qualitative and Quantitative Resistance

Many plants reduce pathogen infection through activation of resistance genes (Thompson and Burdon, 1992). Qualitative resistance is induced by R genes, in which a gene-for-gene interaction between the R gene product and the product of pathogen effector (previously called avirulence) genes. Qualitative resistance follows Mendelian inheritance, suggesting that it is monogenic or oligogenic (Nicholas et al., 2004). Quantitative resistance lacks specificity to a pathogen or group of pathogens, however, and the accumulated effects of quantitatively expressed defense genes result in resistance to the pathogen. Quantitative resistance to a pathogen is predicted to be more durable, which is desirable in crops. On the other hand, qualitative resistance, although highly heritable, is frequently unstable. Despite the genetic differences, both types of resistance can be utilized to increase resistance to pathogenic attack.

2.1. Qualitative and quantitative resistance

Qualitative resistance is also called host resistance, gene-for-gene resistance, qualitative resistance and race specific resistance (Flor, 1971). In qualitative resistance, a set of disease resistance (R) genes in a given plant species can specifically recognize a specific pathogen, which expresses a set of effector (*avr*) genes. The interaction results in disease resistance. In this resistance, disease susceptibility is the result of either the lack of R genes or the absence of the corresponding *avr* genes (Flor, 1971). Isolation of the R genes has been a great success in the past 10 years, with cloning of more than 70 race-cultivar specific resistance genes (Hulbert et al., 2001; Liu et al., 2007; Martin et al., 2003). These genes can be classified into several families, with those sharing common sequences motifs in the same family (Martin et al., 1993; Jones et al., 1994; Staskawicz et al., 1995). So far, cloned R genes have been used in crop breeding programs to increase resistance to specific pathogens (Campbell et al., 2002; Michelmore, 2003; Pedley and Martin, 2003). Although host resistance has been greatly improved, the effectiveness can easily break down when the pathogen population shifts to a different pathogenic strain (McDonald and Linde, 2002).

Quantitative resistance has been extensively sought due to its presumed durability. Quantitative resistance is also known as non-race specific resistance, polygenic resistance, or field resistance, and is predicted to have a broad spectrum resistance against many different non-specific pathogenic strains. Quantitative resistance has the form of approximately normal distribution and is affected by the environment (Paran and Zamir, 2003). Because quantitative resistance is induced by genes at multiple loci, it exhibits additive effects governed by several genes and polygenic inheritance. This multigenic nature of quantitative resistance is believed to

contribute to its durability. Presumably, a plant pathogen would have difficulty breaking down a resistance governed by several genes. To break down the quantitative resistance, the invading pathogen strain would likely have to mutate or modify several genes at the same time. Despite the importance of quantitative resistance in plants, the accumulated molecular mechanisms for resistance by multiple genes remain unclear. Even in quantitative resistance induced by a few genes exerting strong effects, determination of which of the genes are most important for resistance is very difficult due to limited information from the discrete phenotypic segregation of the progeny (Yano and Sasaki, 1997). Elucidation of the genes governing the quantitative resistance is important for understanding molecular plant-pathogen interactions as well as for enhancing crop yield.

2. 2. *Quantitative* trait loci (QTL) mapping and cloning

Variations in resistance for quantitative resistance result from segregation at multiple QTL (Paran and Zamir, 2003). In 1923, Sax first developed the concept for detecting QTL using linked major genes by associating the sizes of the seed (polygenic trait) with the color of the seed coat (monogenic trait) in *Phaseolus* genotypes (Sax, 1923). He found that segregation for quantitative traits was not different from simple Mendelian traits. Therefore, quantitative traits were expected to exhibit similar segregation and recombination properties. Conventional morphological markers, however, were not sufficient to prove this concept. Before the development of the QTL-mapping, studies of quantitative resistance were performed by employing statistical analysis for the progenies from two parental lines, each having different resistant levels.

QTL mapping has been adopted as a standard procedure for quantitative genetics. QTL mapping usually begins with the collection of phenotypic and genotypic data based on molecular markers from a segregating population, followed by statistical analysis which identifies all possible marker loci correlating allelic variation with the phenotype. A primary mapping population is composed of an F2 population or recombinant inbred lines (RIL). This primary mapping can position the QTL of interest within an approximately10-30 cM chromosomal interval, in which several hundred genes may be included (Salvi and Tuberosa, 2005). Paterson et al. (1988) first introduced the idea of QTL mapping using molecular DNA markers. They mapped QTL intervals affecting the soluble solid concentration and pH in tomato fruits and determined the QTL chromosome location and its phenotypic contribution. Following this study, many QTL in crop plants have been clarified using DNA markers (Paterson et al., 1991; Stuber et al., 1992).

Cloning of genes responsible for QTL was finally achieved through development of a technique called positional map-based cloning. Positional map-based cloning assigns a QTL to the shortest possible genetic interval (QTL fine mapping), and then identifies the corresponding interval on the DNA sequence (QTL physical mapping) for evaluating candidate genes (Salvi and Tuberosa, 2005). QTL fine mapping requires another experimental population, QTL-near isogenic lines (QTL-NILs). QTL-NILs are produced by crossing NILs differing only in the allelic constitution harboring the QTL. The target QTL becomes the major genetic source of variation in QTL-NILs because there are no segregating QTLs, therefore, the QTL is considered ‘Mendelized’ and more precise cM distances between a QTL and the nearby molecular markers can be estimated (Salvi and Tuberosa, 2005). The fine mapping step requires increased population size to minimize the size of the candidate gene region. Combinational analysis for the

large segregating population and chromosome-region-specific molecular markers can define a candidate genomic region less than 50 kb (Yano, 2001). The markers closest to the QTL are used for anchoring the genetic map to the physical map, and putative candidate genes in that region are predicted using bioinformatic approaches (Salvi and Tuberosa, 2005).The putative candidate gene(s) co-segregating with the QTL then need to be validated for the real target gene for the QTL (Yano et al., 2001). Many genes responsible for QTLs have been identified, e.g., Arabidopsis QTLs controlling flowering time (ED1 and FLW), glucosinolate structure (GS-elong), root morphology (BRX), and seed dormancy (El-Din, et al., 2001; Werner et al., 2005; Kroymann et al., 2003; Mouchel et al., 2004; Bentsink et al., 2006), tomato QTLs controlling fruit sugar content, fruit shape, and fruit weight, respectively (Fridman et al., 2004; Liu et al., 2002; Frary et al., 2000), and in rice three QTLs controlling heading time (Doi et al., 2004; Yano et al., 2000; Takahashi et al., 2001). To date, there are few examples of the cloning of disease resistance QTL in plants. In one example from rice, a cluster of 12 germin-like protein (OsGLP) genes, were shown to contribute to broad-spectrum disease resistance governed by a QTL on chromosome 8 (Manosalva et al., 2009). Four tandemly duplicated oxalate oxidase genes in rice (*OsOXO1-4*) were detected from a blast resistance QTL in chromosome 3 and one gene, (*OsOXO4*), which was expressed earlier in resistant than susceptible lines (Carrillo et al., 2009), was shown to contribute to a disease reistance QTL on rice chromosome 3 (Davidson, 2009).

3. Major Rice Diseases

Rice is one of the most important cereal crops in the world. It feeds more than three billion people and is cultivated all around the world, including nearly all Asian countries, the United States, Australia, Brazil, Egypt, and Italy (Cantrell and Reeves, 2002). Rice is the

common name of nearly 19 species of annual herbaceous plants in the *Oryza* genus of the gramineae family. *Oryza sativa* (Asian rice) is native to tropical and subtropical Asia, is the most widely cultivated species, and is important for human nourishment (Yano and Sasaki, 1997). In addition to its importance as a food crop, rice serves as a model plant for monocots due to the small genome size (~430 Mb), the availability of complete genome sequence, and the number of tools for functional genomics research (Izawa and Shimamoto, 1996; Shimamoto and Kyozuka, 2002; Yazaki et al., 2004; Han et al., 2007; Krishnan et al., 2009). Rice cultivation requires a warm and wet climate with abundant water. Traditionally, the young rice seedlings are submerged under water to reduce the growth of weeds and pests. Due to climate and cultivation requirements, diseases are inevitably widespread in rice paddies, and this often places major constraints on production. More than 70 diseases are caused by fungi, bacteria, viruses, or nematodes (Ou, 1985). Rice blast caused by the fungus *Magnaporthe oryzae* (*Mo*), bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), and sheath blight caused by the fungus *Rhizoctonia solani* are the most serious diseases impacting rice production (Ou, 1985).

Rice blast is estimated to destroy enough rice to feed more than 60 million people (Agrios, 2005). *Mo*, also known as rice blast fungus, is an ascomycete fungus (Agrios 2005). *Mo* also infects a number of other cereal crops including wheat, rye, barley, and pearl millet, causing diseases called blast disease or blight disease. An extremely effective plant pathogen, *Mo* has specialized structures, such as appressoria that infect aerial tissues and hyphae that can infect root tissues. *Mo* has seven chromosomes and a genome size of 40 Mb, with approximately 9,000 genes (Dean et al. 2005).

Xoo causes bacterial blight (BB), reducing rice yields through wilting of seedlings and yellowing and drying of leaves (Swing et al. 1990). BB is the most destructive rice bacterial disease of rice, occurring in tropical, subtropical, and warm temperate regions of the world. The symptoms start at leaf tips then increase in length and width with a wavy margin, making lesions which turn from yellow to white as the disease advances. Severely infected leaves tend to dry quickly. BB significantly changes the physiology of rice in many aspects, such as increasing or decreasing component substances, changes in enzyme activities, increase in respiratory rate, accumulation of photosynthetic assimilates, decrease in photosynthetic efficiency, and increase in water permeability of leaf cells (Misawa and Miyazaki, 1972; 1973).

The fungus *Rhizoctonia solani* causes rice sheath blight, which occurs throughout temperate and tropical rice production areas, and is especially prominent in growth areas under intense production (Lee and Rush, 1983). Sheath blight disease is very destructive under conditions of high humidity and warm temperatures. The disease also develops vigorously with high nitrogen application. First symptoms of sheath blight appear under water droplets on the plant surface, turning to spots or lesions mostly on the leaf sheath.

4. Candidate gene Approach for Quantitative Resistance in Rice

4-1. Candidate gene approach

In plant and animal genetics, the candidate gene (CG) approach has been extensively used in the past decade for the characterization and cloning of QTL (Pflieger et al. 2001). Three main approaches have been successfully used to identify and isolate genes governing important traits, such as positional cloning (Rommens et al. 1989), insertional mutagenesis (Bechtold et al. 1993), and the candidate gene (CG) approach. Positional cloning and insertional mutagenesis are

classical methods, limited by genome size and/or by the lack of transposons in the species being studied. In the CG approach, the main assumptions are that genes of known function could correspond to major loci (Mendelian trait loci or QTL) (Pflieger et al. 2001). In plant genetics, Byrne et al. (1996) first used the CG approach to find the maize QTL for resistance to corn earworm by linking candidate genes in the flavone synthesis pathway with the host defense response phenotype.

The candidate gene approach in rice was used to investigate many candidate disease responsive genes with quantitative resistance against rice blast, bacterial bight (BB), and sheath blight (SB) (Wang et al., 1994; Prashanth et al., 1998; Chen et al., 2001; Chen et al., 2003; Li et al., 1995; Li et al., 1999; Zou et al., 2000; Ramalingam et al., 2003). The most extensively studied candidate gene approach for quantitative resistance in rice is for resistance against rice blast (Wang et al., 1994, Prashanth et al., 1998; Sallaud et al., 2003; Talukder et al., 2005; Chen et al., 2003; Liu et al., 2004, Wu et al., 2004, Carrillo et al., 2005). The QTL mapping populations in these studies are composed of recombinant inbred lines (RILs), double haploid populations (DH), and backcrossing populations (BC3F3, and BC3F4). The association of major genes (*R*-genes), minor genes (DR-genes), environment x QTL interactions, and the concept of durable resistance were all included in the analysis during QTL mapping.

Both qualitative and quantitative resistances contribute to controlling rice blast disease. In a study using Moroberekan, a traditional African rice variety with a durable resistance against rice blast, major genes and QTLs were identified that contribute to durable resistance (Wang et al., 1994). Several QTLs mapped to regions containing major blast resistance genes (*Pi*) and were thought to be a manifestation of major defeated R-genes (Wang et al. 1994). In a study by Talukder et al. (2004), most of the QTLs were race specific and the manifestation of major

defeated R-genes was also reported. Chen et al. (2003) identified 12 rice blast resistance QTLs in a RIL mapping population. The genes underlying the QTLs may be divided into two categories of defense responses, those affecting a broad range of pathogens and those which conferred resistance to limited number of pathogens, e.g., exhibited race specificity.

A QTL study using a DH population developed from IR64, an *indica* variety and Azucena, a *japonica* variety was used to map QTLs for leaf and neck blast resistance. The identified QTLs had non-race specific effects and stability for blast resistance (Prashanth et al. 1998). Localization studies of expressed sequence tags (ESTs) using the same DH mapping population placed disease resistance genes or defense response genes onto the map (Wang et al. 2001). They localized 109 ESTs, clustering them into three groups mapped to the QTL regions on chromosome 1, 2, and 3. Among seven ESTs mapped at the region on chromosome 1, where a QTL for partial resistance against rice blast was identified, three ESTs have high homology with the gene *NPR-1* from *Arabidopsis*, and two ESTs encode LRR and receptor-like kinase domain, respectively. Six ESTs mapped at the region on chromosome 2, where partial resistance to sheath blight is located (Li et al., 1995). The genes for the ESTs encode PAL, Pto-like kinase, stromal ascorbate peroxidase, PR protein, and thaumatin-like protein, respectively. Two ESTs, corresponding to serine/threonine kinase domain homologs, mapped to the QTL region on chromosome 3, a region with partial resistance to three major rice diseases, rice blast (Wang et al., 1994), sheath blight (Li et al., 1995), and bacterial blight (Li et al., 1999). A total of 118 candidate gene makers were placed on the pre-existing genetic map generated from same DH population (Ramalingam et al. 2003). The candidate gene markers associated with rice blast, BB, SB, and brown plant-hopper are resistance gene analogs (RGAs), leucine-rich repeat (LRR), and

DR genes composed of aldose reductase, dihydrofolate reductase thymidylate synthase, JAMyb, oxalate oxidase, and peroxidase.

Association of candidate genes with blast resistance QTL was also tested using a set of recombinant inbred lines (RILs) derived from a cross between Sanhuangzhan 2 (SHZ-2), an *indica* cultivar exhibiting broad spectrum blast resistance, and Lijiangxin-tuan-heigu (LTH), a highly susceptible *japonica* cultivar (Liu et al. 2004). Five putative candidate genes associated with resistance against rice blast in the RILs were identified- the genes encoding for oxalate oxidase-like proteins, dehydrin, PR-1, chitinase 2a, and P14-3-3 proteins (*GF14-e*). The *oxalate oxidase-like* protein gene contributed the highest levels of resistance. Increased disease resistance was positively correlated with the accumulation of the five QTL regions (Liu et al. 2004). Two genes, *oxalate oxidase* and *P14-3-3*, were also confirmed to correlate with rice blast resistance QTL using an advanced backcross population BC3F3 derived from rice varieties Vandana/Moroberekan (Wu et al. 2004).

Approaches using functional genomics allowed confirmation of the roles of some of the DR genes on rice diseases (Manosalva 2006). Three genes, germin-like proteins, phenylalanine ammonia-lyase, and P14-3-3 proteins (*GF14-e*) were selected to measure their effect on the rice disease resistance. Among nine rice *OsPAL* genes encoding phenylalanine ammonia-lyase, *OsPAL4* colocalized with a QTL on chromosome 2; the contribution of *OsPAL4* to resistance against a virulent strain of *Xoo* was demonstrated using a reverse genetics approach (Manosalva 2006). A gene silencing approach, wherein all of the 12 *OsGLP* genes clustered on chromosome 8 were silenced, resulted in increased susceptibility to *Mo* and *R. solani*, suggesting that the *OsGLP* gene cluster contributes to rice blast and sheath blight resistance (Manosalva et al., 2009). Silencing of *GF14-e,* which co-localized to a blast disease resistance QTL on chromosome 2, did

not increase susceptibility to blast (Manosalva 2006). Instead, reduced expression of *GF14e* correlates with enhanced resistance to a virulent strain of *Xoo* and *R. solani*, suggesting that *GF14e* is a negative regulator of resistance (Manosalva 2006; Manosalva, Bruce et al. in prep).

CG approaches have been used successfully to find QTL-associated DR genes in other plant species including wheat, bean, and potato. In the study of Faris et al. (1999), the application of a CG approach for mapping disease resistance QTL in wheat revealed several DR genes, such as oxalate oxidase, peroxidase, superoxide dismutase, chitinase, P14-3-3, and thaumatin. In bean, genes corresponding to PAL and hydroxyproline-rich glycoprotein (HRGP) have been mapped to a QTL region conferring resistance against *Colletotrichum lindemuthianum* (Geffroy et al. 2000). Genes from the phenylpropanoid pathway (PAL, chalcone synthase, chalcone isomerase), cytocrome P450, osmotin (PR-5), and the WRKY gene transcription factor were co-localized with QTLs encoding resistance to *Phytophthora infestans* in potato (Trognitz et al., 2002).

4-2. RNAi for the validation of the candidate gene function

RNA interference (RNAi) is a highly conserved mechanism in all eukaryotes, serving as an effective defense mechanism against viral infection. RNAi in plants results in silencing of genes by RNA-guided gene regulatory mechanisms that include post-transcriptional gene silencing (PTGS) (Ding et al., 2004). Gene silencing induced by RNAi was observed in transgenic plants with ectopic but highly homologous transgenes, resulting in silencing of the expression of the transgenes and also of the endogenous gene (Napoli et al., 1990). There are two types of RNAs playing major roles in RNA silencing, double stranded RNA (dsRNA), which acts as a trigger for RNA breakdown, and small interfering RNA (siRNA, 21~24 nt) which are

involved in the actual degradation of the target mRNA (Hannon, 2002). Dicer, an RNAse III like enzyme cleaves dsRNAs into siRNAs (Bernstein et al., 2001). The newly generated siRNAs are incorporated into a multi-subunit protein complex, the RNAi-induced silencing complex (RISC complex). The RISC complex directs the siRNAs to the appropriate target mRNA. The RISC complex may unwind the siRNA to help interactions with the target mRNA, or serve as a primer for an RNA-dependent RNA polymerase to create many the siRNA molecules for signal amplification (Martinez et al., 2002). Miki et al. (2005) showed that RNA silencing with an inverted repeat (IR) construct with two members of the *OsRac* gene family suppressed the expression of members of the entire gene family. RNAi is now useful and widely used tool in plant biotechnology for discovering or validating gene functions (Brodersen et al., 2006; Margis et al. 2006; Vaucheret et al., 2006). RNAi has been also used for gene discovery in nematode, human and mouse (Berns et al. 2004; Moffat et al., 2006; Silva et al. 2005; Sonnichsen et al., 2005). Although problems with efficacy, stability and validation limit the uses of RNAi, the rapid pace of discovery will lead to continuous improvements biotechnological uses of RNAi (Small, 2007).

5. Oxalate oxidase and oxalate oxidase-like genes

Two closely-related DR genes associated with QTL conferring resistance to rice blast are *oxalate oxidase gene (OxOa;* now referred to as *OsOXO* genes (Davidson et al., 2009)) and *oxalate oxidase-like protein* (*OxOLP;* now refered to as *OsGLP* genes; (Manosalva et al., 2009) gene. *OsOXO* and *OsGLP* genes belong to the germin-like subfamily in the functionally diverse cupin superfamily (Woo et al., 2000). GLP genes are a multigene family, as demonstrated in barley HvGER4 gene subfamily which has a cluster of at least nine highly conserved duplicated

members (Druka et al., 2002; Wei et al., 1998; Davidson et al., 2009) and wheat TaGLP4 subfamily (Christensen et al., 2004). In the rice genome, more than 40 GLPs were identified using bioinformatics (Carrillo et al. 2005). As multimeric and glycosylated enzymes, germins and possibly some germin-like proteins have oxalate oxidase activity (Lane et al., 1993; Lane, 2000) and superoxide dismutase activity (Bernier and Berna, 2001), respectively. Germins and GLPs have been shown to be involved with plant defense response against pathogen attack (Lane, 2002; reviewed in Davidson et al. 2009). In barley, the genes encoding germins or GLPs are induced during infection of various pathogens including the powdery mildew fungus, *Erysiphe graminis f.*sp. hordei (Zhang et al., 1995; Dumas et al., 1995; Zhou et al., 1998; Zimmerman et al 2006; Schweizer et al., 1999; Wei et al., 1998). Induction of germin-like protein was observed in pepper plants infected with virus and bacterial pathogens (Park et al., 2004).

Overexpression or down-regulation of germin or GLP genes in plants regulates plant resistance to microbial pathogens. The ectopic expression of wheat germin, which has oxalate oxidase enzymatic activity, conferred resistance to pathogens in soybeans (Donaldson et al., 2001), in hybrid poplar (Liang et al., 2001), and in sunflowers (Hu et al., 2003). Transgenic peanut with a barley oxalate oxidase gene showed increased levels of resistance against *Sclerotinia minor* (Livingstone et al., 2005). Among five barley GLP genes, overexpression of two GLP genes (HvGER4 or HvGER5) enhanced resistance to powdery mildew and silencing of HvGER4 increased susceptibility of the pathogen (Zimmermann et al., 2006). Enhanced resistance against *Sclerotinia sclerotiorum* was induced in the transgenic sunflowers with the wheat oxalate oxidase gene, along with elevated levels of H_2O_2, SA, and defense gene expression (Hu et al., 2003). GLP genes were predicted to be important components of quantitative resistance in plants (Christensen et al. 2004).

Bioinformatic analysis using barley *OXO* and *GLP* gene sequences identified more than 40 GLP genes in rice chromosomes (Manosalva et al. 2009). Twelve GLP sequences clustered in a 2.8 Mb section on chromosome 8 in rice are similar to the barley HvOxOLP (accession no. X93171) in the conserved domain structure (Manosalva et al. 2009). These GLPs co-localized with the rice blast QTL for resistance in several mapping populations (Ramalingam et al., 2003; Liu et al., 2004; Carrillo et al., 2005; Wu et al., 2004). Rice *OsGLP*s on chromosome 8 are differentially expressed in response to wounding and *Mo* inoculation of the susceptible and resistant parents (Davidson et al., 2010). Interestingly, of the 12 chromosome 8 *OsGLPs*, one clustered subfamily called *OsGER4* contributes most to rice blast resistance (Manosalva et al. 2009).

Rice blast resistance QTL on chromosome 3 are linked to a putative *OsOXO* gene cluster (Ramalingam et al., 2003; Wu et al., 2004). Further studies identified that the cluster is composed of four tandemly duplicated OXO genes, *OsOXO1*-*OsOXO4*, and may play a role in resistance to *Mo* (Carrillo et al., 2009). Davidson (2009) showed that silencing of *OsOXO4* resulted in enhanced susceptibility to rice blast and sheath blight, confirming a role for the gene in QTL-based disease resistance.

6. Chitinase genes

Chitinase (EC 3.2.1.14) hydrolyzes chitin (poly-β-1,4-N-acetyl glucosamine), which is the main component of the cell walls of fungi and insects (Collinge et al. 1993). Interestingly, chitinases are found in many types of living organisms as well as in some viruses. Chitinases are classified into two groups, endochitinases and exochitinases, depending on their mode of action (Kasprzewska, 2003). Endochitinases cleave chitin polymers randomly at the internal glycosidic

bonds, generating chitin oligomers. Exochitinases generate N-acetyl monomers digesting preferentially the nonreducing end of chitin chains. Chitinases are secreted from microorganisms such as bacteria and fungi, and are produced by higher plants. Chitinases are included in the broad classification of glycosyl hydrolases and have been placed in families 18, 19, and 20 based on the similarities of their amino acid sequences (Henrissat et al. 1991). Family 18 chitinases are found in bacteria, fungi, yeast, viruses, plants and animals. Family 19 chitinases are almost exclusively present in plants. Chitinases of the family18 and family 19 do not share amino acid sequence similarity, therefore, they have completely different 3-D structures and enzymatic mechanisms (Henrissat, 1991; Henrissat and Bairoch, 1993; Iseli et al. 1996). Family 20 chitinases consist of the β-N-acetylhexosaminidases or β-N-acetylglucosaminidases, which are found in bacteria, fungi and humans.

Plant chitinases act as defensive machinery against invading fungi that have cell walls made of chitin (Collinge et al. 1993). Recent investigations also indicate that plant chitinases may be involved in growth and development processes as well as defense-related or general stress responses (De Jong et al. 1992; Goormachtig et al. 1998; Helleboid et al. 2000). Plant chitinases can also be divided into classes I-VII, in which classes III and V belong to family 18 whereas classes I, II, IV, VI, and VII comprise family 19. Pathogenesis-related (PR) proteins are divided into 14 classes and chitinases belong to three of those classes (Neuhaus et al. 1996). Class PR-3 includes chitinases of class Ia, Ib, II, IV, VI and VII. Chitinases of class III belong to PR-8. Chitinases of class V belong to PR-11. Additionally, in class PR-4, some proteins exhibit low endochitinase activity. Plant chitinases can be divided into two totally different families, such as family 18 for PR-8 and PR-11 and family 19 for PR-3 (Collinge et al. 1993; Neuhaus 1999).

