THESIS

ROLE OF *ORYZA SATIVA* CHITINASES IN DISEASE RESISTANCE

Submitted by

Jacob Snelling

Department: Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2010

COLORADO STATE UNIVERSITY

April 5, 2010

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JACOB SNELLING ENTITLED ROLE OF *ORYZA SATIVA CHITINASES IN DISEASE RESISTANCE* BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

_____
Stephen Chisholm

_____
Nora Lapitan

_____
Advisor: Jan Leach

_____
Department Head: Thomas Holzer

ABSTRACT OF THESIS

ROLE OF *ORYZA SATIVA* CHITINASES IN DEFENSE RESPONSE

Plant chitinases have long been implicated in defense responses against invading pests and pathogens. In *Oryza sativa* cv. Nipponbare (rice), chitinases have been shown to co-localize with disease resistance QTL, and are thought to contribute to a multigenic basal defense response. Previous work has shown that overexpression of several *O. sativa* class I chitinases resulted in heightened resistance to the fungal pathogens *Rhizoctonia solani* and *Magnaporthe oryzae* pv. *oryzae.* Here, I examined the responses of several *O. sativa* chitinase classes to the RNAi silencing of two transcriptionally active rice chitinase genes LOC_Os02g39330 and LOC_Os04g41620. Silencing of these genes results in a reduction of expression in several additional rice chitinases, and also shows an increased susceptibility phenotype to fungal rice pathogens *R. solani, M. oryzae* pv. *oryzae,* and bacterial rice pathogen *Xanthomonas oryzae* pv *oryzae*. The relative amounts of silencing of the individual genes LOC_Os02g39330 or LOC_Os04g41620 were not significantly correlated with disease phenotype. Thus, we conclude that silencing of these target genes altered the expression of other chitinases, and perhaps other defense response

genes that were not assayed, and that the combination of altered expression profiles contributed to increased susceptibility to the various pathogens.

Jacob Wayne Snelling
Bioagricultural Sciences and Pest Management
Colorado State University
Fort Collins, CO 80523
Spring 2010

TABLE OF CONTENTS

TABLE LEGEND

Table 1. Chitinase genes used for nomenclature in this study.

Table 2. Primers used for analysis in this study.

Table 3. Expression trends of family 18 (A) and 19 (B) glycosyl hydrolases in response to *M. oryzae and X. oryzae pv. oryzae* in susceptible and resistant interactions. Data are summarized from MPSS (Nakano et al. 2006) database (http://**mpss**.udel.edu/**rice**/). ***LOC_OsXXgXXXXX*** in bold and italic were examined in this study.

FIGURE LEGEND

Figure 1. Estimated location of rice defense response and plant height QTL in 27 mapping studies (Alam and Cohen 1998; Albar et al. 1998; Chen et al. 2003; Courtois et al. 2003; Cui et al. 2008; Fukuoka and Okuno 2001; Han et al. 2002; Hemamalini, Shashidhar, and Hittalmani 2000; Hittalmani et al. 2002; Huang et al. 2001; Li et al. 1995; Li et al. 1999; Liu et al. 2004; Liu et al. 2009; MacMillan et al. 2006; Maheswaran et al. 2000; Miyamoto, Yano, and Hirasawa 2001; Pinson, Capdevielle, and Oard 2005; Ramalingam et al. 2003; Sirithunya et al. 2002; Srinjvaschary et al. 2002; Tabien et al. 2002; Wang et al. 1994; Wu et al. 2004; Xu et al. 2004; Zenbayashi et al. 2002; Zou et al. 2000). Glycosyl hydrolase family 18 and 19 genes with chitinase or chitinase-like structures often show co-localization with regions of the genome strongly linked to defense response and plant height QTL.

Figure 2. Relationships among Family 19 chitinase genes from *Oryza sativa* (LOC_OsXXgXXXXXX), *A. thaliana* (ATXGXXXXX, ARATH), *S. bicolor* (SORBI), *T. aestivum* (WHEAT), *P. trichocarpa* (POPTR), and *O. grandiglumis* (ORYGR) reconstructed using Bayesian analysis of amino acid sequences. Posterior probabilities (scaled to 100) are indicated at nodes.

Figure 3. (A) Os02g39330 gene structure. Silencing construct location and sequence (1), chitinase catalytic domain molecular patterns (2, Prosite-PS00774) (3, PS00773), and chitin binding molecular pattern (4, PS00026). Amino acid residues in bold are highly conserved in the chitin binding domain or catalytic domain. Residues in *red italic* show sequence variations from the molecular

pattern standard. (B) Alignment of family 19 glycosyl hydrolase chitinases over the most conserved 60 bp of the 239 bp region selected for silencing. Dots represent consensus sequence to Os0239330; grey areas represent gaps in the alignment.

Figure 4. Silencing of Os02g39330 compared to wild type control Kitaake (Kit) in T0 (A) lines, and silencing of Os02g39330 and Os04g41620 compared to wild type control Kitaake (Kit) in T1 (B) and T2 (C) lines.

Figure 5. Representative photos of the range of disease phenotypes and corresponding numeric values, on a scale of 0-9, of disease indexes (DI = (lesion length/plant height)*9) in a subset of R. solani inoculated T2 chitinase transgenic and wild type plants. (*) indicates Kitaake wild type plant and DI.

Figure 6. Population distribution of disease phenotypes in T1 (A, C, E) and T2 (B, D) lines relative to wild type control. Transgenic and wild type plants were inoculated with *R. solani* (A, B), *M. oryzae* isolate P06-6 (C), *M. oryzae* isolate KV-1 (D), and *X. oryzae* pv. *oryzae* (E).

Figure 7. Reduced chitinase expression in selected T2 silenced and wild type individuals. Gel bands in each column show variable expression of other rice chitinase genes in the family 18 and 19 glycosyl hydrolases can been seen among transgenic individuals.

Figure 8. Linear regressions of Os02g39330 (A) and Os04g41620 (B) mRNA expression values versus *R. solani* disease index show that disease phenotype does not correlate with silencing in transgenic individuals.

Figure 9. Sheath blight disease (visual index) and plant height (cm) are negatively correlated in T1 (n = 153) and T2 (n = 65) transgenic populations. As plant height increases in the T1 and T2 chitinase silenced lines, disease severity decreases.

Chapter I. Background

1.1 Plant Pathology, an evolving field

The field of plant pathology is entering an era of renaissance. Traditional techniques to characterize disease identity and interactions between plant and pathogen are being increasingly complemented by newly emerging genomic tools that are both powerful and efficient. Complete genome sequencing of *Homo sapiens*, as well as plant model species *Oryza sativa* (rice), and *Arabidopsis thaliana,* have been instrumental in providing new insights to interpret the volumes of genomic information generated by the sequencing process. Identification of the thousands of genes present in a genome, and their function, is only the first step in developing a greater understanding of the biochemical pathways an organism develops in response to its requirements for life, as well as the pathways activated in response to environmental stresses. As new technology integrates into current analytical techniques, the opportunity to look at host-pathogen relations with new perspective is inevitable.

1.2 Rice's global impact

O. sativa is one of the most important agronomic crops worldwide. Close to half the population of the world consumes rice on a daily basis (Cantrell and Reeves 2002; IRRI 2007), making the grain extremely important economically, nutritionally, and scientifically. With ever increasing demand, reliable and sustainable production of rice crops is imperative to feed the world's growing population. *O. sativa* cultivation, over a large range of ecosystems, requires

careful consideration with regard to pest and pathogen management. Yield losses of up to 5% annually (Evenson, Herdt, and Hossain 1998) are attributed to rice pathogens, which significantly reduce global yield each year. Traditional breeding practices have successfully curbed disease impact in resistant rice for decades, but pathogens are constantly reorganizing genetic material during replication, and as a result of selection, eventually overcome incorporated resistance (McDonald and Linde 2002). Understanding genetic interactions between pathogen and host is important both in breeding practices, as well as contributing to the overall understanding of physiological relations between two organisms.

1.3 Rice as a valuable tool for studying plant-pathogen interactions

The fully sequenced genomes of *O. sativa* L. spp. *japonica* cv. Nipponbare, along with its genetically distant relative *O. sativa* L. spp. *indica* cv. 93-11 have opened doors to a wealth of genomic information. The information from the genome project, as well as in previous genetic studies, has given researchers fantastic resources to utilize in breeding practices, disease study, and functional physiology. A caveat to these obtained genomes is the immense diversity within even closely related cultivars of *O. sativa*. Isolating single genes of interest can be useful for short term resistance to specific pathogens, but obtaining sustained resistance to a multitude pathogens may require a suite of genes interacting with one another, in varying contribution, to an overall broad-spectrum defense. Multiple genes, contributing additive effects, are known as quantitative trait loci (QTL) (Liu et al. 2004; Wisser et al. 2005).

1.4 QTL governed resistance

Mapping of QTL has long been the standard for identification of genomic areas of putative multigenic importance. Advancing with the evolution of Sanger sequencing, and newer high-throughput techniques of sequencing, map based cloning has allowed for rudimentary identification of active regions in the genome. QTL are related to many complex plant processes (Hu et al. 2008). For example, QTL are often associated with developmental regulation, defense response, and an assortment of other functions in plants. Identification of disease related QTL has piqued the interest of molecular plant pathologists and breeders alike.

Recent characterizations of genes underlying QTL associated with durable, broad spectrum resistance are providing insights into the complex layers of durable plant resistance. In rice, several disease resistance QTL have been shown to co-localize with defense response (DR) genes (Liu et al. 2004; Ramalingam et al. 2003; Wisser et al. 2005). This supported the hypothesis that these DR genes might be candidates for the function conferred by the QTL. Indeed, using the candidate DR genes as markers, Liu et al. (Liu et al. 2004) demonstrated that accumulation of five different genomic regions associated with QTL into rice varieties provided enhanced resistance to rice blast disease.