The antifungal activity of plant chitinases makes this enzyme an attractive candidate for enhancing pathogen resistance by genetic engineering (Jayaraj and Punja 2007). The first report of success with this approach was transgenic tobacco and canola; increased expression of a bean vacuolar chitinase gene under the control of the CaMV 35S promoter allowed the plants to to survive in soil infected by *Rhizoctonia solani* and delayed development of disease (Broglie et al. 1991). Transgenic carrot plants with a basic tobacco chitinase showed higher resistance level against *B. cinerea, R. solani* and *Sclerotium rolfsii* (Punja 2005). Two separate lines of tobacco plants, transformed with two individual endochitinases (CHIT33 and CHIT42) from *Trichoderma harzianum*, increased a broad level of resistance to fungal and bacterial pathogens (Dana et al. 2006). In rice transformed with a rice-derived chimeric chitinase gene, Chi11, under the control of a CaMV35 promoter, increased resistance to sheath blight was noted (Lin et al. 1995). Transgenic Japonica type rice plants constitutively expressing either chitinase gene Cht-2 or Cht-3 significantly increased resistance against the rice blast pathogen *Mo* (Nishizawa et al. 1999). The high expression levels of chitinase and the higher resistance to the rice blast were stably inherited in the transgenic lines. Transgenic elite indica rice cultivars with a PR-3 rice chitinase gene (RC7) showed higher resistance to rice sheath blight disease caused by *R. solani* (Datta et al. 2001). Ectopic expression of rice chitinase genes in other plant species resulted in higher disease resistance as shown in cucumber (Tabei et al. 1998), grapevine (Yamamoto et al. 2000), rose (Marchant et al. 1998), Italian ryegrass (Takahashi et al. 2005), and chrysanthemum (Takatsu et al., 1999), etc.

7. Conclusion

Crop cultivars containing resistance genes have been extensively used for disease control. Breakdown of the resistance due to the high genetic variability observed in shifting pathogen populations results in only a few years of useful life-span for many resistant cultivars. There is high pressure to develop new strategies for increasing durable resistance, i.e., resistance that provides protection for a long time and over a wide cultivation area. Although single gene sources of resistance frequently are not durable, some R-genes are effective for long periods over large areas relative to others. In the first part of my thesis work, I explore what single disease resistance genes (qualitative resistance) would be good candidates for use in Korean breeding programs by determining their effectiveness against field populations collected over a 6 year period, and by assessing their ability to reduce inoculum loads.

Quantitative resistance is one of the most promising new strategies offering durable disease resistance against a broad-spectrum of pathogens (Song and Goodman, 2001). To identify QTL that might provide sources of resistance, I mapped blast disease resistance QTL in a recombinant inbred rice population developed in Korea, and used a candidate gene approach to associate defense response genes or resistance gene analogs with the QTL.

Previously, our group demonstrated that a subfamily of *OsGLP* genes on chromosome 8 and an *OsOXO* gene on chromosome 3, which co-localize with disease resistance QTL, contribute to rice blast and sheath blight resistance (Manosalva et al., 2009; Davidson et al., 2010; Davidson 2009). We have also demonstrated that *OsPAL* gene on chromosome 2 contributes to bacterial blight resistance (Manosalva 2006; Manosalva et al. in prep). Here I investigate the contributions of a chitinase gene that colocalizes with a QTL on chromosome 2 to resistance to sheath blight and rice blast.

Understanding the contributions of candidate DR genes to disease resistance QTL will increase the tools in our toolkits for resistance. For example, knowing what genes contribute to disease resistance QTL will allow the design of directly linked markers to enable marker-aided selection of these important traits into rice.

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CHAPTER 2 - Analysis of Relationships Between Monogenic Rice Lines And Rice Blast Pathogens In Korea

Summary

Understanding the pathogenic diversity of *Magnaporthe oryzae* populations in target areas is an essential prerequisite for rice breeding programs focused on disease resistance. We used a total of 3,747 isolates of *M. oryzae* collected between 2004 and 2009 from farmers' fields located throughout Korea to screen for disease interactions with eight monogenic rice lines, each harboring a single, major blast resistance gene. The monogenic line harboring *R* gene *Pi-sh* was the susceptible to the greatest number of *M. oryzae* isolates. The line containing the *Pi-9* gene was susceptible to the fewest isolates, ranging from 4.7-12.7% of the total population screened. *Pi-z* was a useful resistance gene until 2006; from 2007 onwards virulent blast isolates increased to more than 90% of the Korean population. Thus, *Pi-z* is no longer an appropriate candidate for breeding rice blast resistant cultivars in Korea. Virulent isolates to the *Pi-b* gene increased over time from 1% to 70% between 1993 and 2009. Isolates virulent to *Pi-i* and *Pi-b* were widely distributed in Gyeonggi and Gyeongbuk provinces, likely due to the cultivation of cultivar Ilpum that possesses *Pi-i* and *Pi-b*. Our data indicate that *Pi-9* may be the best candidate resistance gene for use in breeding programs in Korea

Introduction

Rice blast, caused by *Magnaporthe oryzae*, occurs in all rice growing regions and causes considerable decrease in rice production in temperate regions (Moldenhauer et al., 1992; Ou, 1980). It is one of the major rice diseases world-wide because it infects seedlings, leaves, panicles, and grains. Losses from blast disease can account for over 50% crop loss in Arkansas, USA (Lee, 1994). Sometimes yield losses reach up to 50% in upland conditions in India (Widawsky and O'Toole, 1990). In 2004, yield losses reached 70% in Lampung province, Indonesia (Sobrizal and Anggiani, 2007).

Host resistance is regarded as a cost effective and environmentally friendly means to control the disease (Fjellstrom et al., 2004). However, *M. oryzae* populations exhibit high pathogenic variability, which enables the pathogen to overcome host resistance within a short time (Goto et al., 1961; Iwata et al., 1970; Yamada and Lee, 1978; Chen et al., 1995; Han, 1995). Extensive pathogenic differentiation of rice blast fungus has been reported in many countries, which resulted in breakdown of resistant cultivars (Lee, 1972; Iwano and Yamada, 1973; Lee et al., 1975; Bonman et al., 1986; Ryu et al., 1987; Teng, 1994; Han et al., 1998; Han et al., 2001).

In Korea, the development of Tong-Il rice cultivars, made by crossing Indica type cultivars with Japonica cultivars, resulted in a significant increase in rice production. The Korean government encouraged farmers to cultivate these cultivars nationwide in the mid 1970's to increase rice production (RDA, 1990). Therefore, after 1977, most of the rice fields were planted with these cultivars effectively creating monocultures. The cultivars, Raegyeong and Nopung, were considered to have resistance to rice blast. However, newly adapted pathotypes and favorable environmental conditions in 1977 and 1978 resulted in a huge decrease in rice

production (Cho et al., 2010) and required the importation of expensive rice from many countries. After this outbreak of rice blast, the Korean government attempted to introduce various resistance genes against rice blast. However, efforts changed to focus on high quality traits with good eating quality, thus narrowing the pool of resistance genes in currently used cultivated varieties.

About 60 genes conferring resistance to several blast pathogens have been identified from cultivated rice germplasm (Khush and Jena, 2009) and from wild rice species *Oryza minuta* (Amante-Bordeos et al., 1992). Using a map-based cloning technique, *Pi-9,* which encodes NBS-LRR protein, was isolated as a broad-spectrum blast resistance gene located on chromosome 6 and linked to the *Pi-2* gene (Liu et al., 2002; Qu et al., 2006). *Pi-44* was identified from a cross between CO39 and Moroberekan cultivars (Chen et al., 1999). *Pi-5* is a broad spectrum resistance gene and *Pi-5*-mediated resistance to *M. oryzae* requires *Pi5-1* and *Pi5-2*, which encode N-terminal coiled-coil (CC) motif, a nucleotide-binding (NB) domain, and a leucine-rich repeat (LRR) motif (Jeon et al., 2003, Lee et al., 2009). *Pi-33* located on chromosome 8, was cloned by map-based cloning, and has durability to the Columbian rice blast population (Berruyer et al., 2003). *Pi-b* confers high resistance to most Japanese blast races and belongs to the nucleotide binding and leucine-rich repeat class (Wang et al., 1999). *Pi-ta2* was cloned and demonstrated to interact directly with *Avr–Pita* (Jia et al., 2000; Bryan et al., 2000). Race-specific *Pi* resistance genes are widely used in breeding programs for cultivar development. Information about the resistant spectrum of resistance genes against rice blast isolates is important information to guide the breeding of resistant varieties.

With the cloning and characterization of several *Pi* genes (Qu et al., 2006; Jeon et al., 2003; Jia et al., 2000; Bryan et al., 2000), the understanding of the mechanisms for blast resistance has been gradually improving. However, a comprehensive understanding of why some *R* genes are more durable than others in controlling disease is still elusive. Furthermore, few studies are available where host-pathogen interactions have been measured to assess virulence of *M. oryzae* isolates from large geographic areas. In particular, the virulence of regional populations has not been assessed on monogenic lines harboring known *R* genes. In previous studies, the virulence of *M. oryzae* to monogenic Japonic lines with *Pi-a* (Aichi asahi), *Pi-i* (Ishikao shiroke), *Pi-k* (kanto 51), *Pik-m* (Tsuyake), *Pik-s* (Shin 2), *Pi-ta* (Yashiromochi), *Pi-ta-2* (Pi No4), *Pi-z* (Fukunishiki), and *Pi-zt* (Toride 1) were assessed by Yamada et al. (1976), and Kiyosawa (1984). But, no studies have been undertaken using monogenic Indica lines. Thus, selection of good genes for breeding programs in areas relying on Indica varieties has been hit and miss, and is not guided by experimental data.

The International Rice Research Institute (IRRI) developed Co39, a near isogenic line (NIL), which contained five resistance genes, including *Pi-ta, Pi-4, Pi-z5, Pi-1* and *Pi-3*. Ling et al. (1995) introduced *Pi-b, Pi-k, Pi-km, Pi-kp, Pi-ta2* and *Pi-z* genes into Langxintuangheigu (LTH) cultivar to differentiate Chinese blast pathogen races. Based on these cultivars, IRRI-Japan scientists developed 26 monogenic lines harboring rice blast resistant genes with LTH genetic background (Fukuta et al., 2004). In this study, we analyze the virulence patterns of Korean rice blast isolates on temporal and regional scales using data of the interactions of a geographically comprehensive collection of isolates and eight monogenic lines. These data were compared to information on the virulence of Korean rice blast isolates to major Korean rice cultivars. Second, *R* gene *Pi-9*, which is known to exhibit broad spectrum resistance to rice blast, was tested to

assess its ability to restrict pathogen spread (inoculum buildup) in a simple sequential screening assay. This study identified useful sources of resistance for Korea based on an effective resistance to a diverse collection of *M. oryzae* isolates.

Materials and Methods

Growth conditions for rice monogenic lines

All 24 monogenic lines were provided from the International Rice Research Institute (Table 2.1). These lines each harbor single resistance gene in the background of LTH (Fukuta, 2004). Among the 24 lines, Han et al. (2004) screened the lines against 129 rice blast isolates from 22 representative races at RDA, Korea (Unpublished data). They grouped the 24 monogenic lines into eight groups based on similarities in the resistant or susceptible responses, and selected eight monogenic lines representing each group. In this experiment, the eight monogenic lines, *Pi-9, Pi-5, Pi-z, Pi-ta, Pi-i, Pi-k, Pi-b* and *Pi-sh* as well as the reference variety LTH were used for identification of the interaction. Five pre-germinated seedlings from each of these rice lines were sown in one plastic pot (15 x 8 x 15 cm) and fertilized with 0.5 g of $NH_4(SO_4)2$, 0.5 g of P_2O_5 and 0.2 g of K_2O once. The rice plants were grown for approximately 20 days (3-4 leaf stage) under greenhouse conditions before inoculation.

Collection of rice blast isolates and sporulation

A total of 3,747 rice isolates were collected from leaves or panicles showing typical blast lesions from farmers' fields and blast nurseries from 2004 to 2009 in Korea (Fig. 2.1). The isolates were collected from10 fields per site, and two to three infected leaves or panicles were collected from each field. Annual incidence of disease varied, thus the counties for collection and the number of samples were different from year to year. Isolates were collected from Gyeonggi (510 isolates), Gangwon (525 isolates), Chungbuk (450 isolates), Chungnam (399 isolates), Jeonbuk (402 isolates), Jeonnam (487 isolates), Gyoungbuk (549 isolates), and Gyoungnam (425

isolates). The number of isolates collected per year were 129 (2004), 950 (2005), 617 (2006), 769 (2007), 710 (2008) and 572 (2009).

Cultures of *M. oryzae* were grown on rice polish agar plates (rice polish powder 20g, sugar 20g, agar 20g per1000 mL of distilled water) at 25-28℃ for 7 days. The aerial mycelia was scraped with a sterilized rubber policeman scraper, and placed under continuous light conditions to induce spore formation. Two days after incubation, spores that formed on the medium surface were suspended in 20 ml of 0.02% Tween 20 solution, and filtered through cheese cloth. The concentration was adjusted to approximately $1x10^5$ spores/ml for use in spray inoculation.

Pathogenicity Assay

Rice monogenic seedlings were placed on a rotary platform and inoculated with 20 ml of spore suspension using an electric motor sprayer to ensure an even and uniform distribution of spores. The plants were kept for 24 h in a dew chamber at 26℃, then transferred to a greenhouse, and evaluated for disease response 5-7 days after inoculation. The reactions of the plants were categorized into six groups based on the international standard for disease index of rice blast (IRRI, 1996) as follows; 0- no evidence of infection; 1- presence of pin-point size brown specks; 2- slightly larger brown specks of about 0.5 mm or more in diameter but no necrotic spots; 3- small, round, or elliptical lesions about 1 to 2 mm or slightly more in diameter with grey centers and brown margins; 4- typical spindle-shaped blast lesion on the leaf, 3 mm or more in length, with large necrotic grey center and water soaked or reddish brown margin; 5- many large blast lesions as in 4 or larger and the upper portion of one or two leaves may be killed by collapsed lesions. Scores of 0 to 3 were regarded as resistant, while scores greater than 4 were regarded as susceptible. The average disease index of five plants per cultivar was used per isolate.

Sequential Planting Method

Seed of *Pi-5* and *Pi-9* monogenic lines and a control cultivar Ilpum were directly planted into soil in rectangular plastic containers (40×30×10 cm). Plants were grown until 4-5 leaves had formed. Plants were inoculated with a spore suspension ($1x10^5$ spores/ml) prepared from a mixture of 26 representative Korean *M. oryzae* isolates. Inoculation was by spraying the seedling plants in one plastic container. We used a sequential planting method first described by Roh et al. (2009) to assess the ability of the pathogen to spread naturally. Immediately after inoculation o, a second container with seedlings was set next to the first container, so that infection could occur naturally. Sequential infection was allowed by setting containers alongside at two week intervals up to the 7^{th} seedling container. The rice plants were covered with transparent plastic film during the night to keep humidity high for effective infection.

For resistance evaluation, diseased leaf area (DLA) was measured seven days after inoculation using the standard evaluation system for rice blast (IRRI, 1996). Less than 40% DLA is considered as the baseline for resistance, and, based on this, we scored established a scale ranging from 0 to 7, based on the minimum criterion. If all the seedlings in all 7 containers show less than 40 % DLA, the ability of the *R* gene to reduce inoculum load was defined as 0. If all the seedlings in the 7 containers show more than 40 % DLA, the ability of the *R* gene to reduce inoculum load was defined as 7.

Results

Reaction of monogenic lines to Korean isolates of M. oryzae

The reaction of the eight monogenic lines to a country-wide collection of *M. oryzae* isolates from Korea is summarized in Fig. 2.2. The reference line LTH is susceptible to 99.5% of the collected Korean isolates. The least susceptible monogenic line was *Pi-9*; lines with this gene were susceptible to only 7.1 % of the isolates. Of the *R* genes tested, *Pi-9* is the most effective, and confers resistance against the broadest spectrum of Korean isolates.

The interaction phenotypes of Korean field *M. oryzae* isolates and the eight blast *R* genes were monitored over 6 years to monitor for changes in virulence patterns over time (Fig. 2.2). Interactions with the monogenic line containing *Pi-z* were characterized by a steady increase in susceptibility to blast isolates over the 6 years, i.e., 46% of the blast isolates collected in 2004 were virulent to *Pi-z* compared to 2007 - 2008, where between 70-90% of the isolates were virulent to the line. Therefore, most Korean rice blast pathogens are now virulent to rice with the *Pi-z* gene. Although there were fluctuations in susceptibility over time, most monogenic lines showed an increase in susceptibility to the collection of blast isolates over time. Only lines with *Pi-9* did not show an increase in susceptibility over the 6 years. Lines with some *R* genes, such as *Pi-z, Pi-sh*, and *Pi-k,* were susceptible to more than 80% of the Korean blast isolates by the last year of the study (2009).

To understand regional distribution of *M. oryzae* isolates virulent to particular *R* genes, we monitored interactions with blast isolates collected from eight Korean provinces with eight monogenic lines (Table 2.2). The reference line LTH is susceptible to 98.5% of the isolates

collected from throughout Korea. Only the *Pi-9* monogenic line showed high levels of resistance across regions; the percent of the blast population susceptible to this line ranged from 6~7% , with the most virulent population collected from Gyoungbuk (10.6%) and the least virulent from Gyoungnam (4.3%). The *R* gene susceptible to the greatest portion of the *M. oryzae* population was *Pi-sh.* The distribution of virulence to the other *R* genes over the various provinces ranged from 41->90%, with no *R* genes other than *Pi-9* showing useful levels of resistance across the country.

Korean rice cultivar classification by the monogenic resistance genes

Korean rice cultivars were classified by *R* gene content and the area of cultivation in Korea (Table 2.3). Most of the Korean rice cultivars exhibited a narrow resistant gene spectrum for rice blast. Cultivars with the *Pi-b* gene are found in 57~60% of the cultivated area, followed by *Pi-i* group in 18~23% of the cultivated area. Traditional Korean Japonica-type rices contain *Pi*-a genes, and are used in 15~18% of the cultivated area. The most widely cultivated Korean rice cultivars are Dongjin1, Nampyeong, Junam, Ilmi, and Odae, covering 21.5, 13.1, 8.2, 7.6, and 4.9% of planted areas in 2006, respectively (Table 2.4). Dongjin1 and Junam have only the *Pi-b* gene for blast resistance. Ilmi and Odae also harbor only one blast resistant gene each, i.e., *Pita-2* and *PiK*, respectively. Nampyeong has three *R*-genes, *Pi-b, Pi-ta*, and *Pi-i*. The three most widely grown cultivars, Dongjin1, Nampyeong and Junam, contain the *Pi-b* gene.

Ability of the Pi-9 and Pi-5 gene to reduce inoculum loads

The effectiveness of the *Pi-9* and *Pi-5* genes in reducing inoculum loads was tested by a simple sequential infection method using a mixture of rice blast isolates collected from 1984 to 2009. During 1980's and 1990's, only 1~2 isolates were virulent to *Pi-9*. However, during the 2000's, virulent isolates comprised 7~13 % of the total *M. oryzae* population (Table 2.5). To assess if one reason that *Pi-9* is effective is because it reduces inoculum loads over time, we used a sequential planting method. The *Pi-9* monogenic line showed very small resistant lesions after the first artificial inoculation. After the second planting, the lesion area increased, but did not exceed 40% DLA. The DLA for the line with *Pi-5* did not increase in the sequential plantings. In contrast to *Pi-9* and *Pi-5* lines, the control rice cultivar Ilpumbyeo, which has a low-level of resistance, showed more than 60% DLA at the first inoculation, and >80% DLA by the fourth planting, ultimately withering to death.

Discussion

Several practices have been tested in farmer's fields to reduce rice blast disease and protect sources of resistance, including gene rotation, gene pyramiding, variety mixtures, and the use of multi-line cultivation. Although there has been some success using these methods, they are labor intensive and, so far, have resulted in only a temporary response. Recently, many researchers have aimed for durable resistance over wide geographic areas, and have shown the importance of screening cultivars over many different environments. Identifying which *R* genes will be durable prior to deployment has been a major barrier. An earlier reported protocol, the sequential planting method developed by Roh et al. (2009), offers a simple assay that may improve predictions of *R* gene durability prior to deployment. This method is based on screening rice seedlings harboring different *R* genes to assess the ability of an *R* gene to reduce inoculum loads over time. As reduction in inoculum load over time may contribute to *R* gene durability, this screening method is suggested to assess the potential of *R* gene durability in a short time (within 3 to 4 months). For this sequential planting assay, a diverse set of representative *M. oryzae* isolates made up of diverse pathotypes, genetic differences, and temporal and spatial factors is required.

In previous studies, the representative isolates collected from 1985 to 1998 from different regions were tested repeatedly against major monogenic rice cultivars (SS Han, unpublished). As a result, among 24 monogenic lines, eight were selected to screen for compatibility with a total of 3,747 rice blast isolates to be used for a rice blast-resistant breeding program.

Monogenic lines with the rice blast resistant gene *Pi-9* or *Pi-5* showed very high levels of resistance, therefore, these genes may be considered as target genetic resources for blast

resistance breeding programs. *Pi-9* was reported to be a broad-spectrum resistance gene because no susceptible lesions resulted after exposure to many blast isolates from the Philippines and 43 blast isolates collected from 13 other countries (Liu et al. 2002). However, in Korea, 7.1 % of the *Mo* isolates from 2004 to 2009 were compatible to the IRBL9-W (*Pi-9*) line, with up to 12.7% in 2008. Since most early maturing ecotype commercial varieties harbor the *Pi-9* resistance gene and are used in a relatively small cultivation area in Korea, virulent blast isolates may not have built up in these areas relative to other pathotypes. However, the cultivation area of varieties with *Pi-9* is increasing, since cultivation of early maturing ecotype commercial rice varieties with *Pi-9* have high economic value in Korea.

Although virulence to *Pi-5* is increasing (Fig. 2.3), the gene is still relatively effective in Korea. The *Pi-5* gene had been reported as having broad spectrum resistance against *Mo* races from the Philippines, Indonesia and Korea, and was suggested to be durable (Wang et al., 1994; Chen et al., 2000; Jeon et al., 2003). Durability of *Pi-5* was confirmed again by Roh et al. (2009). In our studies, we would predict that the monogenic line with *Pi-5* gene would confer resistance because less than 40 % DLA was reported up to the 7th sequential planting, suggesting that the *R* gene was confining the amplification of inoculum (Fig. 2.4). Thus, the *Pi-5* gene might be a useful source of durability for a blast breeding program in Korea.

The *Pi-k* and *Pi-b* genes showed moderate levels of resistance (Fig. 2.2, 2.3, Table 2.2), which was verified by screening for 6 years in Korean rice fields. In contrast to these experiments, in the 1960's, the cultivar Kwanok (Kanto51xPungok), which harbors *Pi-k* as the major resistance gene, was devastated by a severe outbreak of rice panicle blast within 3 years of field cultivation (Lee 1979). The *Pi-b* resistance gene is the most highly distributed gene in

commercial cultivars that are grown over the largest area in Korea. In 1985, a new race of *Mo* (KI-409) was was identified, and become the predominant race in Korea in1990's. KI-409 was highly virulent to commercial cultivars with pedigrees that harbor *Pi-b* (BL1 and BL2). For example, Ilpum (BL1 pedigree) harboring *Pi-b* was severely infected by KI-409 after 2 years of cultivation over a large area. Isolates compatible to *Pi-b* were found to be widely distributed in Gyeonggi and Gyeongbuk provinces, possibly caused by the extensive cultivation of Ilpum.

Interestingly, susceptible symptoms were observed on IRBL9-W (*Pi-9*), a line associated with broad-spectrum resistance (Fig. 2.5). In the Roh et al study (2009), the *Pi-9* monogenic line showed increased DLA by the third planting in the sequential planting method, starting from very small lesions characteristic of resistance at the first inoculation, to increased lesion area with emerging susceptible lesions at the second planting. The resistant lesion changed to a typical susceptible lesion with 17% DLA, and reached 57 % DLA in the fourth planting, higher than the minimum criteria of 40% defined by Roh et al. (2009). These high levels of DLA were present until the 7th planting, therefore, this data suggests that the use of *Pi-9* in a breeding program for rice blast resistance may result in a higher probability of pandemic spread of disease. However, our study using the same assay showed that over time the DLA on rice with the *Pi-9* gene did not exceed the 40%DLA threshold. This is consistent with our disease survey data, which show that *Pi-9* was resistant to most (90.3%) *M. oryzae* isolates from throughout Korea. Taken together, these data suggest that *Pi-9* would be an effective gene for Korean breeding programs.

Most of rice blast isolates were virulent to rice lines with *Pi-sh* and distributed throughout Korea. Major rice cultivars grown in Korea have not to date been analyzed for the presence of

Pi-sh. Therefore, there is no information available for the numbers of Korean rice cultivars that possess the *Pi-sh* gene. *Pi-sh* was originated from Shin2 or BL1, and those cultivars were introduced into most of Japonica-type cultivars (Ezuka et al., 1969; Hayashi and Fukuta, 2009). Most of cultivated rice grown in Korea are Japonica-types, therefore, the cultivated rice are assumed to integrate genes from Shin2. In case of BL1, which has *Pi-b* gene in it and has been used for Korea rice breeding programs for high quality rice as a major mother plant, therefore, *Pi-sh* gene is assumed to integrate into many cultivars as well as *Pi-b* gene. *Pi-b* and *Pi-sh* gene is located at chromosome 2 and 1, respectively, therefore, the two genes have not been interacted significantly and classified totally different groups responding to rice blast isolates collected from Korea (unpublished). Therefore, more than 90% of rice blast isolates in Korea are assumed to have built compatibility to *Pi-sh* gene over long period.