Current understanding of gene structure and genomic interactions allow for the manipulation of single gene inheritance. This type of manipulation with respect to disease resistance is associated with qualitative resistance genes (R-gene), or R-gene mediated responses (Wisser et al. 2005). Unfortunately, traits

that are engineered into crops in this manner often allow for only brief periods of resistance to particular pathogens (Liu et al. 2004). It cannot be said that R-gene mediated response does not contribute to the multigenic disease resistance QTL disease resistance QTL (Ramalingam et al. 2003). Conversely, the R-gene alone contributes to a part of the whole defense response mechanism relating to a specific pathogen, or may even have pleiotropic effects. But without the proper timing of the response, as well as the additive contribution of specific genes, offering support in concert to the R-gene, the durability of the resistance is eroded and eventually overcome. A goal for understanding disease resistance QTL is to overcome these time and pathogen specificity barriers.

Using a post-transcriptional RNAi silencing approach to examine gene expression and its correlation with loss of resistance to major rice pathogens, Manosalva et al. (2009) demonstrated that one family of candidate genes, the rice germin like proteins (*OsGLP*) underlying a blast disease resistance QTL, contributed to resistance to the rice blast fungus, *Magnaporthe oryzae*. In addition to the *OsGLP,* candidate oxalate oxidase genes (*OsOXO*) underlying a blast disease resistance QTL on chromosome 3 were also shown to contribute partial broad spectrum fungal disease resistance in rice (Carillo et al., 2009; Davidson, personal communication). The successes in functionally validating *OsGLP* and *OsOXO* gene contribution in disease resistance using RNAi silencing confirm the utility of this approach for assessing the contribution of other DR genes that co-localize with disease resistance QTL.

The type of durable, broad-spectrum resistance that will be useful for agriculture will likely be dependent on the particular pathogen, as different pathogens employ different mechanisms for host entry and host defense repression (Huckelhoven 2007; Ridley, O'Neill, and Mohnen 2001). Collection of data in recent years has successfully identified regions of the rice genome responsible for disease resistance to several major rice pathogens (Liu et al. 2004; Ramalingam et al. 2003; Wisser et al. 2005). Of particular interest to this research, several regions within rice mapping populations have been identified to contribute to resistance to different types of fungal pathogens, such as rice blast (*Magnaporthe oryzae*), and sheath blight (*Rhizoctonia solani* Kuhn), as well as the bacterial pathogen bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) (Liu et al. 2004; Ramalingam et al. 2003). In some rice lines, disease resistance QTL to the aforementioned rice pathogens overlap, and therefore have particular appeal for their broad-spectrum resistance potential. However, studies indicate that QTL need not overlap to physical regions of the genome to act in concert (Liu et al. 2004). Furthermore, it has been shown, utilizing specific recombinant inbred and advanced backcross populations of rice, that pyramiding disease resistance QTL may contribute additively to overall blast resistance within the manipulated line (Liu et al. 2004). Identification of significantly contributing genes to the overall QTL effect is essential for future breeding strategies.

1.5 Plant defense responses

The molecular bases for durable, broad-spectrum resistances are not yet well understood. Preformed defenses may contribute to such resistances, and

would be a first layer of defense encountered by pathogens. Common features of preformed defenses include enhanced physical features such as trichome size, shape, and abundance, or thicker cuticles; these modifications offer complex physical barriers to pathogens (Shepherd and Wagner 2007). Preformed defenses also include constitutively produced antimicrobial proteins and secondary metabolites that are secreted to the apoplast, or may be secreted by glandular trichomes. Such antimicrobial agents can be transported actively, or passively, to the leaf surface via natural plant openings, such as hydathodes, stomata, and trichomes, or into the rhizosphere (Perry et al. 2007; Shepherd and Wagner 2007).

A second layer of defense, also thought to contribute to durable, broad-spectrum resistance, is known as basal resistance. Basal resistance is largely elicitor mediated, requiring specific components of potentially pathogenic microbes, or elicitors of plant origin, which are released during the initial penetration of microbes utilizing cell wall degrading enzymes, to be perceived by the plant (Ma and Berkowitz 2007). The microbial generated elicitors are pathogen-associated molecular patterns (PAMPs), which include a wide variety of sources, e.g., oligosaccarides, glycoproteins, lipids, and (poly)peptides, that are microbe-specific features required for proper physiological function of the microbe (Chisholm et al. 2006; Nürnberger et al. 2004). This perception system is also known as PAMP-triggered immunity (PTI). Elicitor signals are perceived by pattern recognition receptors (PRR), which are largely plasma membrane bound, leucine-rich repeat receptor kinases (LRR-RK) thought to bind to the