The *R* gene content of Korean cultivars is assessed by using specific DNA-marker analysis (Table 2.3; Cho et al., 2010). Although several cultivars have more than two resistant genes, most of the cultivars in Korean rice fields have only a few resistant genes. This is seen in the four groups that are most widely planted in Korea (*Pi-b* group, over 57~60% of the cultivated area; *Pi-ta* group, over 23~32%; *Pi-i* group, over 18~23%; and *Pi*-a group, over 15~18%). The cultivated area for rice cultivars with *Pi-z* gene, which is highly susceptible to Korean rice blast isolates, has decreased from 1.1% to 0.6%. The most widely used cultivars contain the *Pi-b and Pi-i* genes, thus, disease data from these areas can be used to determine the regional distribution of cultivars with different resistant genes. The *Pi-5, Piz-t, Pik-m*, and genes were detected in cultivars cultivated in the past, and may be utilized to diversify the gene pool in new breeding program. Of these, our data suggest that *Pi-5* may be a valuable gene to consider. Interestingly,

the cultivated area for rice cultivars with *Pi-9* gene, which is resistant to most rice blast isolates, has decreased from 3.3% to 0.5%.

All these data can be summarized as that *Pi-sh, Pii, Pb, Pii, Pi-k, Pi-ta*, and *Pi-z* genes had high compatibility to rice blast isolates from Korea, but *Pi-5* and *Pi-9* had durability tested by the sequential planting methods as well as low levels of compatibility to the rice blast isolates. *Pi-5* and *Pi-9* genes, therefore, can be used as a promising gene for breeding program with high levels of rice blast resistance in Korea.

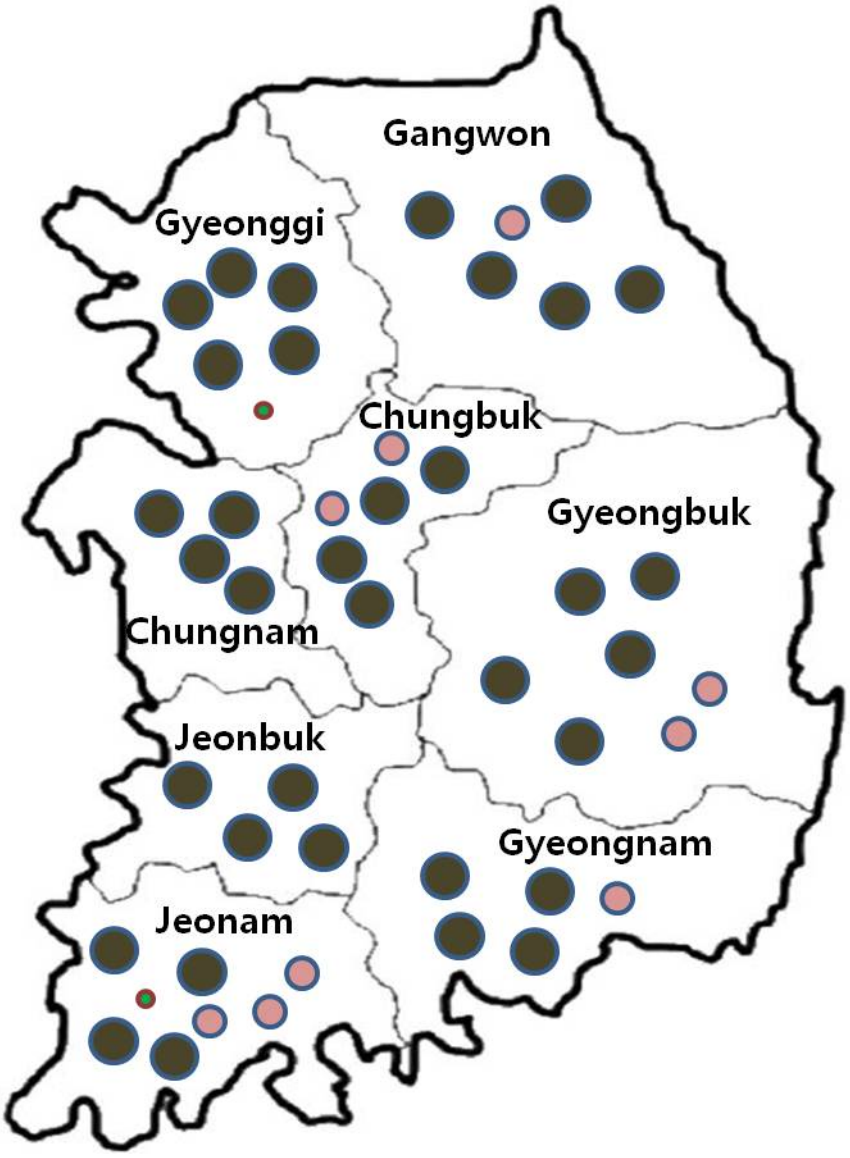

Figure 2.1 Collection sites of *Magnaporthe oryzae* isolates from farmers' fields from 2004 to 2009 in Korea. The different dots represent 10 isolates (), 25 isolates (), and 100 isolates ().

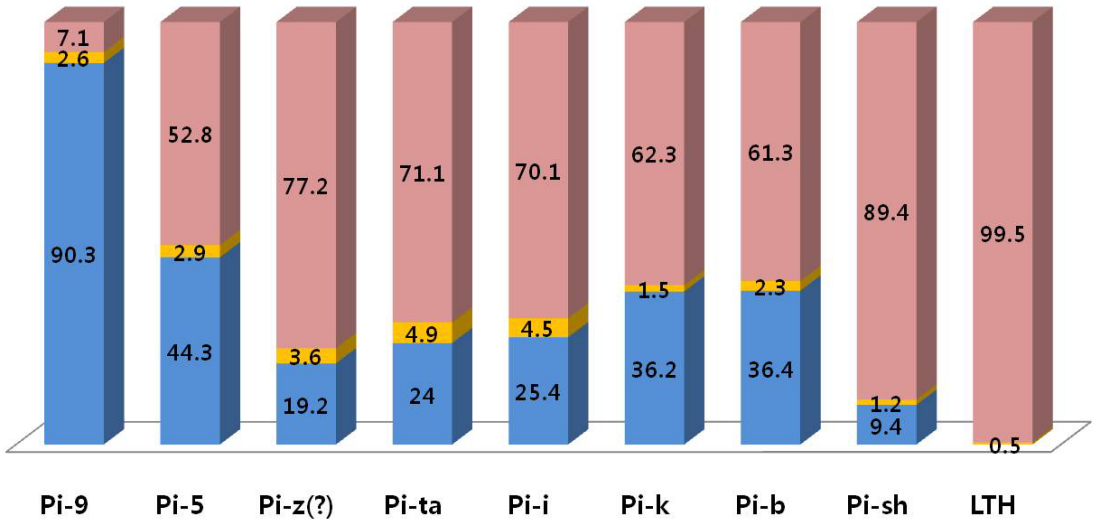

Figure 2.2 Responses of different blast resistance genes to Korean *M. oryzae* isolates collected from 2004 to 2009. Colors within bars represent the percent of total isolates that result in resistant (■), moderately resistant (■), or susceptible (■) interactions with the different *R* genes.

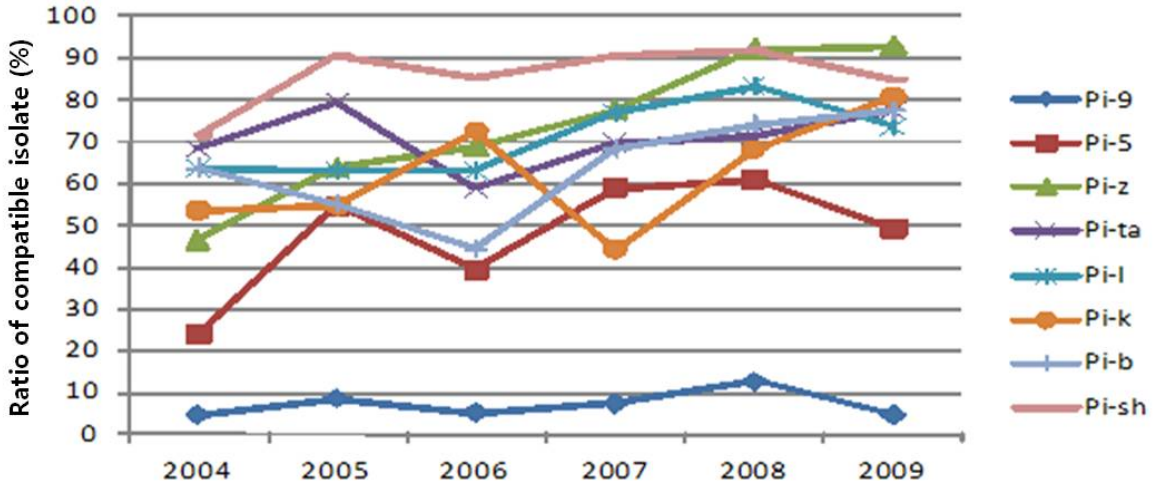

Figure 2.3 Virulence of Korean *M. oryzae* isolates collected from 2004-2009 to rice with different *R* genes.

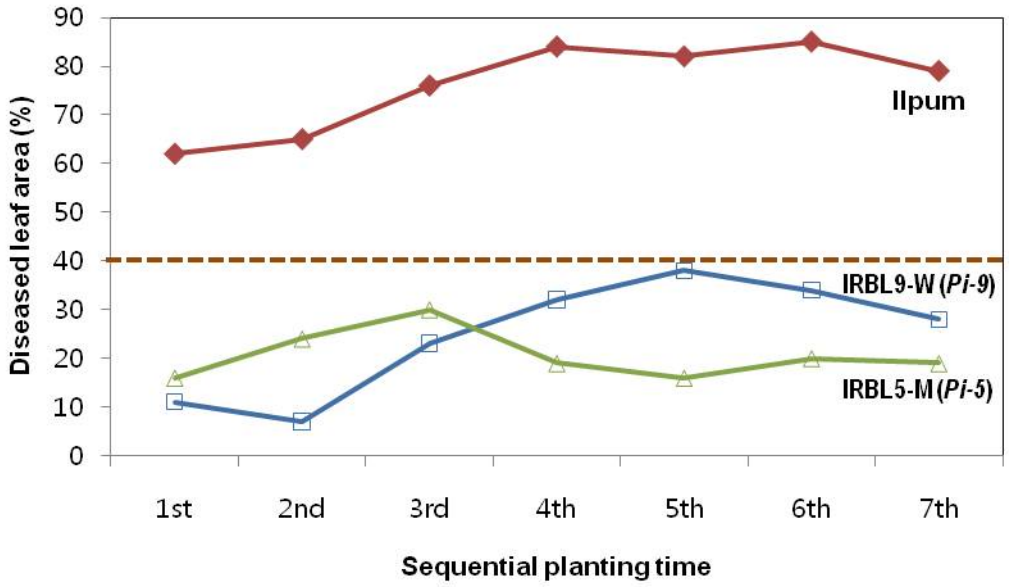

Figure 2.4 Disease severity on IRBL9-W (*Pi-9*) and IRBL5-M (*Pi-5*) lines and the susceptible control Ilpum, as tested using the sequential planting method. Relative to the susceptible control, % DLA on leaves of lines with *Pi-9* and *Pi-5* remained under 40%, suggesting the population of *M. oryzae* had not adapted to virulence under the selection scheme imposed by the experiment.

Table 2.1 Rice blast resistance genes possessing monogenic lines used in this study

Variety name	Resistance genes
IRBL9-W	*Pi-9*
IRBL5-M	*Pi-5*
IRBLz-Fu	*Pi-z*
IRBLta-K1	*Pi-ta*
IRBLi-F5	*Pi-i*
IRBLk-Ka	*Pi-k*
IRBLb-B	*Pi-b*
IRBLsh-S	*Pi-sh*

Table 2.2 Percent of total *M. oryzae* isolates from each Korean province that are virulent to blast resistance genes contained in monogenic rice lines.

Province	Pi-9	Pi-5	Pi-z	Pi-ta	Pi-i	Pi-k	Pi-b	Pi-sh	LTH
Gyeonggi	6.5	63.1	80.2	70.6	85.5	64.1	62.5	87.1	99.4
Gangwon	7	40.8	78.7	67	58.1	55.8	50.9	92.2	98.5
Chungbuk	6.7	46.2	73.8	63.6	62.9	54.4	64.2	88.2	100
Chungnam	6.7	46.2	73.8	63.6	62.9	54.4	64.2	88.2	100
Jeonbuk	7.6	54	76.3	65.7	74.7	73.3	61.4	83.9	99.6
Jeonnam	7	51.1	77.7	76	66.1	73.1	47	88.6	99.5
Gyoungbuk	10.6	74.7	79.2	78.9	86.3	48.1	84.2	95.1	99.8
Gyoungnam	4.3	42.5	76.6	81.6	61.3	74.4	56.9	91.7	99.1

Table 2.3 Classification of Korean commercial cultivars by rice blast resistance genes and the area of cultivation for each group by *R* gene.

R gene	Cultivars	Percent of cultivation area by year				
		2004	2005	2006	2007	2008
Pia	Donjin, Mangeum, Moonjang, Saesangju, Samgwang, Sinseonchal, Daesan, Dongan, Sangju, Dasan, Sambaeg, Gyehwa, Unbong, Chucheong	15.8	15.6	16.8	17.7	18.3
Pib	Mangeum, Palgong, Samgwang, Daepyeong, Daesan, Dongan, Dongjin1, Geuman, Gopum, Hwayeong, Ilpum, Junam, Sangju, Sangmi, Sindongjin, Dasan, Jinpum, Nampyeong, Taebong, Seojin, Sampyeong, Gru, Undoo, Junghwa	58.4	59.5	58.9	58.9	57.4
Pii	Mangeum, Gopum, Ilpum, Hopyeong, Jinpum, Manchu, Nampyeong, Sobi, Taebong, Sangjuchal	23.1	21.3	20.2	19.2	18.1
Pita, Pita-2	Dongjin, Moonjang, Saesangju, Daesan, Dongan, Geuman, Gopum, Sangmi, Dasan, Manchu, Nampyeong, Sambaeg, Seojin, Sampyeong, Dongjinchal, Sangjuchal, Gru, Gyehwa, Ilmi, Jungsan, Hwanam, Jinbu, Junghwa, Seogan	31.8	27.4	26.1	24.8	23.1
Piz	Moonjang, Saesangju, Daesan, Gopum, Sangju, Taebong, Sambaeg, Sangjuchal, Taeseong, Undoo, Goun, Jinbu, Unbong, Geumo	1.1	1.4	1.2	1.2	0.6
Pi9(t)	Saesangju, Sangju, Sangmi, Taebong, Sambaeg, Sangjuchal, Gru, Taeseong, Undoo, Goun, Jinbu, Unbong, Geumo, Junghwa	3.3	2.5	1.9	0.9	0.5
Pik	Moonjang, Gopum, Manchu, Taebong, Sangjuchal, Gru, Taeseong, Undoo, Goun, Jinbu, Unbong, Geumo, Junghwa	1.7	1.5	1.3	1.4	0.7
Pik-m	Seogan	0.1	0.1	0.04	0.05	0.006

Table 2.4 Distribution of rice blast resistance genes in Korean commercial rice cultivars and the percent of total cultivation area in Korea in 2006

Cultivar	C.A(%)*	R-genes	Cultivar	C.A(%)	R-genes
Chucheong	13.6	a	Manchu	0.0011	*ta-2, I, k*
Daepyeong	0.07	*b*	Mangeum	0.001	*a,b,i*
Daesan	0.0021	*a,b,ta,z*	Moonjang	0.13	*a, z, k, ta-2*
Dasan	0.0021	*a, b, ta-2*	Nampyeong	13.1	*b, ta, i*
Dongan	1.82	*a, b, ta-2*	Odae	4.94	*k*
Dongjin	0.03	*a,ta-2*	Palgong	-	*b*
Dongjin1	21.47	*b*	Saesangju	0.37	*a,ta-2,z,9*
Dongjinchal	1.01	*ta*	Sambaeg	0.005	*a,ta,z,9*
Geuman	0.04	*b, ta-2*	Samgwang	0.5	*a, b*
Geumo	0.0032	*z, 9*	Sampyeong	0.0037	*b, ta*
Gopum	0.04	*b, ta, z, I, k*	Sangju	0.06	*a, b, z , 9*
Goun	0.0023	*z, k, 9*	Sangjuchal	0.16	*ta, z, I, k, 9*
Gru	0.01	*b, ta-2, k, 9*	Sangmi	0.27	*b, ta, 9*
Gyehwa	0.01	*a, ta-2*	Seogan	0.05	*ta-2, k, km*
Hopyeong	0.54	*i*	Seojin	0.01	*b, ta-2*
Hwanam	0.0005	*ta*	Seomjin	-	*a,b,ta,z*
Hwayeong	2.46	*b*	Sindongjin	2.37	*b*
Ilmi	7.59	*ta-2*	Sinseonchal	0.22	*a*
Ilpum	5.47	*b, i*	Sobi	0.0021	*i*
Jinbu	0.006	*z, ta, k, 9*	Taebong	0.3	*b,I,z,k,9*
Junam	8.2	*b*	Taeseong	0.02	*z, k, 9*
Jungsan	0.0016	*ta2, k*	Unbong	0.01	*a, z, 9*
Junhwa	0.58	*b,ta-2, k, 9*	Undoo	0.02	*b, z, 9*

*C.A indicates cultivation area

Table 2.5 Number of *M. oryzae* isolates virulent to *Pi-9* per time frame collected

Reaction	Isolates per time frame (%)			
	1980's	1990's	2000-04	2005-09
Resistant	16 (94.1)	78 (97.5)	158 (87.3)	3,364 (93)
Susceptible	1 (5.9)	2 (2.5)	23 (12.7)	254 (7)
Total	17	80	181	3,618

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CHAPTER 3 - Association Of Candidate Genes With Quantitative Blast And Bacterial Blight Resistance Loci In Rice

Summary

A candidate gene approach was applied to a population of 164 recombinant inbred (RI) lines derived from a cross between a Japonica/Indica hybrid derivative (Milyang 23) and a Japonica variety (Gihobyeo) to determine association between defense response genes (DR) and resistance gene analogs (RGA) with quantitative trait loci (QTL) for rice blast and bacterial blight resistance. Of 166 genes tested by gel blot analysis with eight restriction enzyme digests, 96 were polymorphic and the other 70 were not polymorphic between the two parental cultivars. RGA (35) and defense response genes (2) from rice, barley and maize were mapped on the rice chromosomes and analyzed for their association with blast and bacterial blight resistance QTL. All markers produced single loci and were well distributed among all the chromosomes except on 12, where no markers were observed. Based on diseased leaf area, and lesion size using Korean and Philippine blast fungal isolates, a total of 21 putative QTL for blast resistance were identified on chromosomes 1, 4, 5, 6, 8, 9 and 12. Four putative QTL for bacterial blight resistance were identified on chromosome 3, 5 and 10. Thirteen RGA markers were associated with 11 different QTL on chromosome 1, 5, 8, and 9.

Introduction

Rice has been cultivated as a major crop for 7000 years, and it is a grain crop with the second-highest production in the world, sustaining about half the world's population (Watanabe, 1997). Rice has been developed as a model plant for monocotyledons because molecular manipulations such as gene deletion and gene overexpression or silencing are relatively easier than the other monocotyledon model (maize), and a large scale analysis of expressed sequence tags, a highly saturated molecular map, and genetic resources are available (Leung and An, 2004). Large scale mutant libraries (Jeon et al., 2000) and the whole genome sequence (Goff et al., 2002; Yu et al., 2002) are also available. Altogether, these tools are contributing to the accelerated functional analyses of genes through forward and reverse genetics.

Of the many plant pathogens including fungi, bacteria, and viruses that threaten rice production, rice blast and bacterial blight diseases are the most destructive worldwide. In recent years, epidemics by both diseases have occurred in several countries including Korea, China, and Japan. Rice blast disease is caused by the filamentous ascomycete fungus *Magnaporthe oryzae*, which was recently reclassified from *M. grisea* based on multilocus geneology (Couch and Kohn, 2002). Bacterial blight is caused by *Xanthomonas oryzae* pv. o*ryzae*. Comprehensive studies on pathogenesis and development of both pathogens and molecular studies on interactions between the pathogens and rice have been performed by many research groups.

Although chemical and biological methods have been adopted to control the rice blast and bacterial blight diseases, resistant cultivars developed through breeding programs remain the most effective means to control the diseases. Complete resistance carried by a major gene (monogenic or qualitative resistance) is very effective but it is often broken down within a few

years after release of the new cultivars by new races of the pathogen. Polygenic (quantitative) resistance significantly reduces pathogen populations compared to susceptible cultivars. Because multiple genes are involved, quantitative resistance is considered to be more durable than monogenic resistance (Parlevliet, 1995; Boyd, 2006). Although many disease-related quantitative trait loci (QTL) have been reported (Ballini et al., 2008; Wisser et al., 2005), the introduction of these traits into rice cultivars for use in the field is hindered largely because QTL are difficult to precisely locate in the genome and, therefore, to track in breeding populations.

To facilitate the use of QTL in plant breeding programs, the candidate gene approach, which directly tests the effects of variants of a potentially contributing gene in a certain phenotype (Pflieger et al., 2001; Byrne et al., 1996), was proposed as a means to predict genes involved in the disease resistance. This approach has three steps. First, candidate genes, which may include genes induced by a pathogen, or whose expression is correlated with QTL-based resistance, are identified. Second, the candidate genes are mapped to a molecular linkage map or located on a physical map to identify the locations of the genes on chromosomes and to determine if these regions co-localize with disease resistance QTL. Finally, the functions of those genes in disease resistance are confirmed by molecular study including deletion, silencing, overexpression, and complementation. Through this approach, several candidate genes have been shown to affect rice resistance to rice blast and bacterial blight pathogens (Ramalingam et al., 2003; Liu et al., 2004; Manosalva et al., 2009; Davidson et al., in prep).

Although extensive breeding programs to develop rice with resistance to pathogens have been performed since the early twentieth century, rice is still vulnerable to the rice blast and bacterial blight diseases. The targets of those programs were predominantly single gene sources of resistance. Although predicted to be more durable, breeders have been reluctant to adopt

strategies to incorporate quantitative resistance because the effects are smaller, and because absolutely linked markers are not available. In this study, we hypothesized that defense response genes may contribute to QTL-based resistance against rice blast and bacterial blight. As a first step in addressing this hypothesis, we identified chromosomal locations conferring QTL against rice blast and bacterial blight diseases by using a recombinant inbred (RI) population generated from parents that differed in resistance to the two diseases. We used a restriction fragment length polymorphism mapping approach to determine if the defense response and resistance genes co-localized with the QTL. These studies provide a foundation for understanding the molecular basis of QTL-based resistance and provide information to develop resistant rice varieties.

Materials and Methods

Plant materials and phenotypic evaluation

One hundred and sixty two RI lines of a cross between Gihobyeo, a Japonica variety, and Milyang 23, an Indica-Japonica derived variety, were used to analyze association of candidate genes with quantitative resistance to rice blast and bacterial blight diseases. To detect the QTL, four Philippine *M. oryzae* isolates, PO6-6, CA89, 39-1-3-8-1, and C9216-1, and a Korean *M. oryzae* isolate, 00-303 were used for inoculation studies. A single Korean *X. oryzae* pv. *oryzae* isolate, KXO730, was used to detect QTL for resistance to bacterial blight. Lesion size (LS, mm) and diseased leaf area (DLA, %) were used to score blast resistance while lesion length (LL, cm) was used for bacterial blight resistance (IRRI, 1996). The experiments were carried out in the greenhouse at the International Research Rice Institute for the Philippine blast isolates. For Korean isolates of blast and bacterial blight, greenhouse and field experiments were conducted at the National Institute of Agricultural Science and Technology in Korea.

For blast pathogen inoculation, isolates were grown on prune agar (Hayashi, 2009) or rice polish agar medium (Han, 1996) at 26 ºC for 2-3 days. The aerial mycelia were removed using sterilized rubber scraper and then incubated under fluorescent lights for 2-3 days. The spores on the surface of the medium were suspended in sterilized 0.02% Tween 20 solution and the spore concentration was adjusted to approximately 10^5 spores/ml. The spore suspension was sprayed on the leaf surface of 14 day old rice seedlings using artist's spray brush. The inoculated plants were placed in a saturated humidity chamber and then transferred to a greenhouse. Disease evaluation was carried out at 5-7 days after inoculation.

For bacterial blight inoculation, plants were transplanted at 30 days after seeding with a

spacing of 21×27cm in a paddy field. Fertilizer was applied at 220-70-80 (N-P_2O_5-K_2O) kg/ha. Bacteria for inoculum were cultured on PSA media (10 g/L peptone, 10 g/L sucrose, and 1.0 g/L Na-glutamate) for 2 to 3 days at 28ºC. The bacterial cells were suspended in sterile distilled water, and then adjusted to a density of 10^8 CFU/ml. Plant inoculation was conducted by clipping the tip of fully expanded leaves (Lee et al., 1999). The lesion length was measured at 21 days after inoculation (Lee et al., 1999).

Markers and RFLP analyses

A total of 166 defense response (DR) and resistance-related genes from rice, maize and barley were used in this study (Table 3.1, 3.2). Each clone was prepared by restriction enzyme digestion and purification or PCR amplification of relevant sequences to use as probe for hybridization.

To identify probe-enzyme combinations revealing polymorphism between Gihobyeo and Milyang23, DNA was extracted from the leaves of the two parents. Seeds were sown in plastic pots in a greenhouse and after 25 days, fresh leaves were ground in liquid nitrogen, and genomic DNA was extracted from the leaf powder using a modified hexadecetyltrimethylammonium bromide procedure (Saghai-Maroof et al. 1984). Genomic DNAs were digested with eight restriction enzymes, namely, *EcoR* I, *EcoR* V, *Hind* III, *Xba* I, *Dra* I, *BamH* I, *Sca* I and *Bgl* I. The digested DNAs were subjected to electrophoresis on 0.9% agarose gels and transferred to Hybond N+ membranes (Amersham Corp., Chicago) according to the manufacturer's instructions. Probe labeling and detection of hybridized fragments in DNA blot analysis was performed with an ECL kit (Amersham, Piscataway, NJ, U.S.A.).

Only markers and restriction enzyme combinations that clearly showed polymorphisms in

both parents and progeny lines were used for linkage mapping using an F_{11} RI population. Markers showed heterozygous genotypes at low frequency. The heterozygous genotypic data were treated as missing data in the construction of the linkage map.

Linkage Map Construction and QTL Detection

Markers were placed on a linkage map consisting of 168 RFLP-markers (Cho et al. 1998). To place the chromosomal location of the candidate genes, Mapmaker program version 3.0 (Lander et al., 1987) was used to perform the pseudo-linkage group separation and marker interval estimation with Kosambi function (Kosambi, 1944) based on anchor marker positions. Single marker QTL analysis (Falconer and Mackay, 1996) was carried out to determine the association between markers and disease resistance traits. PROC GLM was used to estimate the R^2 values for phenotypic variance of each marker for each trait. Based on the single marker QTL analysis result, simple interval mapping (Lander and Bostein, 1989; Haley and Knott, 1992) was conducted for QTL mapping using the PLABQTL (Utz and Melchinger, 1999) program.

Results

Inheritance of disease resistance in the Milyang23/Gihobyeo RI population

A total of 164 individuals of a recombinant inbred (RI) population and their parents (Milyang23 and Gihobyeo) were used for disease resistance analysis. Distribution of lesion size (LS) and diseased leaf area (DLA) percentage were assessed for each RI line using four Philippine and one Korean isolate of *M. oryzae*. In addition, lesion lengths occurring after inoculation with one isolate of *X. oryzae* pv. *oryzae* from Korea were assessed (Fig. 3.2). The parental cultivars, Milyang23 and Gihobyeo, showed clear compatible and incompatible reactions, respectively, to four Philippine blast isolates, suggesting resistance was controlled by single genes. For both LS and DLA in response to four Philippine isolates, the distribution of the RI population was skewed towards the resistance reaction (Gihobyeo). For example, inoculation with *M. oryzae* PO6-6 resulted in lesions of 6.0 mm on Milyang23 and 0.6 mm on Gihobyeo. Measurements of the progeny were skewed towards Gihobyeo with score of 0.7. The population mean of DLA after inoculation with *M. oryzae* PO6-6 ranged from 0.2 to 31.0%, while the parents were 4.2% (Milyang23) and 0.7% (Gihobyeo), respectively, showing genetic skewing (2.3) towards Gihobyeo (Table 3.3, Fig. 3.2). The Korean isolate (00-303) was incompatible with Milyang23, and the measured DLA of the RI population is skewed towards Milyang23. Among the eight traits, the DLA of 39-1-3-8-1 (4.50±6.38) showed the widest range of variation.

Both parental cultivars showed susceptible reactions, 11cm and 12.4 cm, respectively, to *X. oryzae* pv. *oryzae* isolate KXO730. The mean lesion length of the RI population was 8.55cm, and ranged from 1.1 to 25.8 cm (Table 3.3, Fig. 3.2). For the lesion length, the population showed a normal distribution, but was slightly skewed towards Milyang23 (skewness 0.6). The

frequency distribution of lesion length in the RI progenies varied, and showed transgressive segregation (Fig. 3.2).

Polymorphism of candidate gene markers and linkage map construction

A restriction fragment length polymorphism (RFLP) survey between Milyang23 and Gihobyeo was carried out using each of the 166 candidate genes as probes against the genomic DNAs after digestion with eight different restriction enzymes (Fig. 3.1). Among 166 candidate genes and based on the restriction patterns of eight different enzymes, 96 clones showed polymorphisms between the two parents, while polymorphisms were not detected by the other 70 clones. Less polymorphism was detected from defense response genes compared with resistance gene analogs (NBS clones). In the Milyang23/Gihobyeo population, the average estimated copy number of the NBS clones derived from rice was 2.41.

Of the 96 clones, any producing faint and/or complicated band patterns were not included in the progeny analysis (Fig. 3.1). A total of 37 candidate gene markers were added on an existing linkage map consisting of 168 RFLP-markers (Cho et al. 1998), giving total 205 markers placed throughout the twelve rice chromosomes with an average distance of 7.46 cM between markers. Each of the 37 markers is single locus and all were well distributed among eleven of the twelve rice chromosomes. None of the 37 was mapped on chromosome 12 (Fig. 3.3). Mixed R gene homolog clusters (RHCs, Leister 1998) were detected; rNBS29 and rNBS46 on chromosome 4, yac3, rNBS4, mNBS1, r13 and b4 on chromosome 5, rNBS28, rNBS52 and rNBS53 on chromosome 8, rNBS5 and r8 on chromosome 10, and rNBS8 and rNBS12 on chromosome 11 (Fig. 3.3).

Correlation among the traits

The phenotypic correlation between blast and bacterial blight resistance traits were calculated based on means of the RI population phenotype for each of the eight traits. Significant positive correlations were found between lesion size and diseased leaf area for the same *M. oryzae* isolates, PO6-6 and 39-1-3-8-1 (Table 3.5). Single locus ANOVA and simple interval mapping also detected similar results (Table 3.6, Fig. 3.3). There were significant correlations among responses to the Philippine *M. oryzae* isolates except with CA89 DLA, while no correlation was found between responses to the Korean and Philippine *M. oryzae* isolates. Interestingly, responses to the Korean *X. oryzae* pv. *oryzae* isolate, KXO730, were correlated with the Philippine *M. oryzae* isolates except CA89 DLA. The strongest correlation was detected between *M. oryzae* isolate 39-1-3-8-1 LS and DLA ($r=0.83$, $P\leq0.01$).

Segregation analysis of the markers

A total of 205 informative markers were located in a linkage map of the Milyang23/Gihobyeo RI population. All 205 markers were tested for segregation distortion using χ^2-test for goodness-of-fit to compare the observed and expected allelic frequency of a 1 : 1 ratio at $P \leq 0.01$. The actual allele frequencies were 51.4 and 48.6% for Milyang23 (maternal parent) and Gihobyeo (paternal parent), respectively. The majority of the markers segregated into a 1 : 1 ratio for the maternal and paternal parent. Of 205 mapped markers, 17 loci were skewed towards Milyang23 alleles and four towards the Gihobyeo alleles (Table 3.4). Among 37 candidate gene markers, only rNBS81 on chromosome 6 was skewed in favor of the Milyang23 allele. Totally, 10.24% of the markers showed a distorted segregation ratio and this ratio is at a relatively low level compared to other mapping populations. The allelic frequencies for Milyang23 ranged from

31.9 to 94.81%, and half of the chromosomes (3, 5, 6, 7, 9, and 12) showed distorted segregation (Table 3.4). Most of distorted markers were clustered and these were located in chromosome 3, 6, 7, 9, and 12 at P≤0.01 and P≤0.05 (Table 3.4, P≤0.05 data not shown). Distortion in chromosome 3, 6, 9, and 12 were skewed toward Milyang23, while the cluster region on chromosome 7 was skewed in favor of Gihobyeo.

QTL for blast and bacterial blight resistance

Single marker QTL analysis (Table A.1) provided the putative QTL location of markers for each trait. There was a high similarity in putative QTL location between that estimated by single marker QTL analysis and simple interval mapping. Based on simple interval mapping, we determined the putative locations of the QTL for resistance to blast and blight diseases on the chromosomes of the Milyang23/Gihobyeo RI population (Fig. 3.3, Table 3.6). Using the five *M. oryzae* and one *X. oryzae* pv. *oryzae* isolates, a total of 25 putative QTL were detected with log10 likelihood ratio (LOD) score threshold of 2.0 or above. Of the 25 identified QTL, 20 putative resistance alleles were contributed by Gihobyeo and five alleles were contributed by Milyang23. Progeny lines showed significant difference (P≤0.01) of trait average between Milyang23- and Gihobyeo-carrying genotypes (Table 3.6).

Seven of the 25 total QTL were identified on chromosome 1, 5, and 9 for rice blast resistance by lesion size and diseased leaf area in response to inoculation of PO6-6 isolate (Fig. 3.3, Table 3.6). Three PO6-6 LS QTL (qRBR1-1, qRBR9-1 and qRBR9-3) were detected with LOD scores of 10.07, 3.40, and 2.84, respectively. These QTL explained 24.9%, 9.9%, and 7.7%, respectively, of the observed phenotypic variance. Four QTL for PO6-6 DLA (qRBR1-2, qRBR1-9, qRBR5, and qRBR9-2) with 3.05, 2.19, 3.74, and 2.43 LOD scores, respectively,

combined explained 31.9% of the total phenotypic variance. The qRBR1-1 coincided with qRBR1-2 at the peak location of RG519 marker. These alleles from Gihobyeo probably increased the resistance against the PO6-6 blast isolate.

Nine QTL were detected by three different traits, 39-1-3-8-1 LS, 39-1-3-8-1 DLA, and C9216-11 DLA. These QTL were placed on chromosomes 1 and 12 at the same positions where markers RZ513, rNBS84 (on chromosome 1), and GS117 (chromosome 12) are located. The QTL qRBR1-4, qRBR1-8 and qRBR12-1, based on 39-1-3-8-1 LS, accounted for 6.8%, 10.4%, and 18.7% of the phenotypic variation with 2.49, 3.78, and 7.26 LOD scores, respectively. Three QTL for 39-1-3-8-1 DLA (qRBR1-6, qRBR1-7, and qRBR12-2) explained 29.8% of total phenotypic variation with LOD values between 2.55 to 5.24. Together, qRBR1-3, qRBR1-10 and qRBR 12-3, explained 34.4% variation for resistance to *M. oryzae* C9216-11 diseased leaf area. Interestingly, these nine QTL, each of three QTL by each of three traits, mapped to the same location on the chromosomes, and also provided similar patterns of LOD, R^2, and additive effect values and were significantly correlated. Resistance for all these nine QTL was most likely contributed by Gihobyeo alleles. For C9216-11 QTL (qRBR1-3, qRBR1-10 and qRBR12-3), progeny lines with Gihobyeo- or Milyang23-derived alleles, showed significant differences of diseased leaf area (1.3% vs. 35.4%).

Based on CA89 DLA, three QTL, qRBR1-5, qRBR6 and qRBR12-4, were detected on chromosome 5, 6 and 12 with LOD scores of 3.35, 4.18 and 3.51, respectively. These three QTL were estimated to explain 30.4% of the total variation in diseased leaf area. For resistance against CA89 isolate, qRBR1-5 allele was contributed by Gihobyeo, while qRBR6 and qRBR12-4 were by Milyang23. Using a Korean *M. oryzae* isolate (00-303), two QTL were identified, one on chromosome 4 and another on chromosome 8. These QTL account for 6.1% and 6.8%

phenotypic variation with 2.17 and 2.29 LOD scores, respectively. qRBR8 mapped on the rNBS52 marker position, showed 5.22 additive effect in diseased leaf area. Several blast resistance QTL co-localized on the same chromosomal region. Ten rice blast resistance loci were located on chromosome 1 between marker RG303 and rNBS84. Four rice blast resistance loci with relatively high LOD scores, qRBR12-1, qRBR12-2, qRBR12-3, and qRBR12-4, coincided with the positions of markers RG869 and GS117 on chromosome 12.

Four bacterial blight QTL were detected on chromosome 3, 5 and 10 using a Korean *X. oryzae* pv. *oryzae* isolate (KXO730). QTL qBB5-1 flanked by rNBS74 is significant with a 4.39 LOD score, and this one QTLaccounts for 11.9% phenotypic variation in bacterial blight resistance. Though isolate KXO730 was virulent to both parents (on average, Milyang23 and Gihobyeo exhibit lesion lengths of 11cm and 12.4 cm, respectively), resistance loci were detected by both parental alleles through transgressive segregation (Fig. 3.2, Table 3.3, 3.6). For example, progeny lines harboring the Gihobyeo genotype for qBB5-2 QTL showed average lesion lengths of 6.8 cm after inoculation with KXO730, while the lines with the Milyang23 genotype had 10.8 cm lesion lengths.

Association between candidate genes and disease resistance QTL

Of the twenty-five blast and bacterial blight resistance QTL detected in this study, 11 QTL were associated with 13 markers of 37 R-gene homologs in chromosome 1, 5, 8 and 9 (Fig. 3.3). Some NBS-LRR homologs are associated with more than one disease resistance QTL. rNBS84 on chromosome 1 is associated with four rice blast resistance QTL for lesion size and diseased leaf area. Two QTL for *M. oryzae* isolate PO6-6 LS and DLA are associated with

rNBS7 and rNBS13. Interestingly, a mixed resistance gene homolog cluster region (yac3, rNBS4, mNBS1, r13, b4) on chromosome 5 is associated with both blast and bacterial blight resistance.

Discussion

Incorporation of disease resistance genes into elite lines has been a focus of many researchers and breeders because it is the most efficient way to protect plants from pathogens. A major gene is often responsible for complete resistance against specific pathogens but virulent variants of the pathogen often occur within a few years of deployment of resistance sources. Quantitative resistance which is contributed by minor genes is predicted to be more durable, but it is not easy to introduce the genes into a plant because the genes are often distributed across chromosomes and are involved in complicated interactions with other genes. To overcome these constraints, we identified QTL regions responsible for resistance against rice blast and bacterial blight, and we showed the association of the QTL with other known genes that contribute to resistance (defense response genes).

In total, 21 markers (10.24%) placed on the Milyang23/Gihobyeo RI population linkage map showed distorted segregation from the expected 1:1 segregation ratio at $P \leq 0.01$. Since the genetic backgrounds of Milyang23 (Indica×Japonica hybrid) and Gihobyeo (Japonica) are so different, a certain proportion of segregation distortion was expected. Distorted segregation is a common phenomenon in genetic mapping populations and has been reported in diverse plants (Lu et al., 2002; Konishi et al., 1992; Kazan et al., 1993; Pillen et al., 1993). In rice, segregation distortion ratios ranging from 5% to 37% have been reported in double haploid and recombinant inbred populations (Xiao et al., 1996; Matsushita et al., 2003; Yamagishi et al., 1998; Yamagishi et al., 1996; Xu et al., 1997). Sirithunya et al. (2002) reported extremely distorted segregation ratios (77%) in one rice recombinant inbred population. Significant segregation distortion may result to false linkage; we used published RFLP markers in linkage map construction as anchored

marker which is relatively stable. The locations of distorted segregation markers were consistent with other reported position that from indica/japonica F2 populations, a double haploid population, and a recombinant inbred population (Xu et al., 1997; Xiao et al., 1996).

Recombination suppression and chromosome rearrangement such as large heterozygous inversions could be the possible reasons for segregation distortion. The skewness of distorted marker clusters in Milyang23/Gihobyeo RI population showed the same result as was observed for a LH422 (Japonica)×9024 (Indica) RI population (Xiao et al., 1996), i.e., that the region in chromosome 7 skewed toward to that of the *japonica* type rice while the other regions (chromosome 3, 6, 9, 12) were more like that of *indica*. Because the short arm of the chromosome 3 region affected by two gametophyte genes, ga-2 and ga-3, and the chromosome 6 region was related with several gametophyte and sterility genes, segregation distortion of Milyang23/Gihobyeo RI population could be caused by gametophyte and sterility related genes as well as other genetic and environmental factors.

Of 166 candidate genes tested with eight restriction enzyme digests, 96 were polymorphic whereas the other 70 were not polymorphic between the two parent cultivars. Since we used RFLP analysis to identify polymorphisms between the two parental cultivars, small sequence differences, including single nucleotide polymorphisms (SNPs) and short indels (deletions or insertions), may not be detected. Cleaved amplified polymorphic sequences (CAPS) and identification of SNPs through sequencing PCR amplicons could identify additional polymorphic markers. Recent advances that are making whole genome sequencing relatively easy and inexpensive could ultimately enable genome-wide comparisons between the two parental cultivars, and would solve the limited number of markers that we encountered in this study.

Interestingly, less polymorphism was found in defense response genes compared with the NBR resistance genes. Many of these types of resistance genes are responsible for qualitative resistance against specific groups of pathogens, and they have been extensively introduced into rice by conventional and molecular breeding. The major genes have coevolved with avirulence genes of pathogens; these genes may have evolved differently depending on geographical regions and pathogen populations. One of parental cultvar, Gihobyeo, is a Japonica variety while the other, Milyang 23, was generated from crossing between Japonica and Indica varieties. Both Milyang23 and Gihobyeo showed clear compatible and incompatible reactions, respectively, to four Philippine *M. oryzae* isolates while the Korean isolate was incompatible with Milyang23. Such compatible and incompatible reactions are generally mediated by major genes and our results are consistent with the interpretation that polymorphisms in major genes being responsible for the pattern of reactions shown in the parental cultivars.

In the Milyang23×Gihobyeo population, a total twenty-five blast and bacterial blight resistance QTL were identified on chromosomes 1, 3, 4, 5, 6, 8, 9, 10, and 12. Most of detected resistance QTL were co-localized to the same chromosomal regions in other genetic populations with resistance against blast, bacterial blight, sheath blight and brown plant-hopper (Ramalingam et al., 2003; Talukder et al., 2004; Pinson et al., 2005; Noenplab et al., 2006; Ballini et al., 2008). In particular, a region on the long arm region on chromosome 1 between markers RG303 and rNBS84 that colocalizes with 10 blast QTL in the Milyang23×Gihobyeo population was identified in many othe populations; these QTL were identified to be effective against many different *M. oryzae* isolates in a blast nursery for leaf and neck blast resistance (Noenplab et al., 2006; Prashanth et al., 1998; Wang et al., 1994; Ramalingam et al., 2003; Lopez-Gerena, J., 2006; Talukder et al., 2004; Talukder et al., 2005). In addition, four blast LS and DLA QTL

mapped together on the centromere-containing mid region of chromosome 12. This region was also identified to harbor QTL to many different *M. oryzae* isolates in blast nurseries (Talukder et al., 2004; Talukder et al., 2005; Noenplab et al., 2006). These QTL on the chromosome 1 and 12 region have relatively high LOD scores and the parental lines exhibited distinct differential reactions to *M. oryzae* isolates. Significanlty, the RI population segregated to resistance and susceptible at nearly a 1:1 ratio. Several major resistance genes, including *Pi-6(t), Pi-ta, Pi-ta2, Pi-20(t), Pi-19(t), Pi-31(t), Pi-12(t), Pi-21(t), Pi-32(t), Pi-tq6, Xa25(t), Bph9, Bph2, Bph1,* are located in the QTL region on chromosome 12 (Sallaud et al., 2003; Ramalingam et al., 2003; Hayashi et al., 1998; Imbe et al., 1997; Chen et al., 2002), suggesting that many QTL identified in this region might contain both major gene(s) and major QTL. Importantly, these QTL with high LOD value and which exhibit a broad spectrum resistance will be valuable in marker aided selection breeding.

Four QTL on chromosome 3, 5, and 10 are responsible for bacterial blight resistance. The QTL on chromosome 5 that is linked with markers rNBS74 and AJ226 was commonly identified to Korean *X. oryzae* pv. *oryzae* isolates, and accounts for 11-15% of the reduction in lesion length. RILs with the Gihobyeo genotype at this locus showed more resistance than those with Milyang 23. Since both parental cultivars Milyang23 and Gihobyeo were susceptible to bacterial blight, the increased resistance of RILs might be from a transgressive segregation effect such as epistasis.

There was a high similarity in the chromosomal location of molecular markers, including genomic DNA (RG, G) and cDNA markers (RZ, C, BCD), between Milyang 23×Gihobyeo and other genetic populations (Tabien et al., 2002; Ramalingam et al., 2003; Wang et al., 1994). In particular, the Milyang 23×Gihobyeo and Azucena×IR64 populations (Ramalingam et al., 2003)

exhibit common genomic DNA and cDNA markers, as well as candidate genes (R gene homolog and DR genes) located in the same chromosomes and in the same order. This indicates that the candidate gene markers might have similar function, since they originated from conserved domains of resistance genes. Moreover, several blast resistance QTL were mapped to similar positions in the two genetic populations, suggesting that resistance and defense mechanism to diseases might be conserved in rice plant. This also suggests that those QTL commonly found in various genetic populations might function in diverse genotypes and therefore, would be useful for resistance breeding programs.

Thirteen markers of 37 resistant genes and defense response gene homologs were associated with 11 QTL for resistance. The association level was 35.13%, which indicates that the R-gene and DR-gene molecular markers were much more effective in identifying resistance QTL than non-specific molecular markers. The demonstration that accumulation of DR genes increases blast resistance in a different (SHZ-2 X LTH) RIL population supports the usefulness of candidate gene approach to identify resistance QTL (Liu et al., 2004).

In conclusion, we mapped 35 resistance gene homolog and two defense response gene markers in this study. Some of the QTL identified in the study appear to be controlled by single genes that confer broad spectrum resistance. Compatible or incompatible interactions between rice and the blast or bacterial blight pathogens depended on the rice cultivars and geographical origins of the pathogens tested in this study, suggesting the genes and loci responsible for resistance have coevolved. Chromosomal mapping of more candidate genes may provide more precise information of quantitative disease resistance in the Milyang23/Gihobyeo RI population, and the screening of more pathogen isolates in field studies are needed to determine the importance of each chromosomal region to resistance. The QTL and markers identified in this

study may provide sources of broad spectrum or durable resistance sources. Further studies, including functional analyses, will provide a more comprehensive understanding of broad spectrum resistance and its development during the evolution of rice and its key pathogens.

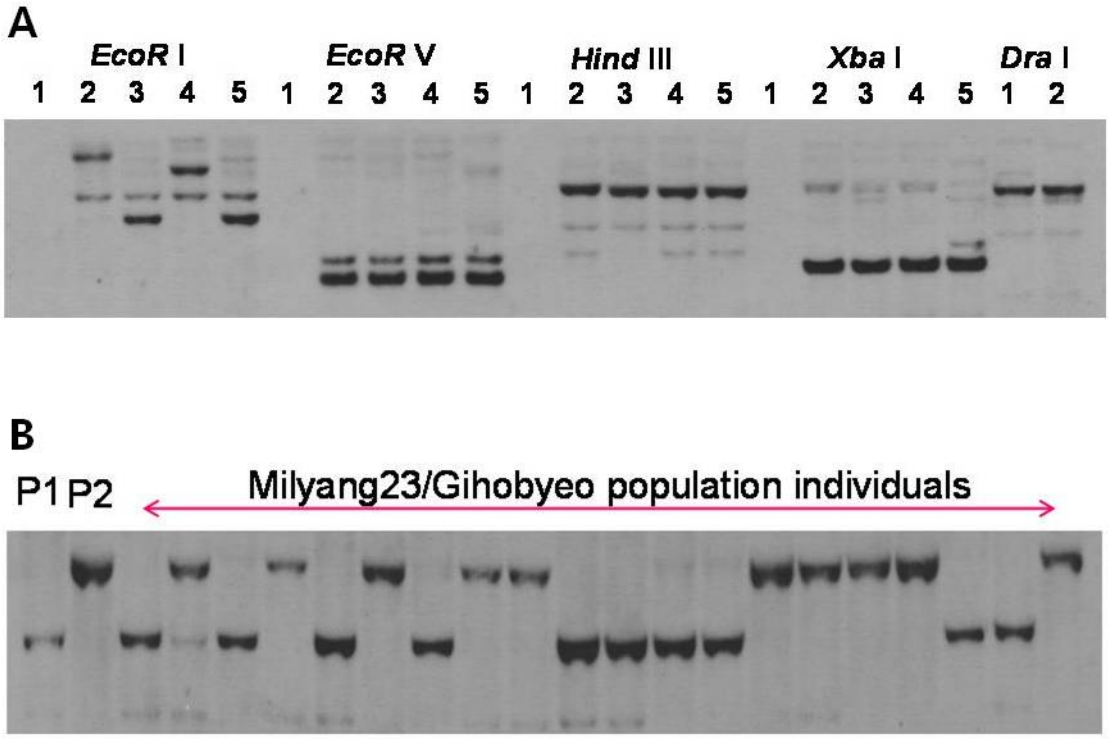


Figure 3.1 Sample RFLP analysis performed on maize and rice varieties including the parental lines, Milyang23 and Gihobyeo and their recombinant inbred progenies using rNBS52 (A) and rNBS36 (B) as probes. (A) genomic DNA from maize CM37 (lane 1), Azucena (lane 2), IR64 (lane 3), Gihobyeo (lane 4), and Milyang23 (lane 5) were digested with five different restriction enzymes. (B) RFLP pattern of Milyang23(P1)/Gihobyeo(P2) recombinant inbred population digested with *EcoR* I.

Figure 3.2 Frequency distribution of bacterial blight and rice blast resistance traits (LS-lesion size, DLA-diseased leaf area, LL-lesion length) in the rice Milyang23/Gihobyeo RI population. Arrows indicate the mean values of parental varieties.