elicitor ligand (Boller and He 2009), or in the case of fungal chitin elicitors, are membrane-bound proteins with peptidoglycan-binding LysM domains (Panstruga, Parker, and Schulze-Lefert 2009; Wan et al. 2008). Perception of PAMP components by PRR results in the activation of specific mitogen-activated protein kinase (MAPK) signal transduction cascades, which in turn convey the signal to the nucleus through WRKY transcription factors, and lead to increased production of perception components, activation or suppression of defense response genes, cell wall reinforcement at the infection site by callose deposition, and the induction of reactive oxygen species (Chisholm et al. 2006; Nürnberger et al. 2004; Zhao and Qi 2008). Along with perception of PAMP components, fluctuations in biochemical components across the plasma membrane, such as Ca^{2+} signaling, are also thought to be early components to the defense response and critical for induction of several downstream defense responses (Ma and Berkowitz 2007; Ma et al. 2009).

Plants utilize three hormone responsive signaling pathways, salicylic acid (SA), jasmonic acid (JA), and gaseous ethylene (ET), to mediate different reactions specific to wounding, herbivore attack, and microbial interactions (Panstruga, Parker, and Schulze-Lefert 2009). In microbial defense response, the pathways can be broken into responses against biotrophic (SA mediated) or necrotrophic (JA and/or ET mediated) pathogens (Glazebrook 2005). Basal defense responses use these pathways to tailor the response to a given stimulus. There is a tremendous amount of complex cross-talk between these pathways (Panstruga, Parker, and Schulze-Lefert 2009). Pathogens have

evolved to take advantage of these complex signaling systems, and a third layer of defense, intrinsically woven into the basal defense response, has co-evolved to meet phytopathogen adaptations to broad-spectrum defenses; this response is known as effector triggered immunity (ETI) (Postel and Kemmerling 2009). ETI can be broad-spectrum, but requires specific host/pathogen interactions with microbial derived avirulence effector proteins (Avr-proteins) and corresponding plant produced resistance proteins (R-proteins), which are another type of pattern recognition receptor, to trigger ETI (Chisholm et al. 2006; Jones and Dangl 2006; Postel and Kemmerling 2009). Therefore, ETI may be specific to species that have an intimate evolutionary history. Natural selection rewards those organisms most suited to their environment, effectors are pathogen produced transcription products, or chemicals, that increase the pathogen's fitness in its environment, and thus increase the microbe's success in susceptible host environments (Boller and He 2009). Effectors can be delivered by bacterial type III secretion systems, or fungal haustoria, to the apoplastic space between the plant's cell wall and plasma membrane (Chisholm et al. 2006; Zhao and Qi 2008). Effectors then target components of the PTI directly by enzymatic activity (effector proteins can be activated after delivery by prokaryotic chaperones or even endogenous plant enzymes) on defense response signaling components, or by targeting the plant's hormonal defense response system using chemical homologs of plant hormones, such as coronatine (a jasmonic acid mimic), to interrupt a specific response and effectively shut down PTI mediated resistance (Boller and He 2009). Plants have co-evolved mechanisms to directly recognize

effectors by physical gene product interactions, or indirectly, by surveillance of plant components that the effector targets within the plant cell (Chisholm et al. 2006). Upon effector recognition, the plant reengages defense response pathways, which results in an amplified response to the pathogen (Jones and Dangl 2006). Characteristic ETI responses include the rapid induction of reactive oxygen species and downstream defense response genes, and are often followed by programmed host cell death (PCD) at the site of pathogen invasion(Ma and Berkowitz 2007). PCD is widely thought to release chemical and biologically active components which create a hostile environment for the pathogen, thus inhibiting further invasion of the host (Ma and Berkowitz 2007).

Comprehensive understanding of the complex relationships between preformed defenses, PTI, ETI, and the overarching defense signaling response pathways, is only now becoming a reality. Improvements in database curation, genome sequencing and annotation, and quantitative functional characterization of genes and their products, open the subject to a much higher resolution picture of plant defense responses. The research presented in this thesis is centric about understanding components of basal responses in the plant/pathogen system of interactions.

1.6 Chitinase in Plant Defense Responses

Chitin is a linear polysaccharide composed of β-1,4- linked *N*-acteylglucosamine residues, and is a structural component to both fungal cell walls and insect exoskeletons (Neuhaus 1999; Zhu et al. 2009). Plant chitinases, which are thought to act both directly and indirectly in plant defenses, as well as

in plant physiological functions, are placed into two very distinct families of glycosyl hydrolases (families 18 &19). Chitinases hydrolyze the β-1,4 linkages between *N*-acetylglucosamine residues of chitooligosaccarides through either a substrate-assisted (family 18) or acid (family 19) catalysis (Kasprezewska 2003). Plant chitinases show a range in size from 27-34 kD and many isoforms in plant tissues have been observed.