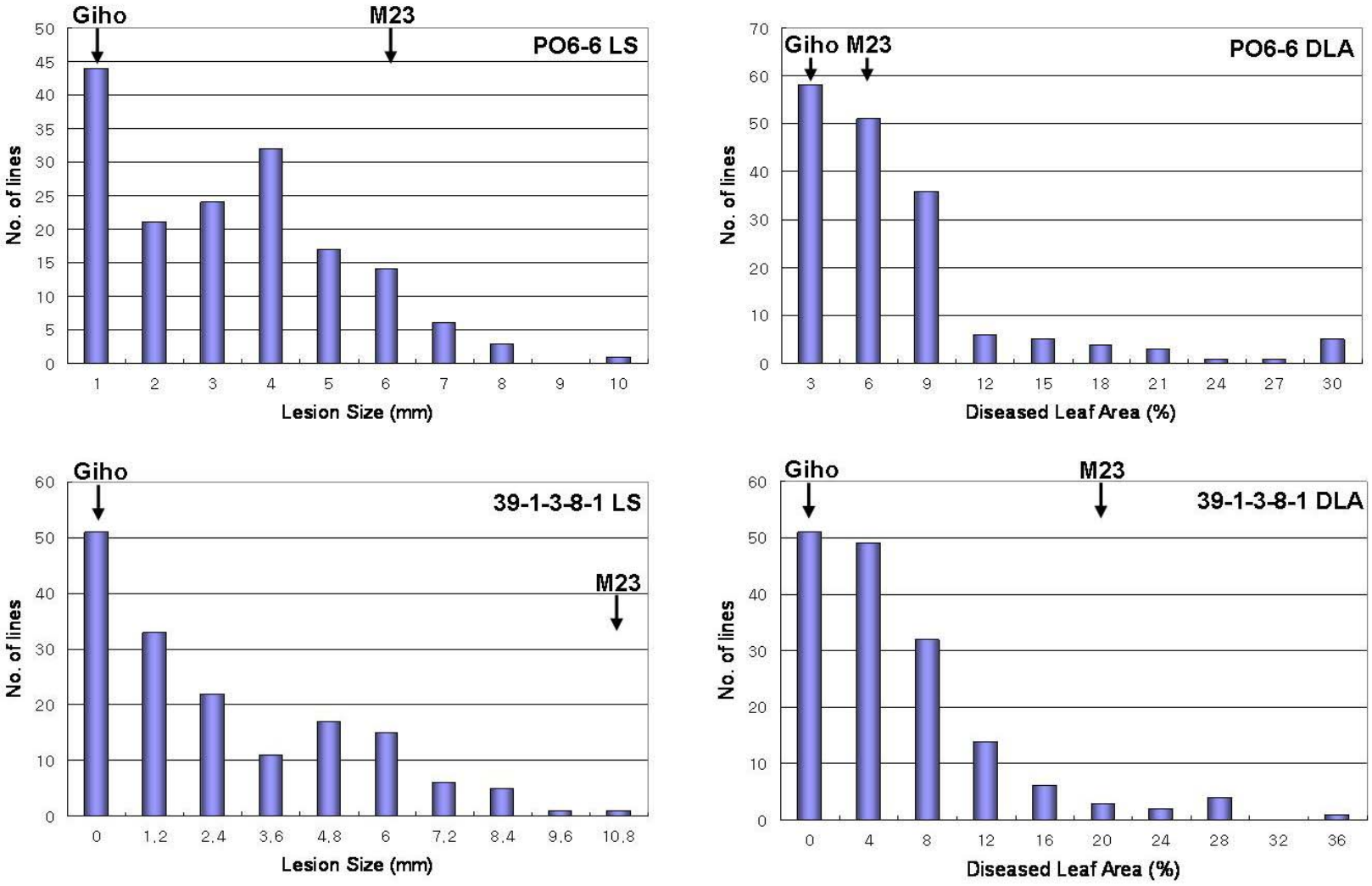
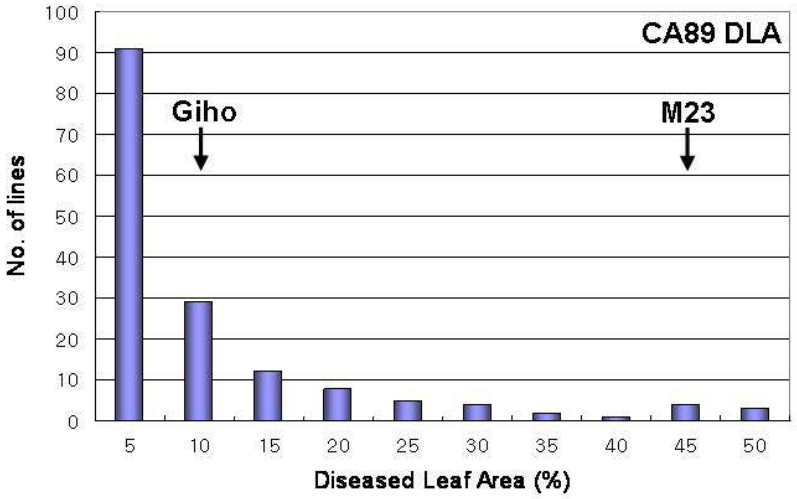
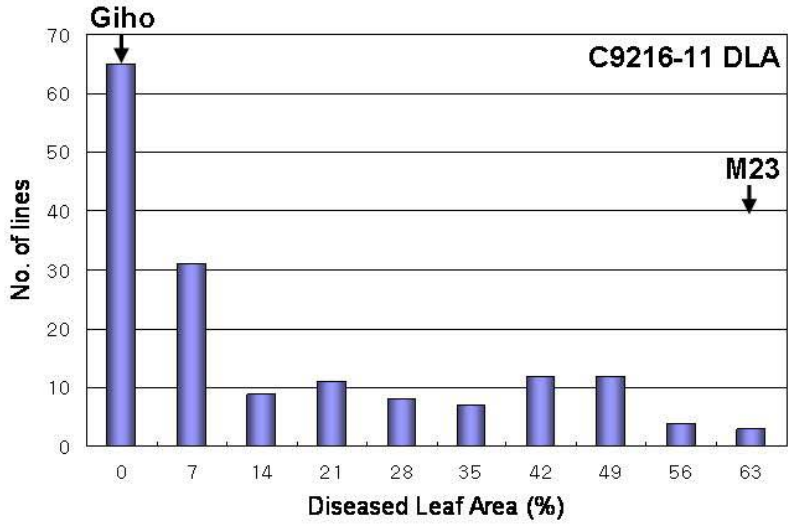
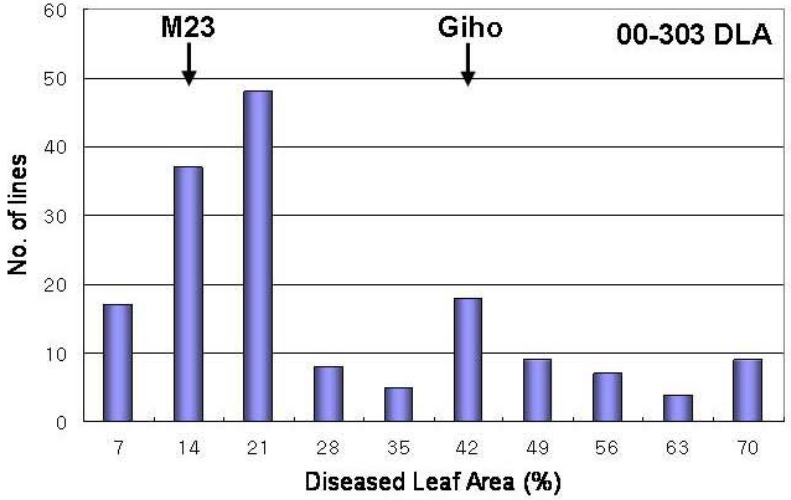


Figure 3.2 Continued







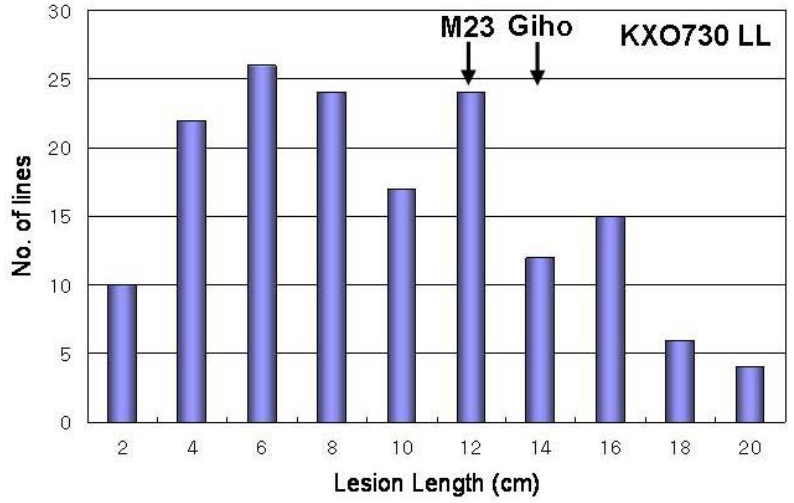


Figure 3.3 Linkage map of the RI population derived from a cross between rice cultivars Milyang23 and Gihobyeo. Two hundred five markers representing RFLP-markers (168), resistance gene analog and defense response genes (37, red color) were placed on an existing genetic linkage map of the RI population using Mapmaker V3.0. Map positions of blast and bacterial blight resistance QTL are shown in bar. QTL were claimed at LOD>2.0.

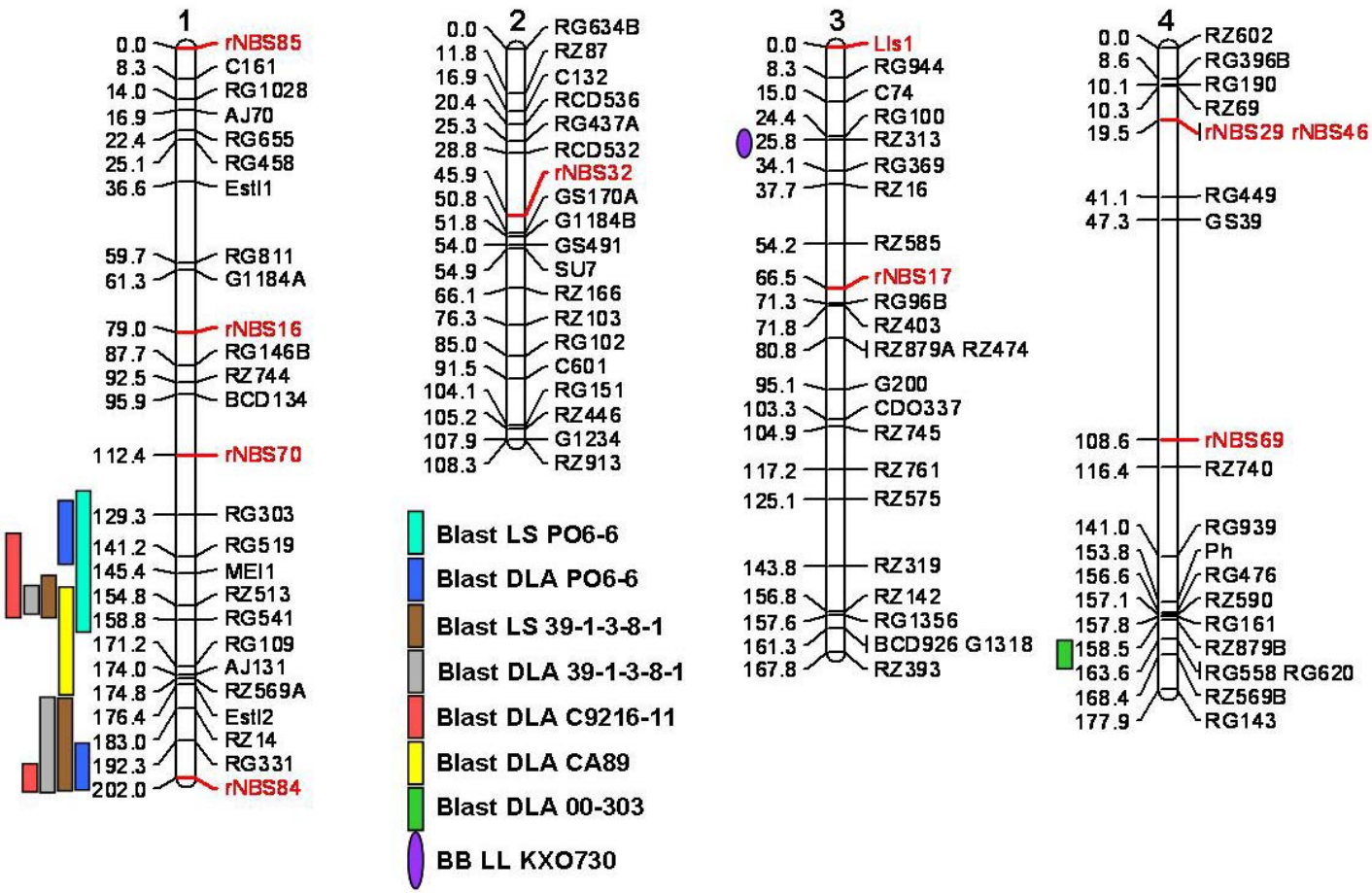


Figure 3.3 Continued

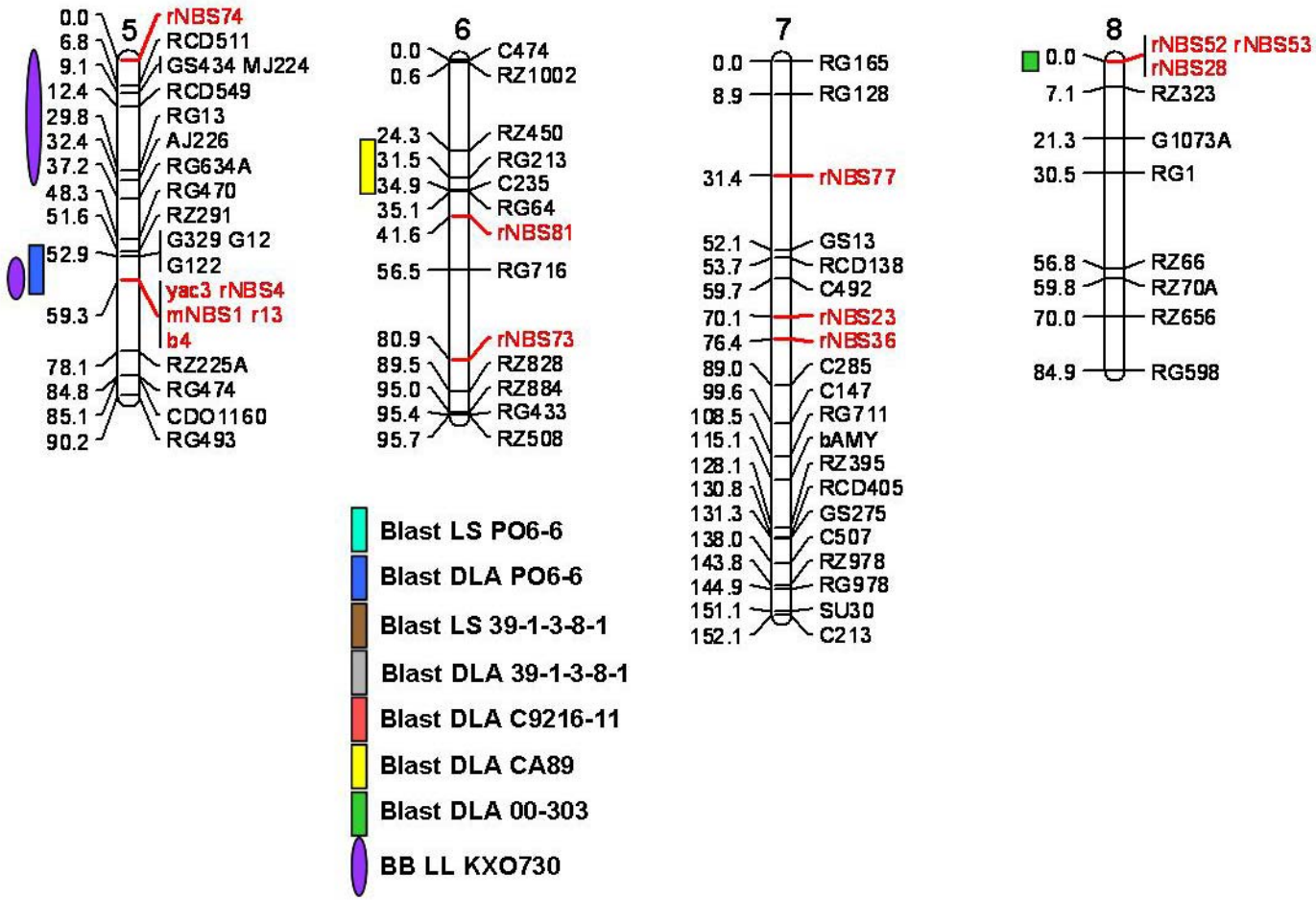


Fig. 3.3 Continued

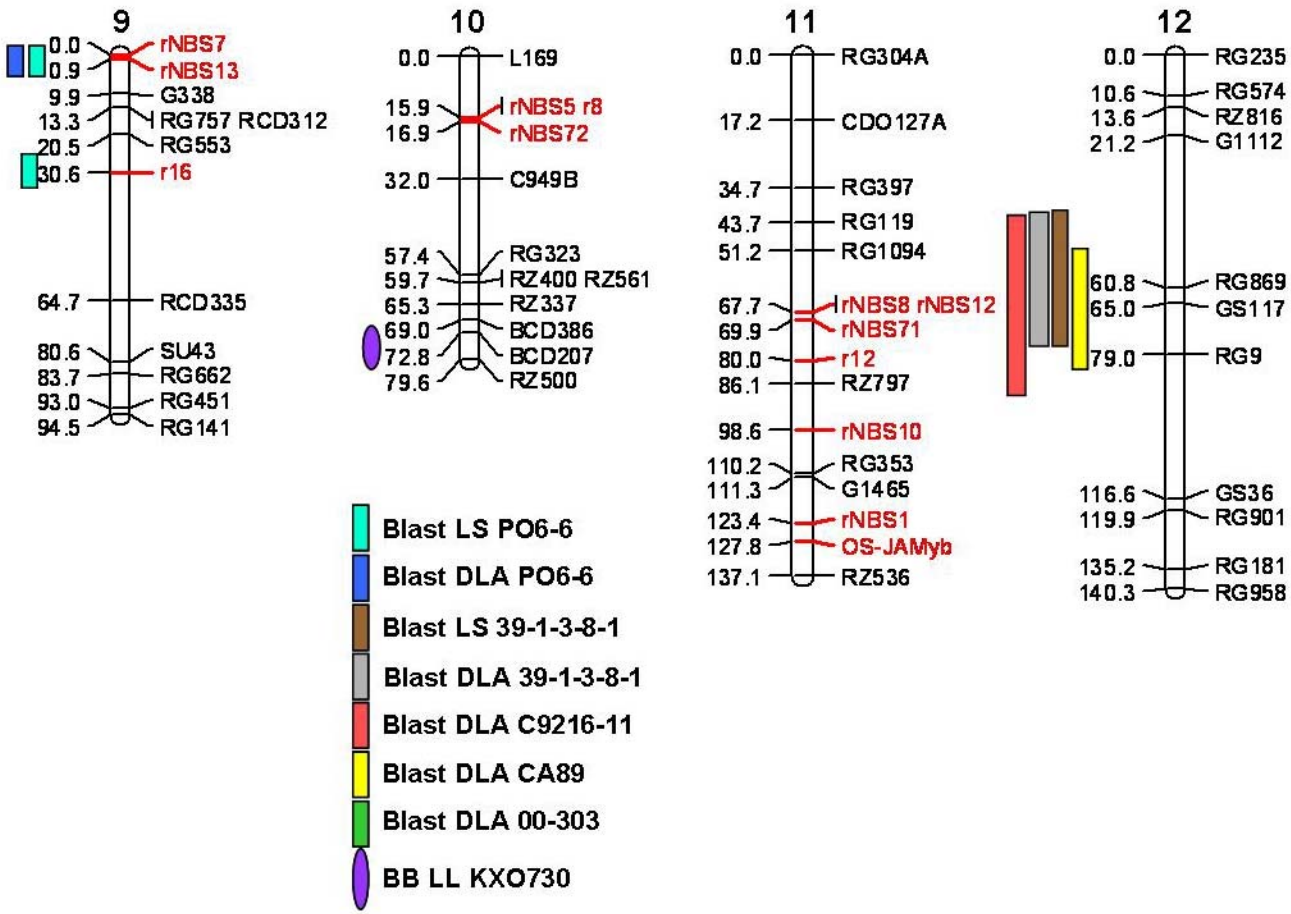


Table 3.1 List of candidate genes used for polymorphism survey between two rice cultivars, Milyang23 and Gihobyeo.

Predicted function or pathway	Number of clones
Resistance gene analogue	112
Genes for protein involved in amino acid	4
Genes that encode protein involved in hormone response	1
Genes that encode protein involved in detoxification	3
Genes that encode protein involved in lipid modification	2
Genes that code for DNA binding	3
Genes that encode PR protein	14
Genes for phenylpropanoid pathway	10
Miscellaneous genes	17

* Complete information of these clones can be found at the KSU defense gene collection (www.ksu.edu/ksudgc)

Table 3.2 List of candidate genes mapped on rice Milyang23/ Gihobyeo linkage map.

Clone designation	Gene categories	Identical or related sequences	Source
rNBS1	NBS-LRR	AB017914	Rice
rNBS4	NBS-LRR	IN2882	Rice
rNBS5	NBS-LRR	IN15065	Rice
rNBS7	NBS-LRR	OSM144390, IN19918	Rice
rNBS8	NBS-LRR	OSM151991	Rice
rNBS10	NBS-LRR	OSM1922	Rice
rNBS12	NBS-LRR	OSM148064	Rice
rNBS13	NBS-LRR	OSM120301, IN7733	Rice
rNBS16	NBS-LRR	OSM 148567, IN15839	Rice
rNBS17	NBS-LRR	OSM116395	Rice
rNBS23	NBS-LRR	OSM133645, AP003839b, IN1044	Rice
rNBS28	NBS-LRR	IN20419	Rice
rNBS29	NBS-LRR	OSM1916, AP3575b	Rice
rNBS32	NBS-LRR	OSM11430, IN1053	Rice
rNBS36	NBS-LRR	OSM13352	Rice
rNBS46	NBS-LRR	OSM15354	Rice
rNBS52	NBS-LRR	OSM15936, IN14071	Rice
rNBS53	NBS-LRR	OSM15929, AF220740, IN17130	Rice
rNBS69	NBS-LRR	OSM12066, IN130	Rice
rNBS70	NBS-LRR	OSM140512	Rice
rNBS71	NBS-LRR	AC083751, IN15208	Rice
rNBS72	NBS-LRR	AC074354, IN3585	Rice
rNBS73	NBS-LRR	OSM133123, IN41851	Rice
rNBS74	NBS-LRR	AL442107, IN48	Rice
rNBS77	NBS-LRR	OSM15610, AP003753, IN28943	Rice
rNBS81	NBS-LRR	OSM128851, IN10755	Rice
rNBS84	NBS-LRR	APO03269-2, IN1507	Rice
rNBS85	NBS-LRR	APO3219, IN112357	Rice
r8	NBS-LRR	AF032695	Rice
r12	NBS-LRR	AF032699	Rice
r13	NBS-LRR	AF032700	Rice
r16	NBS-LRR	AF032703	Rice
b4	NBS-LRR	AF032682	Barley
mNBS1	NBS-LRR		Maize
yac3	NBS		Maize
Lls1	Lethal leaf spot		Rice
OS-JAMyb	JAMyb	LOC_Os11g45740	Rice

Table 3.3 Descriptive statistics of measured rice blast and bacterial blight resistance traits in Milyang23/Gihobyeo recombinant inbred lines.

Trait (Isolate)[a]	Descriptive statistics of the RI population[b]							Means of parental lines	
	Mean	Std. dev	CV (%)	Skewness	Kurtosis	Min.	Max.	Milyang23	Gihobyeo
LS (PO6-6)	2.88	2.04	70.76	0.7	0.4	0.2	11.0	6.0	0.6
DLA (PO6-6)	5.98	6.15	102.88	2.3	5.7	0.2	31.0	4.2	0.7
LS (39-1-3-8-1)	2.14	2.58	120.48	1.4	2.4	0	14.4	10.9	0
DLA (39-1-3-8-1)	4.50	6.38	141.74	2.2	5.3	0	33.6	16.4	0
DLA (C9216-11)	13.67	17.84	130.50	1.0	-0.3	0	60.0	58.7	0
DLA (CA89)	8.12	11.20	137.91	2.2	4.7	0	51.2	44.8	7.2
DLA (00-303)	23.90	18.20	76.17	1.0	-0.1	0.2	70.0	13.5	42.0
LL (KXO730)	8.55	4.78	55.90	0.6	0.1	1.1	25.8	11.0	12.4

[a] LS: lesion size, DLA: diseased leaf area, LL: lesion length, Rice blast isolates: PO6-6, CA89, 39-1-3-8-1, C9216-11, and 00-303, Bacterial blight isolate: KXO730

[b] Std. dev: standard deviation, CV: coefficient of variance, Min: minimum, and Max: Maximum

Table 3.4 Segregation ratio of markers on individual rice chromosomes in the Milyang23/ Gihobyeo recombinant inbred (RI) population

Chromosome	No. of markers	No. of markers skewed to the maternal parent	No. of markers skewed to the paternal parent	No. of markers for distorted segregation*
1	26	0	0	0
2	19	0	0	0
3	24	4	0	4
4	20	0	0	0
5	22	0	2	2
6	13	8	0	8
7	20	0	2	2
8	10	0	0	0
9	12	1	0	1
10	12	0	0	0
11	16	0	0	0
12	11	4	0	4
Total	205	17	4	21

*χ^2-test to expected allelic frequency of 1:1 ($P \leq 0.01$).

Table 3.5 Phenotypic correlation coefficients of eight disease resistance traits in rice Milyang23/Gihobyeo recombinant inbred (RI) population.

	PO6-6 LS	PO6-6 DLA	C9216-11 DLA	39-1-3-8-1 LS	39-1-3-8-1 DLA	CA89 DLA	00-303 DLA	BB KXO730
PO6-6 LS[a]	-	0.69**	0.47**	0.48**	0.41**	0.28**	0.15	0.37**
PO6-6 DLA		-	0.42**	0.41**	0.50**	0.15	0.17*	0.30**
C9216-11 DLA			-	0.79**	0.74**	0.09	0.11	0.23**
39-1-3-8-1 LS				-	0.83**	0.10	-0.01	0.25**
39-1-3-8-1 DLA					-	0.10	-0.02	0.25**
CA89 DLA						-	0.04	-0.01
00-303 DLA							-	0.14
BB KXO730								-

[a] Lesion size (LS), Diseased leaf area (DLA) and Bacterial blight (BB)

*, ** are significance at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 3.6 Putative quantitative trait loci (QTL) for rice blast and bacterial blight resistance in rice Milyang23/Gihobyeo recombinant inbred (RI) population.

Isolate[a]	Trait	QTL[b]	Interval[c]	NML[d]	LOD	R^{2} [e]	Additive effect	Trait mean by genotype	
								Milyang23	Gihobyeo
PO6-6	LS	qRBR1-1	RG303 ~ RG541	RG519	10.07	24.9	-1.17	3.7	1.7****
		qRBR9-1	rNBS7 ~ rNBS13	rNBS7	3.40	9.9	-0.65	3.5	2.2****
		qRBR9-3	RG553 ~ r16	r16	2.84	7.7	-0.64	3.4	2.1****
	DLA	qRBR1-2	RG303 ~ RG519	RG519	3.05	8.3	-2.10	7.5	4.0***
		qRBR1-9	RG331 ~ rNBS84	rNBS84	2.19	6.2	-1.68	7.7	4.7**
		qRBR5	G329 ~ b4	rNBS4	3.74	10.2	-2.07	8.5	4.4****
		qRBR9-2	rNBS7 ~ rNBS13	rNBS7	2.43	7.2	-1.69	7.7	4.3***
39-1-3-8-1	LS	qRBR1-4	RZ513	RZ513	2.49	6.8	-0.70	3.0	1.5***
		qRBR1-8	RZ14 ~ rNBS84	rNBS84	3.78	10.4	-0.95	3.2	1.4****
		qRBR12-1	RG869 ~ GS117	GS117	7.26	18.7	-1.18	3.1	0.8****
	DLA	qRBR1-6	RZ513	RZ513	2.55	7.0	-1.76	6.5	2.9***
		qRBR1-7	RZ14 ~ rNBS84	rNBS84	3.22	9.0	-2.18	6.8	2.9****
		qRBR12-2	RG869 ~ GS117	GS117	5.24	13.8	-2.51	6.5	1.8****

Table 3.6 Continued

Isolate[a]	Trait	QTL[b]	Interval[c]	NML[d]	LOD	R^{2e}	Additive effect[f]	Trait mean by genotype[g]	
								Milyang23	Gihobyeo
C9216-11	DLA	qRBR1-3	RG519 ~ RZ513	RZ513	2.54	7.0	-5.39	18.8	9.8**
		qRBR1-10	rNBS84	rNBS84	2.73	7.6	-5.38	20.0	9.0****
		qRBR12-3	RG869 ~ RG9	GS117	7.71	19.8	-8.41	20.7	4.2****
CA89	DLA	qRBR1-5	RZ513 ~ Estl2	AJ131	3.35	9.3	-3.82	12.4	5.0***
		qRBR6	RZ450 ~ RG64	C235	4.18	11.4	8.22	7.4	22.6****
		qRBR12-4	RG869 ~ RG9	RG869	3.51	9.7	3.68	5.5	11.9***
00-303	DLA	qRBR4	RG558 ~ RZ569B	RZ569B	2.17	6.1	4.59	19.5	29.5***
		qRBR8	rNBS52	rNBS52	2.29	6.8	5.22	19.4	29.3***
KXO730	LL	qBB3	RG100 ~ RZ313	RZ313	2.11	5.9	1.22	7.6	10.2****
		qBB5-1	rNBS74 ~ AJ226	RCD549	4.39	11.9	-2.06	10.7	7.2****
		qBB5-2	G329 ~ yac3	rNBS4	4.36	11.9	-1.74	10.8	6.8****
		qBB10	BCD207 ~ RZ500	BCD207	2.34	7.1	-1.35	9.9	7.6***

[a] *M. oryzae* isolates: PO6-6, 39-1-3-8-1, C9216-11, CA89, 00-303, *X. oryzae* pv. *oryzae* isolate: KXO730
[b] QTL nomenclature is according to McCouch et al.(1997). The names imply trait abbreviation and chromosome number.
[c] QTL interval were claimed at LOD >2.0
[d] Nearest marker locus of the QTL
[e] Phenotypic variation explained by each QTL
[f] Effect of Milyang23 allele by the corresponding Gihobyeo allele
[g] Means of trait (LS mm, DLA %, LL cm) of all the progeny lines harboring Milyang23 and Gihobyeo alleles. **, *** and **** are levels of significant difference between Milyang23 and Gihobyeo lines at $P \leq 0.01$, 0.001, and 0.0001, respectively.

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Supplementary Materials

Marker intervals of the linkage map and the statistic of single marker QTL analysis on traits

Order		Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]															
					PO6-6 LS		PO6-6 DLA		3-9-1-3-8-1 LS		3-9-1-3-8-1 DLA		C9216-11 DLA		CA89 DLA		00-303 DLA		BB KXO730	
Chr1	1	0	0	rNBS85	1.461		0.351		2.692		0.861		0.203		1.861		0.008		0.374	
	2	8.3	8.29	C161	2.182		2.994		3.259		4.850	*	1.587		4.722	*	0.067		0.863	
	3	14.0	5.7	RG1028	3.626		2.030		2.862		2.057		0.185		4.712	*	1.845		0.112	
	4	16.9	2.9	AJ70	3.558		3.692		0.252		0.136		0.023		2.216		0.231		0.275	
	5	22.4	5.5	RG655	2.570		3.886		1.873		2.346		0.330		1.363		1.367		0.811	
	6	25.1	2.7	RG458	0.242		0.708		0.015		0.103		0.440		0.858		0.817		2.077	
	7	36.6	11.5	EstI1	3.228		0.476		0.040		0.002		0.747		0.317		0.038		0.318	
	8	59.7	23.1	RG811	0.262		1.417		0.075		0.206		0.030		1.144		1.878		2.301	
	9	61.3	1.6	G1184A	1.491		1.035		0.562		0.230		0.025		0.213		0.332		0.910	
	10	79.0	17.7	rNBS16	0.723		4.218	*	0.591		0.007		1.147		0.102		4.323	*	0.251	
	11	87.7	8.7	RG146B	0.002		2.260		0.353		0.366		0.641		1.021		2.722		0.529	
	12	92.5	4.8	RZ744	0.221		0.645		0.237		0.639		0.467		1.049		0.071		1.209	
	13	95.9	3.4	BCD134	0.005		1.823		0.021		0.087		0.335		1.418		1.145		0.358	
	14	112.4	16.5	rNBS70	5.027	*	0.372		6.382	*	0.503		4.599	*	0.430		0.499		0.447	
	15	129.3	16.9	RG303	23.957	****	7.625	**	2.589		1.473		4.476	*	2.230		0.199		0.756	
	16	141.2	11.9	RG519	46.039	****	12.516	***	6.727	*	2.672		8.590	**	3.453		1.060		5.276	*
	17	145.4	4.2	MEI1	35.943	****	5.321	*	3.612		1.476		7.687	**	7.712	**	0.182		5.178	*
	18	154.8	9.4	RZ513	28.624	****	13.515	***	11.201	**	11.610	***	8.365	**	15.759	***	0.243		7.448	**
	19	158.8	4	RG541	28.743	****	3.776		4.498	*	2.468		4.801	*	8.275	**	0.148		2.225	
	20	171.2	12.4	RG109	3.840		3.193		9.473	**	6.475	*	9.409	**	10.315	**	0.148		6.474	*
	21	174.0	2.8	AJ131	8.156	**	4.248	*	9.486	**	5.826	*	6.189	*	13.676	***	0.495		7.691	**
	22	174.8	0.8	RZ569A	2.116		3.429		6.728	*	3.757		7.120	**	11.649	***	0.598		4.519	*
	23	176.4	1.6	EstI2	2.339		3.982	*	7.473	**	5.357	*	6.704	*	13.438	***	0.152		3.200	
	24	183.0	6.6	RZ14	0.224		4.820	*	9.752	**	9.527	**	4.548	*	4.169	*	2.137		0.995	
	25	192.3	9.3	RG331	1.977		6.262	*	12.444	***	9.554	**	4.129	*	4.603	*	1.336		0.814	
	26	202.0	9.7	rNBS84	9.537	**	9.669	**	16.532	****	14.091	***	13.933	***	4.738	*	0.000		4.447	*

Continued

Order		Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]													
					PO6-6 LS		PO6-6 DLA		3-9-1-3-8-1 LS		3-9-1-3-8-1 DLA		C9216-11 DLA		CA89 DLA		00-303 DLA	BB KXO730	
Chr2	1	0	0	RG634B	0.646		1.756		0.934		1.420		1.364		0.000		0.057	0.445	
	2	11.8	11.79	RZ87	0.000		1.482		3.892		5.338	*	9.803	**	1.528		1.543	0.790	
	3	16.9	5.1	C132	0.042		2.862		3.001		5.869	*	7.486	**	4.455	*	0.351	0.010	
	4	20.4	3.5	RCD536	0.905		0.000		0.644		1.314		3.760		3.609		1.468	0.419	
	5	25.3	4.9	RG437A	0.532		1.772		6.669	*	7.237	**	10.045	**	2.356		0.195	0.754	
	6	28.8	3.5	RCD532	0.186		0.429		2.834		4.267	*	4.053	*	3.324		0.202	0.055	
	7	45.9	17.1	rNBS32	3.658		0.247		2.993		0.522		2.408		0.593		0.567	3.039	
	8	50.8	4.9	GS170A	4.981	*	3.055		4.590	*	3.469		5.421	*	0.324		0.251	4.487	*
	9	51.8	1	G1184B	7.540	**	3.458		4.730	*	3.692		5.965	*	0.000		0.633	2.097	
	10	54.0	2.2	GS491	6.450	*	2.956		4.738	*	2.779		5.670	*	0.002		0.299	3.559	
	11	54.9	0.9	SU7	3.475		0.884		4.454	*	2.127		7.055	**	0.001		0.338	1.185	
	12	66.1	11.2	RZ166	12.380	***	4.700	*	0.953		0.768		1.311		0.868		0.526	3.852	
	13	76.3	10.2	RZ103	4.405	*	1.960		0.611		0.431		0.337		0.361		0.282	2.836	
	14	85.0	8.7	RG102	2.554		0.008		0.873		2.338		1.558		0.042		1.504	1.520	
	15	91.5	6.5	C601	2.078		0.217		0.663		3.876		3.741		0.376		0.161	3.043	
	16	104.1	12.6	RG151	0.066		0.444		0.740		6.921	**	2.957		1.731		0.015	0.824	
	17	105.2	1.1	RZ446	0.364		0.083		0.244		4.995	*	1.912		0.917		0.754	0.993	
	18	107.9	2.7	G1234	0.163		0.278		0.264		3.204		2.133		0.116		0.394	0.745	
	19	108.3	0.4	RZ913	0.410		0.075		0.150		3.326		2.307		0.386		0.005	1.202	
Chr3	1	0	0	Lls1	0.734		0.000		2.725		0.539		3.988	*	1.068		0.138	3.617	
	2	8.3	8.29	RG944	0.027		0.368		0.061		0.970		0.001		0.068		1.902	9.215	**
	3	15.0	6.7	C74	0.288		0.584		0.277		1.118		0.190		0.409		1.457	5.598	*
	4	24.4	9.4	RG100	0.035		0.499		0.013		0.096		0.008		0.013		4.208	*	3.349	
	5	25.8	1.4	RZ313	1.582		0.130		0.056		0.299		0.002		0.322		0.661	11.510	***
	6	34.1	8.3	RG369	0.759		0.944		0.423		0.454		0.344		0.671		0.872	4.849	*

Continued

Order		Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]							
					PO6-6 LS	PO6-6 DLA	3-9-1-3-8-1 LS	3-9-1-3-8-1 DLA	C9216-11 DLA	CA89 DLA	00-303 DLA	BB KXO730
Chr3	7	37.7	3.6	RZ16	1.713	1.779	1.330	3.204	1.665	0.738	3.094	3.832
	8	54.2	16.5	RZ585	0.727	0.022	0.029	0.147	0.005	0.204	0.024	0.015
	9	66.5	12.3	rNBS17	0.045	0.153	0.054	0.085	0.327	0.051	1.686	0.139
	10	71.3	4.8	RG96B	0.000	0.042	0.147	0.003	0.552	1.279	1.273	0.161
	11	71.8	0.5	RZ403	0.108	0.056	0.020	0.002	0.624	1.694	1.152	0.079
	12	80.8	9	RZ879A	0.474	0.014	1.608	0.901	0.972	3.682	0.039	0.065
	13	80.8	0	RZ474	0.712	0.152	2.109	1.222	1.664	3.213	0.014	0.004
	14	95.1	14.3	G200	0.187	1.977	0.037	0.427	0.059	2.280	0.499	5.186 *
	15	103.3	8.2	CDO337	2.435	3.283	3.362	1.664	1.717	1.660	0.181	5.908 *
	16	104.9	1.6	RZ745	0.993	1.718	1.819	0.545	0.356	3.497	0.130	5.677 *
	17	117.2	12.3	RZ761	0.175	0.349	1.147	0.185	0.433	2.173	2.411	4.752 *
	18	125.1	7.9	RZ575	0.001	0.201	1.265	0.194	0.562	1.294	0.413	3.556
	19	143.8	18.7	RZ319	0.041	0.055	0.378	0.152	0.325	0.351	1.162	3.682
	20	156.8	13	RZ142	0.021	0.074	0.011	0.318	0.262	0.071	0.046	2.483
	21	157.6	0.8	RG1356	0.455	0.651	0.085	0.018	0.162	0.042	0.628	2.944
	22	161.3	3.7	BCD926	1.769	1.899	0.141	0.086	0.017	0.119	0.197	0.404
	23	161.3	0	G1318	1.005	1.014	0.005	0.267	0.000	0.115	0.002	0.368
	24	167.8	6.5	RZ393	0.592	0.021	0.170	0.491	0.087	0.564	0.277	0.748
Chr4	1	0	0	RZ602	0.240	0.005	0.828	0.070	0.263	6.282 *	0.003	1.204
	2	8.6	8.59	RG396B	1.157	0.034	0.361	0.055	0.072	6.818 **	1.061	0.268
	3	10.1	1.5	RG190	0.175	0.060	0.209	0.004	0.200	4.337 *	1.665	0.127
	4	10.3	0.2	RZ69	0.014	0.309	0.679	0.117	0.002	3.180	1.608	0.249
	5	19.5	9.2	rNBS29	0.033	3.680	1.253	1.437	0.229	2.609	0.486	0.092
	6	19.5	0	rNBS46	0.099	3.429	1.130	1.312	0.150	2.868	0.401	0.089
	7	41.1	21.6	RG449	0.006	0.317	0.434	0.120	0.149	3.260	1.991	0.004

Continued

Order	Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]											
				PO6-6 LS		PO6-6 DLA		3-9-1-3-8-1 LS		3-9-1-3-8-1 DLA		C9216-11 DLA		CA89 DLA	00-303 DLA		BB KXO730	
Chr4 8	47.3	6.2	GS39	0.306		0.002		3.157		2.126		0.000		0.824	0.005		0.184	
9	108.6	61.3	rNBS69	0.210		0.304		0.193		1.053		0.072		3.234	2.816		0.028	
10	116.4	7.8	RZ740	0.004		0.814		0.505		0.319		2.395		0.598	0.132		0.554	
11	141.0	24.6	RG939	0.364		0.752		0.838		0.999		1.018		0.003	5.166	*	0.065	
12	153.8	12.8	Ph	0.391		0.480		0.491		0.187		0.224		0.753	7.379	**	4.328	*
13	156.6	2.8	RG476	0.194		0.000		0.030		0.091		0.212		0.177	6.162	*	2.227	
14	157.1	0.5	RZ590	2.224		0.192		0.445		0.425		1.459		0.424	4.774	*	2.139	
15	157.8	0.7	RG161	0.028		0.006		0.007		0.104		0.316		1.172	6.754	*	4.357	*
16	158.5	0.7	RZ879B	0.173		0.517		0.234		0.953		1.140		0.047	5.445	*	3.341	
17	163.6	5.1	RG558	0.314		0.016		0.005		0.060		0.601		1.973	5.421	*	4.088	*
18	163.6	0	RG620	0.477		0.003		0.078		0.076		0.312		1.623	6.545	*	5.561	*
19	168.4	4.8	RZ569B	2.923		0.195		0.000		0.154		0.010		1.928	11.588	***	5.116	*
20	177.9	9.5	RG143	0.765		0.013		0.613		0.160		0.116		2.070	3.445		1.340	
Chr5 1	0	0	rNBS74	4.357	*	6.025	*	5.940	*	5.371	*	3.994	*	1.268	5.927	*	19.498	****
2	6.8	6.79	RCD511	2.651		3.162		1.572		2.029		2.891		1.039	6.407	*	11.747	***
3	9.1	2.3	GS434	2.648		3.221		3.009		2.048		4.164	*	0.791	7.866	**	18.763	****
4	9.1	0	MJ224	4.019	*	4.025	*	3.931	*	2.268		3.838		0.917	6.641	*	16.597	****
5	12.4	3.3	RCD549	3.452		4.929	*	2.826		2.893		6.492	*	1.341	8.303	**	16.473	****
6	29.8	17.4	RG13	2.815		6.864	**	0.502		3.179		1.707		0.008	0.687		12.249	***
7	32.4	2.6	AJ226	2.345		9.257	**	2.716		6.396	*	4.450	*	0.003	0.381		17.837	****
8	37.2	4.8	RG634A	2.353		6.973	**	0.620		3.164		2.801		0.132	0.308		5.999	*
9	48.3	11.1	RG470	0.105		5.998	*	0.328		0.511		0.247		2.832	0.207		6.097	*
10	51.6	3.3	RZ291	0.920		9.694	**	0.067		0.978		1.372		2.969	0.046		7.727	**
11	52.9	1.3	G329	1.184		8.956	**	0.050		1.464		3.670		3.114	0.010		6.823	**
12	52.9	0	G12	1.062		9.163	**	0.095		1.606		3.725		2.738	0.108		8.088	**

Continued

Order	Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]													
				PO6-6 LS		PO6-6 DLA		3-9-1-3-8-1 LS		3-9-1-3-8-1 DLA		C9216-11 DLA		CA89 DLA		00-303 DLA		BB KXO730	
Chr5 13	52.9	0	G122	0.004		2.279		1.003		0.036		1.152		0.471		0.010		2.377	
14	59.3	6.4	yac3	4.067	*	18.334	****	3.819		8.074	**	6.483	*	1.188		0.078		30.921	****
15	59.3	0	rNBS4	3.723		17.231	****	3.000		7.046	**	5.357	*	1.419		0.007		36.490	****
16	59.3	0	mNBS1	4.514	*	18.537	****	3.494		7.534	**	6.219	*	1.078		0.107		33.071	****
17	59.3	0	r13	5.228	*	18.417	****	3.551		7.494	**	6.074	*	1.901		0.067		30.246	****
18	59.3	0	b4	5.099	*	19.685	****	3.972	*	8.077	**	6.979	**	1.009		0.105		34.563	****
19	78.1	18.8	RZ225A	0.215		2.225		0.110		0.267		2.464		2.263		0.750		1.129	
20	84.8	6.7	RG474	0.187		2.411		0.088		0.423		1.071		0.906		0.930		0.917	
21	85.1	0.3	CDO1160	1.281		3.976	*	0.044		0.198		0.955		0.586		0.067		0.100	
22	90.2	5.1	RG493	0.086		3.229		0.095		0.012		0.090		0.312		0.079		0.825	
Chr6 1	0	0	C474	1.265		1.665		0.330		0.094		0.856		0.530		0.939		0.012	
2	0.6	0.59	RZ1002	0.690		0.946		0.015		0.008		0.667		0.141		2.106		0.234	
3	24.3	23.7	RZ450	2.827		1.505		0.068		0.137		0.005		9.368	**	0.444		2.396	
4	31.5	7.2	RG213	1.802		2.084		0.973		0.110		1.362		3.360		2.900		0.573	
5	34.9	3.4	C235	2.891		2.147		0.648		0.562		1.716		17.103	****	0.001		3.240	
6	35.1	0.2	RG64	0.108		0.056		0.012		0.000		0.003		13.029	***	0.196		1.820	
7	41.6	6.5	rNBS81	0.003		1.612		0.004		0.507		0.006		5.944	*	4.629	*	0.849	
8	56.5	14.9	RG716	0.110		0.040		0.161		0.001		3.164		0.423		0.011		3.595	
9	80.9	24.4	rNBS73	2.253		0.087		4.474	*	2.232		2.450		0.375		0.230		2.308	
10	89.5	8.6	RZ828	0.677		0.048		0.073		0.283		0.160		0.256		0.078		1.581	
11	95.0	5.5	RZ884	0.987		0.140		0.000		0.146		0.448		0.056		0.029		0.417	
12	95.4	0.4	RG433	0.114		0.286		0.157		0.535		1.215		0.432		0.044		0.237	
13	95.7	0.3	RZ508	0.085		0.306		0.207		0.597		1.383		0.548		0.055		0.298	
Chr7 1	0	0	RG165	3.993	*	3.999	*	0.036		0.010		0.065		0.060		0.000		0.102	

Continued

Order		Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]							
					PO6-6 LS	PO6-6 DLA	3-9-1-3-8-1 LS	3-9-1-3-8-1 DLA	C9216-11 DLA	CA89 DLA	00-303 DLA	BB KXO730
Chr7	2	8.9	8.89	RG128	0.524	0.193	0.077	0.010	0.251	0.161	0.005	0.154
	3	31.4	22.5	rNBS77	4.649 *	0.434	0.040	0.266	1.504	0.300	0.037	0.000
	4	52.1	20.7	GS13	3.191	1.778	0.050	0.046	0.363	1.259	0.026	2.133
	5	53.7	1.6	RCD138	3.397	2.704	0.011	0.192	0.627	1.859	0.028	1.000
	6	59.7	6	C492	4.818 *	3.560	1.006	0.816	0.516	0.058	0.202	3.892
	7	70.1	10.4	rNBS23	4.103 *	1.465	0.862	0.000	0.725	0.552	0.032	1.123
	8	76.4	6.3	rNBS36	0.042	0.002	0.196	0.496	0.011	0.542	1.549	0.632
	9	89.0	12.6	C285	2.627	3.116	0.545	0.312	0.027	0.001	3.367	0.383
	10	99.6	10.6	C147	1.105	0.192	1.702	0.759	0.599	0.876	5.241 *	0.000
	11	108.5	8.9	RG711	1.569	0.022	0.039	0.088	0.092	2.397	4.593 *	1.460
	12	115.1	6.6	bAMY	0.760	0.170	0.116	0.249	0.138	0.012	5.479 *	0.001
	13	128.1	13	RZ395	3.270	0.040	0.556	0.254	0.868	0.114	0.009	1.390
	14	130.8	2.7	RCD405	1.735	0.090	0.104	0.051	0.585	1.907	3.328	1.323
	15	131.3	0.5	GS275	0.940	0.335	0.220	0.173	0.200	0.261	0.485	1.703
	16	138.0	6.7	C507	1.365	0.341	0.012	0.029	0.363	0.023	6.875 **	0.995
	17	143.8	5.8	RZ978	0.034	1.565	0.899	1.335	0.171	0.009	1.333	4.971 *
	18	144.9	1.1	RG978	0.432	3.355	2.667	2.220	1.358	1.128	0.491	3.997 *
	19	151.1	6.2	SU30	0.609	1.269	3.972 *	1.777	2.761	0.150	0.531	2.012
	20	152.1	1	C213	0.053	1.646	2.436	2.214	1.743	0.000	0.661	1.454
Chr8	1	0	0	rNBS52	2.524	0.452	1.466	3.956 *	4.130 *	1.110	11.074 **	3.459
	2	0.01	0	rNBS53	2.767	0.055	0.820	2.355	2.638	2.206	9.452 **	3.612
	3	0.01	0	rNBS28	2.229	0.171	1.202	2.738	3.669	3.848	8.415 **	3.445
	4	7.1	7.09	RZ323	0.208	0.000	1.655	1.314	1.396	5.049 *	6.932 **	1.839
	5	21.3	14.2	G1073A	0.156	0.078	0.827	0.243	0.746	0.056	1.339	1.304
	6	30.5	9.2	RG1	0.062	1.065	0.404	0.001	0.703	0.119	0.008	2.703