Chitinases have been implicated in defense response research for well over 45 years (Young and Pegg 1982). Early research centered around crude plant exudates showing an inhibitory effect on fungal growth and the ability of those exudates to degrade chitin preparations. As research methods evolved, chitinases purified from crude plant extracts, or from transgenic plants where chitinase genes were overexpressed, were shown to retain their antifungal activity, especially in combination with another pathogen-induced enzyme that attacks glucan components of the fungal cell wall, 1,3-β-glucanase (Mauch, Mauch-Mani, and Boller 1988; Young and Pegg 1982; Zhu et al. 1994).

The last 20 years of chitinase research has given rise to many intriguing plant/pathogen chitinase interactions. Genetic and biochemical techniques have allowed researchers to identify chitinase activity associated with not only defense responses, but an overwhelming number of plant stresses and developmental activities. For example, exogenous stresses such as wounding, cold, high salinity, UV radiation, and ozone have been shown to increase chitinase transcription (for review see (Collinge et al. 1993; Kasprezewska 2003; Passarinho and de Vries 2009)). Chitinase activities increase during

developmental activities such as root nodulation, flowering, embryogenesis, seed storage, fruit ripening, signal degradation or formation, and plant growth (for review see (Collinge et al. 1993; Kasprezewska 2003; Passarinho and de Vries 2009)). Some chitinases have demonstrated sensitivity to defense response hormonal regulation of ET and JA (Boller 1988; Boller et al. 1983; Robinson, Jacobs, and Dry 1997; Zhao and Chye 1999), but many chitinases have not.

Early research showed low constitutive expression of chitinases in healthy plants. The enzymes are induced to much higher levels upon infection or wounding (Boller 1988). These observations lead to the hypothesis that higher constitutive chitinase activity could increase resistance to pathogen invasion. Transgenic overexpression lines, using family 19 chitinases from bean, tobacco, and rice confirmed that higher constitutive expression of some chitinases indeed does contribute to increased fungal resistance (Broglie et al. 1991; Datta et al. 2001; Lin et al. 1995; Nishizawa et al. 1999; Vierheilig et al. 1993).

Many chitinases are developmentally regulated in different tissues, at different times, and many may only show induced expression under certain circumstances. It is widely hypothesized that different chitinases involved in perception and induced direct defense against invading pathogens may also share a dual role with plant physiological function (Collinge et al. 1993; Hanfrey, Fife, and Buchanan-Wollaston 1996; Kasprezewska 2003; Minic 2008). Many chitinases, while having similar structure to defense responsive chitinases, do not show antifungal activity and are therefore attributed to either plant physiological or novel functions. In rice, chitinases have been shown to co-localize with

disease resistance QTL (Figure 2 in research chapter 2, also (Liu et al. 2004). Many rice chitinases are constitutively active in root, shoot, leaf, and meristem tissues (Nakazaki et al. 2006). The timing and relative abundance of constitutive and induced expression of chitinases correlates with resistant interactions in *R. solani* inoculated cultivars (Shrestha et al. 2008). These studies suggest that chitinases are important to basal defense responses in rice.

1.7 Chitin perception as a PAMP

PAMPs are critical to a microbe's functionality. For example, flagellin monomers, the basic building blocks of flagellum, are required for bacterial motility. Similarly, chitin oligosaccharides, which are integral to most fungal cell wall formation and expansion, are recognized by plant plasma membrane receptors as foreign (Chisholm et al. 2006; Nürnberger et al. 2004). Recent identification of CeBip (NCBI accession no. AB206975), a chitin oligosaccharide elicitor-binding protein containing two extracellular LysM motifs, although lacking in cytoplasmic signaling components, confirmed the presence of such chitin elicitor related plasma membrane receptors in rice (Kaku et al. 2006). Related studies in *Arabidopsis thaliana* independently identified a LysM receptor-like kinase; LysM RLK (also known as CERK1), which possesses an intercellular kinase domain that is a putative complementary component to the chitin oligosaccharide perception-signaling complex. LysM associates with CeBip-like proteins, and is absolutely necessary for perception and response of chitin oligosaccharide elicitors (Miya et al. 2007; Wan et al. 2008). Identification of specific chitinases, generating elicitor signal components, is important to

understanding chitin recognition and its role in basal defense response, as well as integral to breeding research aimed at heightening plant defense perception interactions.

1.8 Chitinase Classification

Chitinases are identified by a fairly complex nomenclature that includes reference to their classification as pathogenesis-related (PR) proteins, glycosyl hydrolase family members, chitinase classification, and gene family name (Neuhaus 1999). Nomenclature for rice chitinases examined in this study conforms to this classification system (Figure 3). Chitinases are represented by four PR protein designations of PR-3, PR-4, PR-8, and PR-11.PR-4 chitinases were not examined in this study due to their apparent lack of glycosyl hydrolase activity (Neuhaus 1999). PR-3 chitinases are made up solely of family 19 glycosyl hydrolases, which are plant-specific. These chitinases are further divided into classes relating to their chitin binding domain abundance and structure, and their catalytic domain structure. Classes I and II share catalytic domain homology and differ by the presence (class I) or absence (class II) of the chitin binding domain. Classes IV and VII are also differentiated by the presence (class IV) or absence (class VII) of the chitin binding domain. However, several deletions in their homologous catalytic region, and the chitin binding domain of class IV chitinases, identify these chitinases as unique from classes I and II. Class V and VI chitinases are unique in their chitin binding domains. Class V contains two chitin binding domains, and class VI, not observed in the *O. sativa* L. spp. *japonica* cv. Nipponbare genome in this study, contains half a chitin

binding domain followed by an extremely long proline hinge sequence. Genes in the PR-3 class are given the designation *Chia(1-7)* corresponding with their chitinase class. For example, in rice, a class IV chitinase would be *OsChia4*. Specific characterization of multiple class IV chitinases would lead to an additional character behind the numerical value, e.g. *OsChia4a, OsChia4b, OsChia4c.* PR-8 and PR-11 chitinases are family 18 glycosyl hydrolases, each of these PR class chitinases are also assigned a chitinase class, but only a single designation for each, class III and class I respectively. The PR-8 chitinases were given the chitinase class III designation because the class was very well established in earlier literature, so to avoid confusion was carried through to the latest nomenclature (Neuhaus 1999). Genes in the PR-8 and PR-11 classes are given the designation *Chib1* and *Chic1* respectively.

1.9 RNAi silencing, a powerful tool for examining gene contribution to defense response

RNA interference (RNAi) has been shown to be a very effective tool in assessing a genes function. Initially observed in plants, post-transcriptional modification of gene expression was originally thought to be a novel process by which plants were able to defeat viral presence within the host (Baulcombe 2004). While this theory is still upheld, recent data indicate a much broader function to the silencing machinery working in plant cells. It is thought that short sequence, double stranded RNAs are actually contributing to a very complex network of transcript identification, enzymatic modification, and eventual recycling of cellular components. These interactions can be taken advantage of

with careful planning. While the mechanisms of detection still elude researchers, RNAi is triggered by the presence of overabundant double-stranded RNA transcripts, which are complementary to the mRNA transcript being over-expressed. Small 20-22 bp sequences are cleaved from the double stranded RNA by ribonuclease III Dicer (Baulcombe 2004; Sontheimer 2005). The small sequence is then further degraded into single stranded RNA, which in turn forms a duplex with specific mRNA transcripts underway to translational sites. The duplex is once again identified and targeted for degradation in the RISC complex (Hannon 2002). The continual regeneration of 20-22 bp sequences effectively interrupts the mRNA message.

To use RNAi effectively one must have the mRNA sequence of the target gene in question. The idea is to essentially create your own constitutive double-stranded RNA that will be processed by Dicer and then silence your mRNA of interest. A process utilizing short hairpin RNA (shRNA) to generate small interfering RNA (siRNA) is currently utilized by scientists to silence genes of interest (Meister and Tuschl 2004). The sequence of interest is inserted into a vector plasmid and eventually transformed into the plant genomic DNA. The vector contains several known enzymatic splicing sites, as well as a region of the target sequence that contains both the sense and antisense directional sequence; the sense and antisense are linked by a sequence that will allow for a hairpin fold so that the complex may anneal and form a double-stranded RNA (Meister and Tuschl 2004). The successful vector is inserted into *Escherichia coli* for selection against antibiotic resistance**.** Colonies passing the selection

screen are once again manipulated into a second vector, which can then be transformed into tissue culture calli, for example, by *Agrobacterium tumefaciens*-mediated transformation. Plants grown from tissue culture can then be screened for the presence of a separate constitutive promoter that was also inserted from the vector into the DNA for insertion recognition. If there is a phenotype associated with silencing, this can also be a use tool for silencing confirmation.

RNAi silencing of a specific gene, relating to resistance against a particular pathogen, may often contribute to an adverse response in resistant levels against a different pathogen. Silencing may also activate genes that would not normally be responsive when the silenced gene was functioning properly, also contributing to the altered response to the pathogen. By comparing susceptibility among the different disease phenotypes, one may be able to judge if contribution of the individual genes additively enhances resistance/susceptibility to a particular pathogen. This approach was taken, in the research component of this thesis, to examine the effects of silencing chitinase genes in rice.