Continued

Order		Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]													
					PO6-6 LS		PO6-6 DLA		3-9-1-3-8-1 LS		3-9-1-3-8-1 DLA		C9216-11 DLA		CA89 DLA		00-303 DLA		BB KXO730	
Chr8	7	56.8	26.3	RZ66	1.013		1.719		1.062		0.557		0.011		0.629		0.054		0.210	
	8	59.8	3	RZ70A	0.030		1.356		0.440		0.204		0.027		0.013		0.075		0.305	
	9	70.0	10.2	RZ656	0.002		0.022		0.474		0.263		0.466		0.805		2.250		0.025	
	10	84.9	14.9	RG598	0.000		0.019		0.696		0.447		0.584		0.008		0.076		0.003	
Chr9	1	0	0	rNBS7	16.278	****	11.464	***	3.950	*	3.987	*	7.578	**	7.590	**	6.703	*	1.222	
	2	0.9	0.89	rNBS13	10.823	**	7.699	**	1.336		2.689		4.773	*	8.560	**	5.640	*	0.257	
	3	9.9	9	G338	3.707		0.648		0.183		0.001		0.313		1.580		0.263		0.315	
	4	13.3	3.4	RG757	3.731		0.652		1.167		0.469		1.597		1.440		4.373	*	0.548	
	5	13.3	0	RCD312	5.744	*	1.822		1.318		0.507		2.292		1.188		4.273	*	1.382	
	6	20.5	7.2	RG553	7.929	**	2.199		2.772		0.882		0.744		2.058		2.929		3.492	
	7	30.6	10.1	r16	14.286	***	6.888	**	2.472		1.573		4.278	*	1.104		1.189		3.964	*
	8	64.7	34.1	RCD335	0.019		0.164		0.000		0.476		0.472		1.108		2.175		1.362	
	9	80.6	15.9	SU43	0.878		1.129		1.179		1.103		1.682		4.438	*	0.065		1.205	
	10	83.7	3.1	RG662	0.257		0.150		0.503		0.713		1.827		2.042		0.000		2.466	
	11	93.0	9.3	RG451	0.239		3.457		0.185		1.613		3.043		0.892		0.257		0.422	
	12	94.5	1.5	RG141	0.084		4.897	*	0.532		3.408		2.946		0.780		0.082		1.015	
Chr10	1	0	0	L169	1.351		0.211		4.440	*	2.188		7.522	**	3.326		1.676		0.969	
	2	15.9	15.89	rNBS5	2.411		0.890		2.338		2.365		1.544		0.000		0.350		0.915	
	3	15.9	0	r8	2.560		0.799		2.400		2.260		1.804		0.000		0.156		0.661	
	4	16.9	1	rNBS72	3.832		1.878		3.101		2.384		1.127		0.031		0.291		0.127	
	5	32.0	15.1	C949B	5.568	*	1.837		9.935	**	6.081	*	11.121	**	0.045		0.605		0.025	
	6	57.4	25.4	RG323	2.559		0.736		2.129		0.789		0.766		0.982		2.483		3.483	
	7	59.7	2.3	RZ400	2.328		0.472		3.519		1.493		1.532		0.375		1.351		4.271	*
	8	59.7	0	RZ561	2.665		0.908		4.087	*	1.998		1.898		0.622		1.599		6.831	**

Continued

Order	Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]							
				PO6-6 LS	PO6-6 DLA	3-9-1-3-8-1 LS	3-9-1-3-8-1 DLA	C9216-11 DLA	CA89 DLA	00-303 DLA	BB KXO730
Chr10 9	65.3	5.6	RZ337	3.460	0.002	0.679	0.097	0.033	0.634	0.198	8.074 **
10	69.0	3.7	BCD386	2.879	0.185	0.293	0.057	0.015	0.294	2.262	5.541 *
11	72.8	3.8	BCD207	5.128 *	0.248	1.435	0.646	0.003	0.314	1.210	9.991 **
12	79.6	6.8	RZ500	4.502 *	2.408	0.314	0.184	0.007	0.187	4.219 *	7.386 **
Chr11 1	0	0	RG304A	4.811 *	1.887	3.940 *	3.893	5.805 *	0.044	1.196	0.964
2	17.2	17.19	CDO127A	1.354	0.459	0.024	0.065	0.314	0.559	0.378	0.162
3	34.7	17.5	RG397	13.662 ***	0.828	3.207	0.813	1.635	0.265	0.052	4.675 *
4	43.7	9	RG119	5.135 *	0.208	0.259	0.100	0.478	2.149	0.293	2.493
5	51.2	7.5	RG1094	2.511	1.321	0.037	0.037	0.099	0.296	0.127	3.070
6	67.7	16.5	rNBS8	2.932	0.144	0.337	0.646	0.050	2.595	0.054	1.148
7	67.7	0	rNBS12	4.846 *	0.349	0.476	0.402	0.327	3.843	0.285	2.234
8	69.9	2.2	rNBS71	3.445	0.666	0.125	0.385	0.138	0.471	0.004	3.676
9	80.0	10.1	r12	0.042	0.001	0.485	0.470	0.294	0.001	0.061	1.252
10	86.1	6.1	RZ797	0.255	1.587	0.329	1.379	0.509	0.358	1.295	3.188
11	98.6	12.5	rNBS10	1.322	0.470	0.156	0.276	0.734	0.001	1.725	0.093
12	110.2	11.6	RG353	0.363	0.349	0.699	0.029	0.350	0.502	2.446	1.782
13	111.3	1.1	G1465	1.696	1.123	0.915	0.007	1.109	1.421	1.270	1.887
14	123.4	12.1	rNBS1	0.068	0.000	0.204	0.538	0.172	0.161	8.362 **	0.977
15	127.8	4.4	JAMyb	0.162	0.370	0.329	0.500	1.186	0.415	1.321	0.614
16	137.1	9.3	RZ536	0.538	0.034	0.527	0.612	0.156	0.088	0.164	1.146
Chr12 1	0	0	RG235	1.374	0.919	1.442	1.622	0.940	2.069	0.590	0.012
2	10.6	10.59	RG574	0.535	0.085	3.763	3.322	2.961	0.066	0.158	0.161
3	13.6	3	RZ816	2.284	0.042	4.495 *	3.119	2.304	0.001	0.027	0.018
4	21.2	7.6	G1112	0.109	0.369	1.568	0.534	0.727	0.120	0.223	0.254

Continued

Order	Interval (cM)[a]		Locus	Single marker QTL analysis (*F-value*)[b]															
				PO6-6 LS		PO6-6 DLA		3-9-1-3-8-1 LS		3-9-1-3-8-1 DLA		C9216-11 DLA		CA89 DLA		00-303 DLA		BB KXO730	
Chr12 5	60.8	39.6	RG869	0.171		0.249		21.843	****	19.144	****	19.911	****	16.449	****	4.520	*	0.447	
6	65.0	4.2	GS117	0.301		0.025		35.529	****	23.437	****	39.681	****	4.896	*	6.463	*	0.507	
7	79.0	14	RG9	0.842		0.266		4.942	*	7.262	**	8.844	**	10.113	**	1.359		0.782	
8	116.6	37.6	GS36	0.069		0.030		0.017		0.074		0.557		0.077		0.008		9.417	**
9	119.9	3.3	RG901	0.078		0.069		0.083		0.014		0.184		0.018		1.502		7.536	**
10	135.2	15.3	RG181	4.292	*	0.180		2.388		0.215		1.323		0.347		0.385		3.747	
11	140.3	5.1	RG958	1.531		0.007		2.068		0.395		1.679		0.633		0.548		2.414	

[a] Accumulative map size of chromosome and marker intervals distance with cM

[b] *, **, *** and **** are levels of significance at $P \leq 0.01$, 0.001, and 0.0001, respectively.

CHAPTER 4 - Rice Chitinase Gene Contributes To Rice Sheath Blight Disease Resistance

Summary

Rice chitinases co-localize with disease resistance QTL and are implicated in multiple defense responses. Rice lines overexpressing chitinases have been previously shown to exhibit increased resistance to the fungal pathogens *Rhizoctonia solani* and *Magnaporthe oryzae*. Previous work also demonstrated that the class IV rice chitinase LOC_Os02g39330 is linked to a disease resistance QTL on chromosome 2, and that this gene is transcriptionally active in response to pathogen attack. We used an RNAi silencing approach to determine if *Os02g39330* contributes to broad-spectrum disease resistance. The effect of the silencing construct was measured on expression of *Os02g39330* and two closely related chitinases, *Os04g41680* and *Os04g41620* in five transgenic lines after inoculation with *Rhizoctonia solani* and *Magnaporthe oryzae.* All three chitinase genes were induced after infection with both pathogens in the wild type control. Two of the five transgenic lines showed no silencing of *Os02g39330*, *Os04g41680* or *Os04g41620;* sheath blight and rice blast disease scores on these lines were similar to the wild type control plants. Three of the five transgenic lines exhibited high levels of silencing of *Os02g39330*, and little to no silencing of *Os04g41620* and *Os04g41680.* These lines showed increased sheath blight disease, but less rice blast disease, relative to control lines with no silencing. The increase in sheath blight disease in *Os2g39330* silenced lines suggests that this chitinase contributes to *R. solani* resistance in rice. *Os2g39330* was not associated with *M. oryzae* resistance in this study. Enhanced expression of related chitinases *Os04g41680* and *Os04g41620* in the transgenic lines was not correlated with increased resistance to sheath blight or rice blast, suggesting that these genes do not contribute to disease resistance or susceptibility. The demonstration that *Os239330* contributes to sheath blight resistance shows that this class IV chitinase is a valuable source of basal resistance for QTL breeding programs.

Introduction

As one of the most important cereal crops, rice (*Oryza sativa*) feeds close to half the population in the world (Cantrell and Reeves 2002). In addition to the values for human nourishment (Yano and Sasaki, 1997), rice has been used as a model plant for research (Izawa and Shimamoto, 1996; Shimamoto and Kyozuka, 2002). The more than 70 diseases of cultivated rice present serious constraints to the production of rice. Fungal diseases such as rice blast, caused by *Magnaporthe oryzae* (*Mo*), and sheath blight, caused by *Rhizoctonia solani* (*Rs*), are the most serious diseases affecting rice production (Ou, 1985). Classical breeding programs have traditionally incorporated single disease resistance genes into commercial rice cultivars for control of rice blast, however, changes of the pathogen population to new virulence types have often destabilized single gene resistance. Furthermore, for diseases like sheath blight, no single resistance genes are known, thus different strategies for resistance are needed. We have been exploring sources of quantitative disease resistance, which is predicted to be broad spectrum and more long-lasting, as a possible means to reduce the erosion of disease resistance and protect rice crops. Incorporation of quantitative sources of resistance by plant breeding programs has been hindered by the lack of information as to what genes contribute to this type of resistance. By identifying the genes that underly quantitative trait loci (QTL) and demonstrating their contribution to disease resistance, useful molecular markers can be developed to aid in the incorporation of these needed traits into widely used germplasm.

Plant chitinases have been shown to act as defense genes and are linked to a broad spectrum resistance (De Jong et al., 1992; Goormachtig et al., 1998; Helleboid et al., 2000). Chitinase (EC 3.2.1.14) hydrolyzes the β-1, 4-linkage of chitin (poly-β-1,4-N-acetyl

glucosamine), the main component of the cell walls of fungi and the exoskeletons of insects (Collinge et al., 1993; Zhu et al., 2009). Classification of chitinases is based on sequence homology in their catalytic domain and by the presence or absence of a chitin binding domain; these features are used to group chitinases into family 18, 19 and 20 glycosyl hydrolases (Neuhaus, 1999). Plant chitinases are also divided into seven classes, I-VII, in which classes III and V belong to family 18, and classes I, II, IV, VI, and VII comprise family 19 (Collinge et al., 1993; Neuhaus 1999). Analysis of the sequence of the *Oryza sativa* cv. Nipponbare genome revealed several chitinases, including the *OsChia1, 2, 4*, and *7* gene that belong to the family 19 class, and *OsChib1* and *OsChic1*, which belong to family 18 chitinases (Snelling, 2010). In addition to the direct role of plant chitinases in hydrolysis of chitin, they also are implicated in basal disease resistance by generating elicitors in the form of chitin oligosaccharides or lipids from the hydrolyzed cell walls (Chisholm et al., 2006; Huckelhoven 2007; Nürnberger et al., 2004). Binding of the chitin oligosaccharide elicitors to plant membrane-anchored receptors activates downstream defense responses (Desaki et al., 2006). The induced chitin oligosaccharides were shown to be bound by a high-affinity binding protein CeBiP in rice (Kaku et al., 2006)

Several studies have addressed the roles of plant chitinases in plant disease defense. Purified forms of chitinases from tomato, pea, and bean were demonstrated to inhibit fungal growth (Boller et al., 1983; Mauch et al., 1988; Sharma et al., 2011; Schlumbaum et al., 1986; Young and Pegg, 1982). Accumulation of chitinases is correlated with fungal infections. For example, binding of gold-labeled chitinase-specific antibodies increased at the synthesis sites of expanding *Trichoderma* hyphae during invasion of *Pisum sativum* (Arlorio et al., 1992). Finally, overexpression of chitinases is associated with enhanced disease resistance in a number of

studies. Transgenic tobacco and canola transformed with a bean vacuolar chitinase gene under the control of the CaMV 35S promoter survived in soil infected by *R. solani* and exhibited delayed development of disease (Broglie et al., 1991). Transfer of a basic tobacco chitinase to carrots conferred higher resistance to *Botrytis cinerea*, *R. solani* and *Sclerotium rolfsii* (Gilbert et al., 1996; Punja, 2006).

Chitinases have also been implicated to contribute to disease resistance in rice. A rice secretome study, using secretomes acquired from rice calli and leaves treated with *M. oryzae* or an extracted elicitor, revealed differential induction of several chitinases (Kim et al., 2009). Transgenic rice constitutively over-expressing *Chi 11*, a rice-derived chimeric chitinase gene, increased resistance to sheath blight (Lin et al., 1995). Transgenic rice plants with rice *Cht-2* or *Cht-3* chitinase genes exhibited significantly increased resistance against *M. oryzae* (Nishizawa et al., 1999). Indica rice cultivars over-expressing a rice chitinase showed increased resistance to sheath blight disease caused by *R. solani* (Datta et al., 2001). Furthermore, ectopic expression rice chitinase genes in other plant species, including cucumber (Tabei et al., 1998), rose (Marchant et al., 1998), chrysanthemum (Takatsu et al., 1999), grapevine (Yamamoto et al., 2000) and Italian ryegrass (Takahashi et al., 2005), resulted in increased disease resistance.

Of the many rice chitinases, one, *Os02g39330,* co-localizes with a minor blast disease resistance QTL on chromosome 2 in two mapping studies (Liu et al., 2004; Wu et al., 2004). Furthermore, this and other closely related class IV chitinase genes were induced by *M. oryzae* infection (Kim et al., 2009). In this study, we investigate the role of *Os02g39330* in resistance to *R. solani* and *M. oryzae* by using the method of RNAi silencing. We show that *Os02g39330* contributes to resistance to *R. solani,* but apparently not to *M. oryzae.*

Materials and Methods

RNAi silencing

To silence *Os02g39330*, a 239 bp fragment was generated based on the glycosyl hydrolase domain and 3' UTR of *Os02g39330*, which correspond to the last 129 bp of the second Os02g39330 exon and subsequent 110 bp of the 3'UTR (Fig. 4.1). Rice cultivar Kitaake genomic DNA was used the template to generate the fragment. The PCR profile consisted of a 3 min denaturation at 95ºC, then 32 cycles of 20 s at 95ºC, 40 s at 52ºC, and 30 s at 72ºC, using Chitinase F5 (5'-CACCATCCGCGCCATCAACG-3') and Chi3UTRR5 (5'-CTCCTATGCCGCAAACAACG) primers. The PCR product was confirmed to be the correct size by 1 % agarose gel electrophoresis, then subcloned into an entry vector TOPO pENTER (Invitrogen, Carlsberg, USA). *E. coli* transformants were selected by growth on media containing 50 µg/ml Kanamycin (Km). Gateway LR Clonase Enzyme Mix (Invitrogen, Carlsberg, USA) was then used to recombine the entry vector with the destination pANDA vector. The recombined pANDA vector was transformed into *E. coli* strain DB3.1 and selected on 50 µg/ml Km agar media. The purified pANDA vector was transferred to *Agrobacterium tumefaciens* strain EHA105, and used to transform rice calli by the method of Miki and Shimamoto (2004). Successful rice transformants were identified by PCR amplification of the hygromycin selection gene in T0, T1, T2, and T3 generations using HygroF (5'-GAGCCTGACCTATTGCATCTCC-3') and HygroR (5'-GGCCTCCAGAAGAAGATGTTGG-3') primers. Transcript change of chitinase *Os02g39330* compared to wild type Kitaake was analyzed in T0 plants, and only selected lines with reduced expression were further advanced to T1, T2 lines were assessed for the following: (1) reduced accumulation of *Os02g39330* mRNA, (2) presence of the hygromycin gene, and (3) severity of *R. solani* and *M. oryzae* disease symptoms. Initial screening of T1

transgenic lines were done by using Platinum High Fidelity DNA Taq Polymerase (Invitrogen, Carlsberg, USA) to allow amplification of low transcript target genes. T2 transgenic lines were selected by using a primer set, which was developed for use with a standard Taq DNA Polymerase (New England Biolabs). Lines Chi2-5 and Chi2-6 were from the T2 generation and Chi28-8-11, Chi28-12-2 and Chi28-12-10 were selected at the T3 generation and further used in this study.

Plant Growth Conditions and Sheath blight and Blast Disease Evaluations

The RNAi silenced lines and wild type Kitaake seeds were pre-germinated in fungicide Maxim XL (Active Ingredients: 21.0% Fludioxonil and 8.4% Mefenoxam, Syngenta, Swiss) for 2 days prior to seeding. For sheath blight experiments, germinated seeds were sown in a plastic pot (15 x 12 x 15 cm) and fertilized with 0.5 g of $NH_4(SO_4)2$, 0.5 g of P_2O_5 and 0.2 g of K_2O once.

R. solani isolate RM01401 was grown on Difco potato dextrose agar (Becton Dickinson, Franklin Lakes, NJ) containing tetracycline (0.005%, wt/vol) at 26°C and produced active mycelia by transferring mycelium to fresh medium. To evaluate the degree of sheath blight disease, wild-type and silenced lines of 15-day-old Kitaake were inoculated with *R. solani* isolate RM01401 using the microchamber method (Jia et al., 2007). Microchamber conditions during sheath blight infection were 90-100% relative humidity and 34ºC/26ºC day/night temperatures applied with supplementary lighting to maintain a 16 h light/ 8 h dark photoperiods. *R. solani* inoculum consisted of round disks (1 cm in diameter), which were excised from a 3-day-old mycelia culture grown on potato dextrose agar. The first fully emerged plant leaves were collected after 48 h of *R. solani* inoculation on the bottom of rice plants as described (Jia et al.

2007). Sheath blight symptoms were scored using a quantitative disease index (DI) = (lesion length/ plant height) x 9, modified from Groth et al. (1990). For the Visual index (VI) scale (0-9), 0 indicates no lesion and 9 represents lesions covering all leaves and the panicle.

M. oryzae were grown on oatmeal agar in continuous light at 26ºC and stored on sterile filter paper at -20ºC before usage. To evaluate effect of silencing on rice blast responses, 15-day-old transgenic and wild-type Kitaake were inoculated with *M. oryzae* using a spot assay (Jia, 2007). The second youngest leaves collected at 12 days were infected and spotted with 5 µl drops of $1x10^5$ spore/ml suspension. A disease score was determined for the range of phenotypes using a scale from 0-7; 0-1 being a resistant score, with little to no visible sign of the fungus or plant response to the fungus; 2-3 being a moderately resistant score, showing some physical presence of the fungus (single to a few hyphae), but evidence of necrotic areas associated with fungal penetration points; 4-5 being a moderately susceptible score, showing abundant aerial hyphae, and more severe necrosis to plant tissue throughout and extending beyond the site of the inoculation spot; 6-7 being a susceptible score, showing dense hyphal mats, as well as severe necrotic lesions extending beyond the range of the inoculation spot and giving the typical diamond shaped lesion associated with plants susceptible to *M. oryzae*.

Results

OsO2g39330 and other closely-related rice chitinase genes

Only one chitinase gene, *Os02g39330*¸ is located under the chromosome 2 disease resistance QTL. The coding region is separated by one intron (Fig. 4.1a). The gene is composed of total 816 nucleotides and encodes a 271 amino acid protein (Fig. 4.1b). The most closely related chitinases to *Os02g39330* are *Os04g41620*and *Os04g41680*, which are located on rice chromosome 4 (Snelling, 2010).

Silencing of Os02g39330 by RNAi

The 239 bp region of *Os02g39330* used to make the dsRNA in the silencing construct OSChia4SIL potentially contains enough similarity to silence other closely related chitinases in the rice genome, in particular *Os04g41620*and *Os04g41680* (Fig. 4.1c). *Os04g41620*and *Os04g41680* had not yet been discovered when this project was initiated but because of their high similarity to *Os02g39330*, we tested their expression in the silenced lines. The japonica rice cultivar Kitaake, which was used for silencing experiments due to ease of transformation, and exhibits moderate, quantitative-type resistance to *R. solani* isolate RM01401 and *M. oryzae* isolate PO6-6 and susceptible to the Korean isolate KI-197. Kitaake transformants were identified by the insertion of the hygromycin gene by PCR in each generation (not shown). The relative amounts of silencing of the chitinase genes, measured in untreated lines, varied in each generation.

Very little chitinase expression was detected in untreated wild type and transgenic lines in our initial screens (at the limits of detection for RT-PCR), and was highly variable. This is

because *Os02g39330* is very weakly expressed in untreated wild type Kitaake (Figs. 4.2 and 4.3, first lane) and transgenic silenced lines, but is induced after pathogen treatment (e.g., Figs. 4.2 and 4.3, second lane). However, based on our initial assays of untreated lines, a total five transgenics with the silencing construct were selected for further analysis. These were T2 (Chi2.5, Chi2.6, no silencing) and T3 (Chi28.8.11, Chi28.12.2, and Chi28.12.10, silencing).

RNAi silencing of rice chitinase increases R. solani disease

Among the five different lines transformed by the RNAi silencing construct, only three lines, Chi28.8.11, Chi28.12.2, Chi28.12.10, showed reduced expression of *Os02g39330* after inoculation with *R. solani* (Fig. 4.2). Chi29.8.11, which had no expression of *Os02g39330* in response to *R. solani*, exhibited severe drying and death after fourteen days (Fig. 4.3). Chi28.12.2 and Chi28.12.10, which showed very faint bands in after RT-PCR, exhibited very severe symptoms of sheath blight compared to the control plant KitWT (Fig. 4.3). The three T3 silenced lines also showed more disease than controls as measured by two scoring methods, visual index and disease index (Fig. 4.4). T2 transgenic lines that did not exhibit silencing in initial screens, Chi2.5 and Chi2.6, did not show reduced expression of *Os02g39330* after inoculation with *R. solani* (Fig. 4.2), and showed symptoms and levels of sheath blight disease similar to the Kitaake wild type controls (Figs. 4.3, 4.4). Thus, silencing of *Os02g39330* is correlated with enhanced sheath blight disease in rice.

RNAi silencing of rice chitinase does not increase rice blast disease

The five transgenic lines were tested for reaction to *M. oryzae.* In response to *M. oryzae* infection, expression of *Os02g39330* was increased slightly in wild type Kitaake, and in the T2

transgenics Chi2.5 and Chi2.6 (Fig. 4.3). In the T3 lines Chi28.8.11, Chi28.12.2, Chi28.12.10 after inoculation with *M. oryzae, Os02g39330* expression was silenced (Fig.4.3). Plants that did not show reduced expression of *Os02g39330*, i.e., Kitaake wild type and the T2 transgenes (Chi2.5 and Chi2.6), showed moderate disease symptoms after inoculation with *M. oryzae* KI-197 (scores of 4 on a 0-5 scale). On the other hand, the silenced lines showed more variation in disease symptoms, but in general, showed less blast disease than the control plants (scores of 3-4). These data suggest that *Os02g39330* does not contribute to QTL-based resistance to *M. oryzae*.

Effects of silencing of Os02g39330 on expression of Os04g41620 and Os04g41680.

Because of the close similarity of sequence of *Os04g41620* and *Os04g41680* to *Os02g39330*, we asked if the silencing of *Os02g39330* affected the expression of these two genes before and after infection with *R. solani* and *M. oryzae*. As shown in Figure 4.4, both *Os04g41620* and *Os04g41680* are induced by infection, with *Os041620* being induced more by *M. oryzae* than by *R. solani*. The differences in levels of induction may be a result of the differences in pathogen infection styles. Based on the RT-PCR results, *Os04g41620* and *Os04g41680* show very little, if any at all, silencing in the T3 transgenic lines relative to the Kitaake wild type and T2 controls. Given the higher levels of expression of both relative to *Os02g39330* in both the silenced and unsilenced lines, there is no correlation with the silencing of *Os04g41620* and *Os04g41680* and sheath blight disease, suggesting that only *Os02g39330* is involved in resistance to sheath blight. For blast disease, there was no correlation with expression of *Os04g41620* and *Os04g41680* and the observed enhanced resistance, because the same levels of expression were observed in the T2 and Kitaake control lines as in the T3 lines.

Discussion

As pathogen-responsive genes, the expression levels and enzymatic activities of plant chitinases have been correlated to plant defense responses (Kasprezewska 2003; Passarinho and de Vries 2009). In the previous QTL experiments, chitinase genes co-localized with rice disease resistance QTL, suggesting they are candidates for contribution to quantitative resistance (Liu et al., 2004). Among the over 51 identified putative rice chitinase genes in rice (Snelling, 2010), the selected *Os02g39330* chitinase gene belongs to Class IV and Family 19, and its expression was induced following infection of *M. oryzae* and *R. solani* (Snelling, 2010). This gene colocalized with a chromosome 2 disease resistance QTL (Liu et al., 2004).To determine if *Os02g39330* indeed plays a role in pathogen resistance and contributes to QTL-based defense, we developed RNAi lines silenced for *Os02g39330* expression.

The *Os02g39330* RNAi silencing lines exhibited altered disease resistance levels. Among the five lines harboring the RNAi silencing construct, reduced expression of *Os02g39330* was observed in three selected T3 lines (Fig. 4.2). Chi28.8.11, in particular, showed no detectable levels of *Os02g39330* expression even after attack by *R. solani* (Fig. 4.2). Silencing of *Os02g39330* was consistent with increased disease in the T3 lines, as indicated by both visual rating and disease index (Fig. 4.4A and B). Thus, we conclude that the class IV chitinase *Os2g39330* contributes to QTL-based resistance to *R. solani* in rice.