1.10 *Magnaporthe oryzae*

M. oryzae is a hemibiotrophic ascomyceteous fungus affecting rice and several other grass species (Wilson and Talbot 2009). The fungus begins its disease cycle as sympodial conidia; the mechanism for conidia dispersal is still hypothetical, but is thought to result from increased turgor pressure on a small stalk cell at the base of the conidium until it ruptures, thereby launching the conidium to new hosts (Kankanala, Czymmek, and Valent 2007). Nature helps

this process in times of high wind and rain, providing transport to distant new hosts. Spore germination is optimal during times of high humidity and darkness (Ebbole 2007). While it has been shown that *M. oryzae* can infiltrate rice's root system, infection primarily results from conidia landing on leaves and mechanically forcing its way into the inner leaf tissue (Ebbole 2007). The mechanism by which the leaf is penetrated results from the formation of a high pressure, melanized appressorium; the appressorium works in tandem with the *M. oryzae*'s penetration peg to rupture outer cuticle layers and the rice cell wall (Kankanala, Czymmek, and Valent 2007; Wilson and Talbot 2009). The fungus spreads cell to cell by means of infectious hyphae through the clustered pit field plasmodesmata (Kankanala, Czymmek, and Valent 2007). Cells remain alive throughout the growth process and only succumb when hyphal presence within the cell becomes too great (Kankanala, Czymmek, and Valent 2007). The fungus releases several toxic compounds and cell wall degrading enzymes as it spreads through the plant, but there is no obvious transition that differentiates biotrophic hyphae from necrotrophic hyphae as plant cells begin to die (Ebbole 2007). As disease overwhelms the plant, the fungal reproductive cycle begins anew. Understanding multigenic resistance to blast will be integral to combating this resourceful fungus. Chitin is an integral component to the *M. oryzae* cell wall, and is thus potentially interactive with plant chitinases during penetration and invasion of the plant. A greater understanding of individual chitinases and their influence on *M. oryzae* resistance in rice is needed to examine each chitinases' contribution to basal defense response.

1.11 *Rhizoctonia solani*

R. solani is a necrotrophic basidiomycete affecting a wide geography of rice cultivation, as well as several other species of cultivated crops (IRRI 2003; Pinson, Capdevielle, and Oard 2005). Most commonly, *R. solani* uses asexual sclerotia to spread short distances (IRRI 2003). Sclerotia are modified masses of hyphae that develop during stressful periods in the lifecycle. The hyphal mass is surrounded by a type of rind consisting of thickened, melanized hyphae drained of their cytoplasmic contents. The sclerotia drop to the soil in a state of dormancy until proper conditions allow the mycelium to again infect plant tissue. *R. solani* sclerotia require proper temperature and extremely high humidity (96-100%) to break their dormant state (Eizenga 2002). Growth requirements are often met during pretreatment of rice fields, when the land is submerged in water. Infection most commonly occurs at soil or water level when hyphae come in contact with the base of the rice plant and swiftly grow up the surface of the plant (IRRI 2003; Pinson, Capdevielle, and Oard 2005). As the disease spreads, a different type of hyphae begins to infiltrate the cuticle and stomatal slits present in the sheath and eventually the leaves (IRRI 2003). Penetration is facilitated by an appressorium-penetration peg complex. Infection, under optimal conditions, is extremely detrimental to seed fill and formation, which results in high yield loss (Pinson, Capdevielle, and Oard 2005).

Thus far, researchers have identified rice varieties only with partial resistance to *R. solani*. It is hoped that the elements of partial resistance can be identified, and then exploited, for a more effective resistance to the pathogen.

Mapping studies using pure-breeding recombinant inbred line studies have identified QTL co-localizing with resistance to *R.solani* (Li et al. 1995; Pinson, Capdevielle, and Oard 2005; Zou et al. 2000) Chitinases are likely direct interactors with *R. solani* in the early and late infection process. With an apparent lack of R-protein mediated resistance in rice, identification of genes important to the basal defense response, such as chitinases, are likely going to be the key to increasing resistance to this aggressive fungus.

1.12 *Xanthomonas oryzae* pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is one of the most detrimental bacterial pathogens affecting modern rice production. *Xoo* is a gram-negative, rod-shaped bacterium occurring throughout a wide range of cultivated regions throughout the world (Gnanamanickam et al. 1999). The bacterium is motile by means of a polar flagellum, and gains access to the plant via natural openings; the primary entrances are hydathodes, fresh wounds, and stomata (EPPO 1990; Gnanamanickam et al. 1999). *Xoo* is largely disseminated by means of bacterial ooze dripping into irrigation channels that then carry the bacteria to new locations. It has been suggested that the disease may be transmitted by contaminated seed stock (EPPO 1990; Gnanamanickam et al. 1999). The bacterium is a vascular pathogen largely existing and proliferating in plant xylem tissue (Gnanamanickam et al. 1999). Severity of the disease is largely an issue of timing; seedlings younger than 21 days are most severly affected, but inoculation after this period can still have tremendous impact on seed set and fill. There have been several R-genes associated with resistance to various strains

of *Xoo*, but these genes are not present in all rice lines (Ramalingam et al. 2003). Some chitinases are active against bacterial peptidoglycan; if these are targeted extracellularly, they would be ideal candidates for early defense interactions and potential elicitor signal generation.