In contrast to the results with *R.solani*, enhanced susceptibility to rice blast disease was not detected in the *Os2g39330* silenced lines. Indeed, two lines, Chi28.12.2 and Chi28.12.10, exhibited increased levels of resistance to *M. oryzae* relative to controls. One possible explanation is that since the chromosome 2 QTL is a minor-effect QTL (Liu et al., 2004), our assays for blast disease may not have been sensitive enough to see a slight increase in disease in

the transgenic silenced lines, particularly in the Kitaake genetic background. Another plausible explanation is that altered expression of other chitinases may have impacted the disease response. Two other chitinases (*Os04g41620* and *Os04g41680*) that are closely related to *Os02g39330* showed enhanced expression after infection with *M. oryzae;* however, the induction of expression was not correlated with increased resistance because similar levels of expression were observed in the unsilenced lines and Kitaake wild type after inoculation. Thus, *Os02g39330, Os04g41620* and *Os04g41680* are not candidate contributors for resistance to *M. oryzae*. It is possible that the expression of other, less closely related chitinases was altered by the presence of the silencing construct to compensate for the silencing of *Os02g39330*, resulting in resistance. We did not test the expression of unrelated chitinases.

Manosalva et al. (2009) demonstrated that a family of 12 germin-like protein genes (*OsGLP*) collectively contributed to disease resistance governed by a rice chromosome 8 resistance QTL, and that this resistance was effective against both *R.solani* and *M. oryzae*. The contributions of the different *OsGLP* genes varied, with some contributing more to resistance than others. It is possible that rice chitinases also contribute collectively to disease resistance, and, although located on different chromosomes (unlike the *OsGLPs*) they may respond to pathogen signals in concert. Indeed, our work shows that chitinases *Os2g39330, Os04g41620* and *Os04g41680* are all induced upon pathogen infections, although the degree of induction varies with pathogen. Given that there are more than 50 chitinase or chitinase-like genes in rice (Snelling, 2010), co-regulation and redundancy of function are not unlikely.

Increasing evidence is showing that off-target silencing is common in RNAi silencing studies. For example, Manosalva et al. (2009) demonstrated that a single silencing construct with significant variation could silence many related gene family members of a germin-like

protein gene family in rice. Held et al. (2008) targeted a cellulose synthase gene for silencing, but found that several closely related cellulose synthase genes, as well as distantly relate glycosyl transferase genes, were down regulated by the silencing of a single gene. In this study, we show very low level non-target silencing of a highly related chitinases, *Os04g41680* and *Os04g41620.* Because different apparent levels of non-target silencing of these chitinases were observed, we were able to conclude that relative to *Os02g39330*, these genes are less important in resistance to sheath blight disease.

Overall, my results agree with previous studies that certain chitinases do contribute to disease resistance in rice. Furthermore, the association of *Os02g39330* with the disease resistance QTL on chromosome 2 suggests that this gene contributes to resistance conferred by that QTL. Future studies should target differences in *Os02g39330* alleles or their expression in the donors of the chromosome 2 QTL to determine if there are differences that can be exploited by molecular breeders to accumulate this QTL into germplasm. For example, other studies have shown that differences in the promotor region of an oxalate oxidase gene contribute to differences in expression of the gene and QTL-based resistance, and that this variation can be exploited to improve disease resistance (Carillo et al., 2009; Davidson et al., 2010). Other future studies should focus on determining if *Os02g39330* contributes to disease resistance in different genetic backgrounds; either molecular breeding approaches or transgenic lines overexpressing *Os02g39330* could be used to test the effects of genetic background. Finally, silencing *Os02g39330* in Kitaake did not have an effect on rice blast disease resistance, even though *Os02g39330* is co-localized with the QTL on chromosome 2. While it is possible that *Os02g39330* does not contribute to blast disease resistance, there are other possibilities that need to be ruled out. First, our assays may not have been sensitive enough to measure changes in

resistance/susceptibility or the *M. oryzae* isolate used may have been too virulent to see minor differences in host response. Second, altered expression of other non-target chitinases may have compensated for the silencing of *Os02g39330*, and obscured any effect. Testing with other *M. oryzae* isolates and monitoring the expression of other chitinase genes, particularly those that co-localize with other disease resistance QTL, will address these possibilities. The roles other chitinase family members alone and in combination should be explored further.

Table 4.1 Primers used for silencing analysis in this study

TIGR locus Id	Primer Name	Ta	Amplicon(bp)	Primer Sequence (5'-3')
LOC_Os02g39330	T2-2.39330F	60	130	CAGCAACCTCACCTGCTAAT
	T2-2.39330R			ACTCCTATGCCGCAAACAAC
LOC_Os04g41680	4.41680F	59	750	TAACGCTGCCCACTCCTACT
	4.41680R			TCCGTACCAAACTCTTGACG
LOC_Os04g41620	4.41620F	58	550	CAAGAGCAACAAACAGTGGC
	4.41620R			CGCCCTAAGATAGAGTAACATCG

A

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ATCACAATTAAAGTCACAGCATAGTAACCAACCGAACCAATCATCAGCCATGGCGCGAAGGCTCTCGC
TGCTGGCCGTCGTGCTGGCGATGGTGGCGGCCGTGTCGGCGAGCACGGCGGCGGCGCAGAGCTGCGGG
TGCGCGTCGGACCAGTGCTGCAGCAAGTGGGGGTTCTGCGGCACCGGCAGCGACTACTGCGGCACGGG
GTGCCAGGCGGGCCCCTGCGACGTGCCGGCCACCAACGACGTGTCCGTGGCGAGCATCGTCACGCCGG
AGTTCTTCGCGGCGCTCGTCGCCCAGGCCGACGACGGCTGCGCCGCCAAGGGCTTCTACACCCGCGAC
GCCTTCCTCACCGCCGCCGGTGGCTACCCTTCCTTCGGCCGCACCGGCTCCGTCGACGACTCCAAGCG
CGAGATCGCCGCCTTCTTCGCCCACGCCAACCACGAGACCATAAGTAAGCAGAAACACCAAAAAAAAA
ACATACAGTATAATACTACCAGTAGAGACCACGGTATACCTAACATGACGATAACGTTGTTGCAGAGT
TCTGCTACATCGAGGAGATCGACGGACCGAGCAAGAACTACTGCGACGAGACGAGCACGCAGTGGCCG
TGCATGGCGGGGAAGGGGTACTACGGGCGCGGGCCGCTGCAGATCTCGTGGAACTTCAACTACGGGCC
GGCGGGGCAGAGCATCGGGTTCGACGGGCTGGGCGACCCGGACGCGGTGGCGAGGAGCCCCGTGCTGG
CGTTCCAGACGGCGCTCTGGTACTGGACCAACAACGTGCACGACGCCTTCGTCTCCGGCCAGGGGTTC
GGCGCCACCATCCGCGCCATCAACGGCGCGCTCGAGTGCGACGGCAAGAACCCCACCGCCGTCAGCAA
CCGCGTCGCCTACTACCAGCAGTTCTGCCAGCAGTTCGGCGTCGACCCCGGCAGCAACCTCACCTGCT
AATTACAGGGATTGACATATGCTATGTATGTGATTTTGTACGAATAATAATTGTCATAAGTAATCAAA
TAAAATCGTATTGCATTACGTCAACGTTGTTTGCGGCATAGGAGTATATATATTTCCGTGGACAGATT
TTCTTTGTCGTCTCAGTCAAATTTTGTGGCTCCTGAATATTTTCACGTGACTTGTTTGTTTGTTTGTT
GTGTTTTTGGATA
```

B

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atggcgcgaaggctctcgctgctggccgtcgtgctggcgatggtggcggccgtgtcggcg
 M  A  R  R  L  S  L  L  A  V  V  L  A  M  V  A  A  V  S  A
agcacggcggcggcgcagagctgcgggtgcgcgtcggaccagtgctgcagcaagtggggg
 S  T  A  A  A  Q  S  C  G  C  A  S  D  Q  C  C  S  K  W  G
ttctgcggcaccggcagcgactactgcggcacggggtgccaggcgggcccctgcgacgtg
 F  C  G  T  G  S  D  Y  C  G  T  G  C  Q  A  G  P  C  D  V
ccggccaccaacgacgtgtccgtggcgagcatcgtcacgccggagttcttcgcggcgctc
 P  A  T  N  D  V  S  V  A  S  I  V  T  P  E  F  F  A  A  L
gtcgcccaggccgacgacggctgcgccgccaagggcttctacacccgcgacgccttcctc
 V  A  Q  A  D  D  G  C  A  A  K  G  F  Y  T  R  D  A  F  L
accgccgccggtggctacccttccttcggccgcaccggctccgtcgacgactccaagcgc
 T  A  A  G  G  Y  P  S  F  G  R  T  G  S  V  D  D  S  K  R
gagatcgccgccttcttcgcccacgccaaccacgagaccataaagttctgctacatcgag
 E  I  A  A  F  F  A  H  A  N  H  E  T  I  K  F  C  Y  I  E
gagatcgacggaccgagcaagaactactgcgacgagacgagcacgcagtggccgtgcatg
 E  I  D  G  P  S  K  N  Y  C  D  E  T  S  T  Q  W  P  C  M
gcggggaaggggtactacgggcgcgggccgctgcagatctcgtggaacttcaactacggg
 A  G  K  G  Y  Y  G  R  G  P  L  Q  I  S  W  N  F  N  Y  G
ccggcggggcagagcatcgggttcgacgggctgggcgacccggacgcggtggcgaggagc
 P  A  G  Q  S  I  G  F  D  G  L  G  D  P  D  A  V  A  R  S
cccgtgctggcgttccagacggcgctctggtactggaccaacaacgtgcacgacgccttc
 P  V  L  A  F  Q  T  A  L  W  Y  W  T  N  N  V  H  D  A  F
gtctccggccaggggttcggcgccaccatccgcgccatcaacggcgcgctcgagtgcgac
 V  S  G  Q  G  F  G  A  T  I  R  A  I  N  G  A  L  E  C  D
ggcaagaaccccaccgccgtcagcaaccgcgtcgcctactaccagcagttctgccagcag
 G  K  N  P  T  A  V  S  N  R  V  A  Y  Y  Q  Q  F  C  Q  Q
ttcggcgtcgaccccggcagcaacctcacctgctaa
 F  G  V  D  P  G  S  N  L  T  C  -
```

C

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                                1,500                   1,520                   1,540
LOC_Os02g39330 ATCCGCGCCATCAACGGCGCGCTCGAGTGCGACGGCAAGAACCCCACCGC-------CGTCAGCAAC 60
LOC_Os04g41620 .....G..........T..........A....G.........GGT..-------.....A.GCA 60
LOC_Os04g41680 .....G..........T..........A.............GG...-------.....A.GCA 60
LOC_Os06g51060 .C.AA.AT..........GCG.G.....G.CA.GGCGCGGA..G.-------AGA...GC.G.. 60
LOC_Os06g51050 .C.AA.AT..........GTG.G.....GGCA.GGCGCGGA.GA.-------AAG..GGC.G.. 60
LOC_Os03g30470 .C.AA.AT..........G...G.....G.CATGGCG.GGA.GAT-------CG....GC.G.. 60
LOC_Os05g33130 .C.AA.AT.G........G...G.....GGCA.GGCCC.GA.GA.-------CG...GGCG... 60
LOC_Os05g33140 .C.AA.AT..........G.T.G.....GGTT.GGCCC.GA.GA.-------CGG..GGCG... 60
LOC_Os05g33150 .CGAA.AT.C...C.....G....C.....C....GGC...GG.GG.---------------GC.G.. 54
LOC_Os05g04690 .CGAA.AT......T....GA.........A..AA.CGC.C.GG.GG..ATCCCCGG..GGAGG.. 66
LOC_Os01g18400 .C.AA.AT.G........G..........A..---CG.GCAGG.GA..---CCCGG..G.A.... 60
LOC_Os03g04060 .C.AA.AT..........GTGAA.....G.AAGGGCT..T.....A---CAA..C..AGG... 63
LOC_Os10g39680 .C.AA.AT..........GCA.........G..T.GGCC.GAA.GA...-------.AA.GT.G.. 60
LOC_ Os10g39700 .C.AA.AT..........CGCT..........T.GGCC.GAA.GA...-------.AA.GT.G.. 60
LOC_Os08g41100 ..GAA.AT.C.GT.T....AT...ATC..T.GTCAAGGCTC.AT.GATA-------AGA.G.A.GT. 60
LOC_Os09g32080 ..GAA..TGC..T.....AC.AGATC....G.AAGGGCT..AT.GA..-------A.A.G.A.GT. 60
```

Figure 4.1 Gene structure and sequences of rice chitinase *Os02g39330*. (A) Sequence of rice chitinase *Os02g39330* on rice chromosome 2. The coding region (yellow part) is separated by a single intron. The letters in red font are primers used to amplify the region included in the RNAi silencing construct. The underlined letters are primers used for silencing analysis. (B) Nucleotide and amino acid sequence of rice chitinase *Os02g39330*. (C) Alignment of the most highly conserved region of the 239 bp fragment used for silencing with the most closely related rice chitinases. Dots indicate bases identical to the top sequences and dashes represent gaps in the sequences.

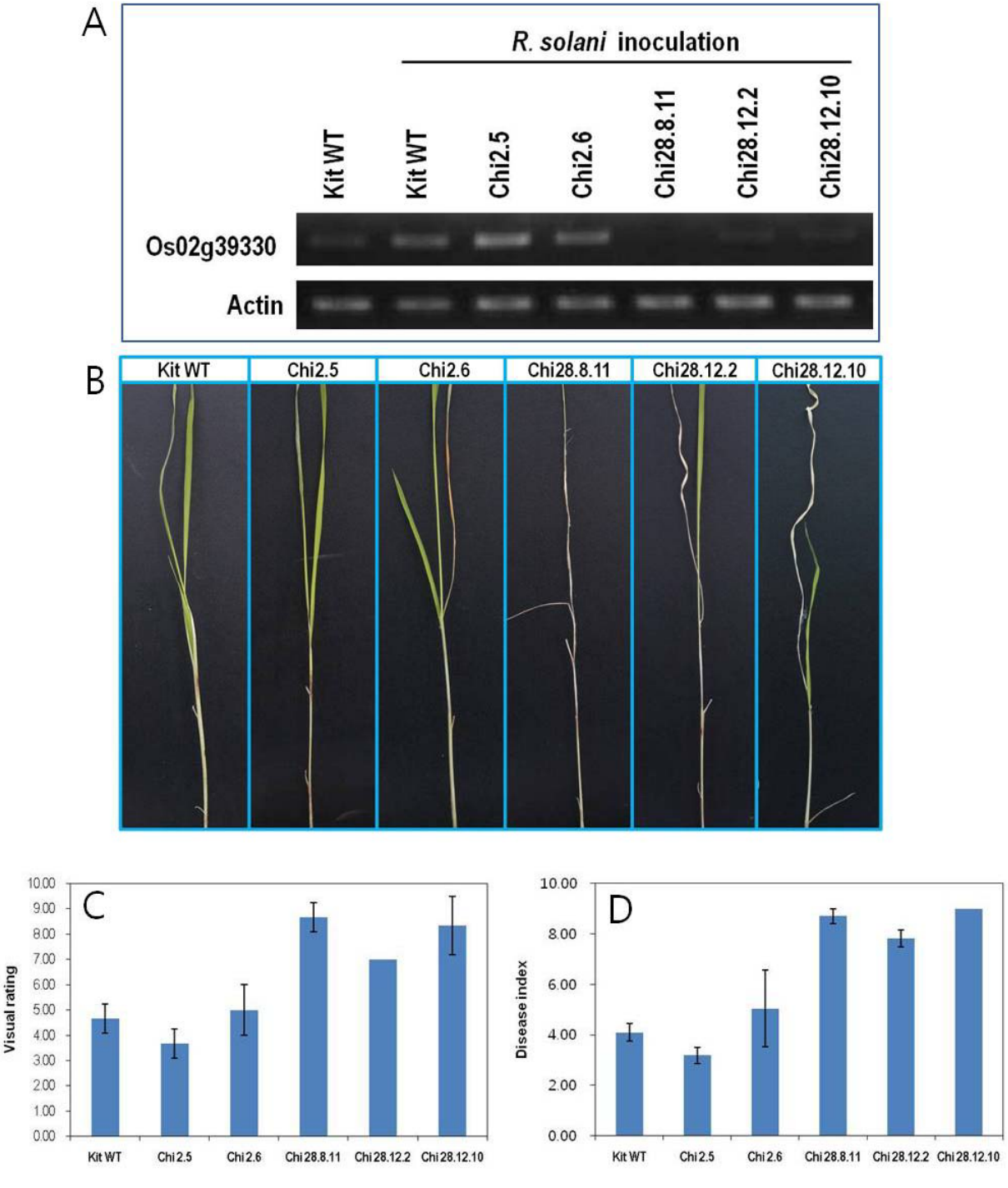
A
R. solani inoculation
Kit WT
Kit WT
Chi2.5
Chi2.6
Chi28.8.11
Chi28.12.2
Chi28.12.10
Os02g39330
Actin
B
Kit WT
Chi2.5
Chi2.6
Chi28.8.11
Chi28.12.2
Chi28.12.10
C
Visual rating
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Kit WT
Chi 2.5
Chi 2.6
Chi 28.8.11
Chi 28.12.2
Chi 28.12.10
D
Disease index
10.00
8.00
6.00
4.00
2.00
0.00
Kit WT
Chi 2.5
Chi 2.6
Chi 28.8.11
Chi 28.12.2
Chi 28.12.10

Figure 4.2 Silencing of *Os02g39330* and disease phenotypes. (A) Semi-quantitative RT-PCR data showing suppression of the expression of rice chitinase *Os02g39330* in T2 (Chi2.5, Chi2.6) and T3 (Chi28.8.11, Chi28.12.2, Chi28.12.10) generation transgenic plants harboring the silencing construct. First lane: *Os02g39330* expression in Wild type Kitaake (KitWT), not inoculated. Second lane: *Os02g39330* expression in KitWT in response to *R. solani* infection. Third to seventh lanes: Differential expression of *Os02g39330* chitinase in transformants in response to *R. solani* infection. RT-PCR amplification of an actin gene was used to demonstrate comparable mRNA concentrations. (B) Symptoms of disease caused by *R. solani*. KitWT and five transgenic rice lines (two T2, not silenced; three T3, silenced) were rated visually on a scale of 0-9. The dried rice parts were caused by the infection of *R. solani*. C, D) Visual rating and disease index for the sheath blight disease phenotype in KitWT and five RNAi transgenic lines. (C) Visual rating 0-9 scale, in this scale, visual index 0 indicates no lesion and 9 shows lesions covering all leaves and panicle. (D) Disease index. Disease index = (lesion length/ plant height) x 9. Three different plants were used to calculate the visual ratings and disease index. The means and standard deviations are shown.

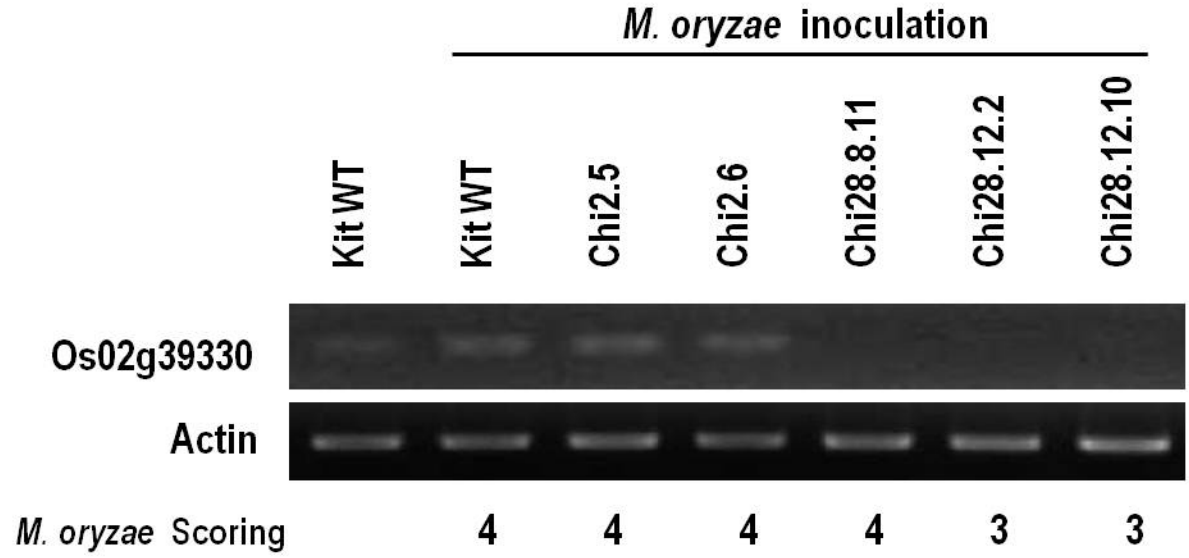

Figure 4.3 Semi-quantitative RT-PCR data showing expression of rice chitinase *Os02g39330* in Kitaake wild type (KitWT), T2 (Chi2.5, Chi2.6) and T3 (Chi28.8.11, Chi28.12.2, Chi28.12.10) transgenic lines containing the *Os02g39330* silencing construct. Plants in lanes 2-7 were inoculated with *M. oryzae.* The disease scores are shown below the gels; the ranking scale is 0-2: Resistant reaction, 3-4: Moderately susceptible, 5: Susceptible reaction.

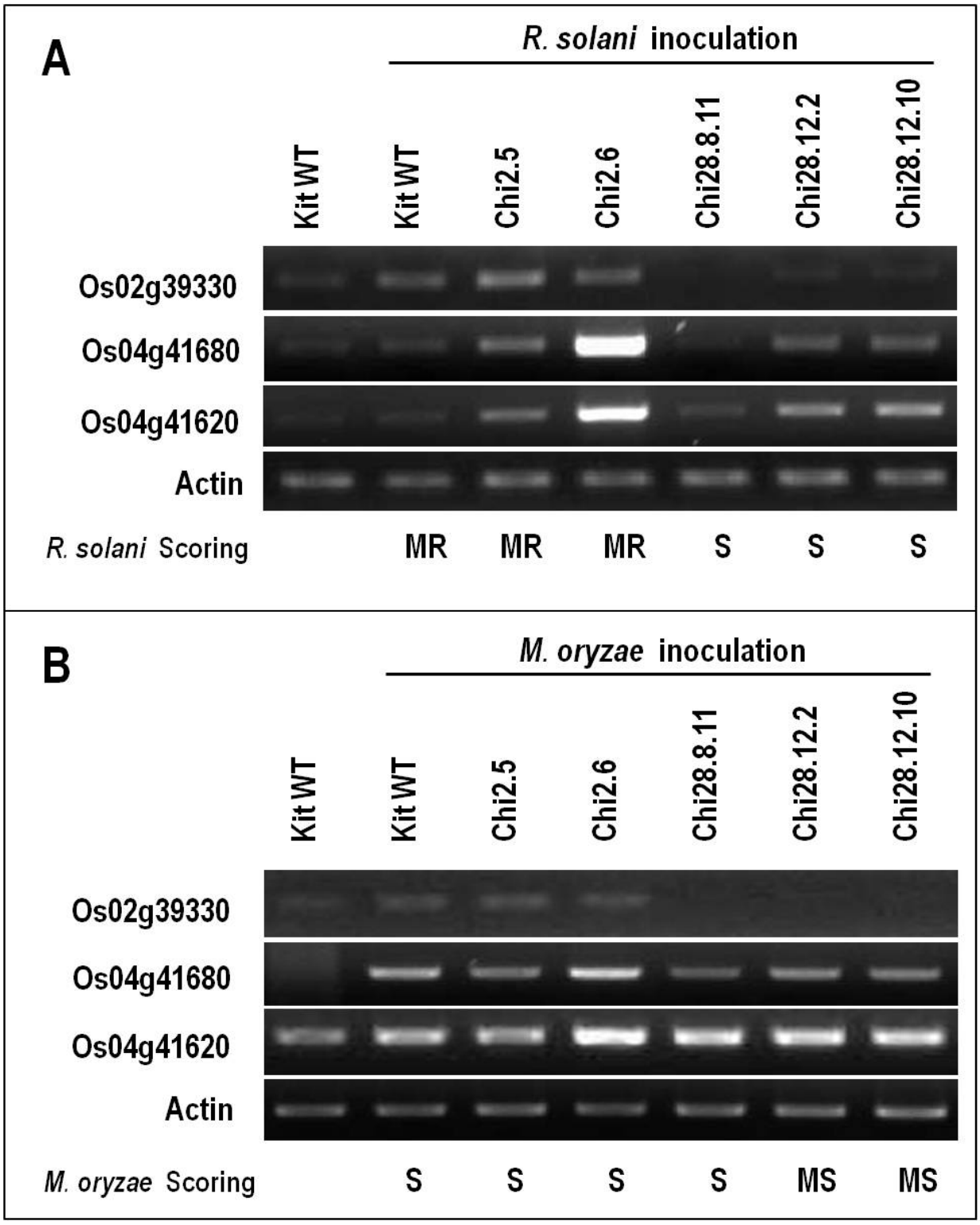
A
R. solani inoculation
Kit WT
Kit WT
Chi2.5
Chi2.6
Chi28.8.11
Chi28.12.2
Chi28.12.10
Os02g39330
Os04g41680
Os04g41620
Actin
R. solani Scoring
MR
MR
MR
S
S
S
B
M. oryzae inoculation
Kit WT
Kit WT
Chi2.5
Chi2.6
Chi28.8.11
Chi28.12.2
Chi28.12.10
Os02g39330
Os04g41680
Os04g41620
Actin
M. oryzae Scoring
S
S
S
S
MS
MS

Figure 4.4 Expression changes in chitinases *Os04g41620* and *Os04g41680* after infection by *R. solani* and *M. oryzae*. Expression of both genes is induced after inoculation of wild type Kitaake and transgenic lines expressing the *Os02g39330* silencing construct with (A) *R. solani* and (B) *M. oryzae*. *Os04g41620* and *Os04g41680* are slightly silenced in the induced T3 lines, but the effect of silencing is much less relative to *Os2g39330.* The disease scores represent resistant reaction (R), Moderately resistant (MR), Moderately susceptible (MS), and Susceptible reaction (S).

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