1.13 Family 18 glycosyl hydrolase expression and significance in the rice genome.

The research presented in this thesis focuses largely on family 19 glycosyl hydrolase chitinases because of their close relationship to our silencing targets Os02g39330 and Os04g41620. MPSS mRNA expression data was collected for all rice glycosyl hydrolase 18 and 19 family chitinase sequence signatures. It is important to understand, due to their large presence in the rice genome, that the family 18 chitinases are also very relevant to defense response interactions, but have received much less attention in rice research. I report here trends in the Massively Parallel Signature Sequencing (MPSS) expression data collected for 35 family 18 chitinases, during interactions with *M. oryzae* and *X. oryzae* pv. *oryzae* (refer to Table 3 A in the next chapter), to add pertinent information to the total chitinase story in rice. These observations are based on trends in the induction profiles; no statistical analyses were applied.

Of the 35 survey family 18 chitinases, 17 were shown to be differentially expressed within the host during the first 48 hour post-inoculation. Six rice chitinase protein sequences were identified that have structural homology with hevamine, the archetypal plant family 18 chitinase. The putative rice hevamine homologs do not possess a C-terminal extension required for vacuolar

localization, and based on subcellular signal analysis, using WoLF PSORT (Horton et al. 2007; Nakai and Horton 2007), these chitinases possess target signals for secretion to the apoplastic space.

Four of the six rice hevamine homologs showed differential expression in *X. oryzae* pv. *oryzae* and *M. oryzae* interactions in the MPSS library; two of these have a basic pI, and two have an acidic pI, research have shown hevamine-homolog pI to be an important factor in lysozyme activity against bacterial pathogens (Majeau, Trudel, and Asselin 1990; Nielsen et al. 1993). A recent proteomics study of rice xylem and phloem exudates showed that the protein product of LOC_Os01g64110, which was also differentially expressed in the pathogen MPSS libraries, and is most similar to hevamine in sequence and pI, occurred in the rice xylem sap (Aki et al. 2008). Rice xylem sap has a natural pH of 5.6-6.0 (Mitani, Ma, and Iwashita 2005). This makes an appealing case for possible lysozyme activity of this enzyme against xylem specific pathogens such as *X. oryzae* pv. oryzae.

Four of the remaining family 18 glycosyl hydrolases that show differential expression in the MPSS library are specific to the *M. oryzae* interaction. These genes are members of a newly emerging subfamily in family 18 glycosyl hydrolases, the chitinase-like xylanase inhibitor. Many proteins related to the xylanase inhibitors have been identified in rice, and exhibit both constitutive and differential organ-specific expression in root and shoot, as well as by treatment of wounding, methyl jasmonate, and pathogen interaction (Kim et al. 2009; Lee et al. 2006; Tokunaga and Esaka 2007). As the hemicellulose component of rice

cell walls is largely of arabinoxylan (Takeuchi, Tohbaru, and Sato 1994), these xylanase inhibitors may be necessary in plant growth and development processes. Like chitinase, these enzymes may have a dual function within the plant. Interestingly, a rice xylanase inhibitor, *OsXip* (LOC_Os05g15880), was shown to have little or no effect on endogenous rice xylanases, as well as no effect on grain development and germination in RNAi mediated silencing studies, but strongly inhibited a *Trichoderma longibrachiatum* glycosyl hydrolase family 11 xylanase, indicating a probable role in defense response (Tokunaga et al. 2008). Trends in MPSS expression data suggest that some family 18 chitinases may be involved in plant defense response.

1.14 Relevance of thesis research

Chitinases have a well established role in plant interactions, especially defense response. Significant efforts have been taken to characterize the structure and function of several chitinases in rice. Relatively little has been done to examine how expression of specific chitinases affects basal defense response to several pathogens, or, to what effect altering chitinase expression has on the expression of other chitinase family members in the rice genome. The research examined in this thesis presents a comprehensive picture of silencing family 19 rice chitinases with the class IV catalytic domain and the resultant effect on disease resistance to *M. oryzae*, *R. solani*, and *Xoo*. I also examine the effect of the silencing on many other chitinases in the rice genome.

REFERENCES

Aki, T., M. Shigyo, R. Nakano, T. Yoneyama, and S. Yanagisawa. 2008. Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. *Plant Cell Physiol* 49 (5):767-90.

Baulcombe, D. 2004. RNA silencing in plants. *Nature* 431 (7006):356-63.

Boller, T. 1988. Ethylene and the regulation fo antifungal hydrolases in plants. *Oxford Surveys of Plant Molecular and Cell Biology* 5:145-174.

Boller, T., and S. Y. He. 2009. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324 (5928):742-4.

Boller, T., A. Gehri, F. Mauch, and U. Vogeli. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* 157:22-31.

Broglie, Karen, Ilan Chet, Mark Holliday, Robert Cressman, Phyllis Biddle, Susan Knowlton, Jeffry C. Mauvais, and Richard Broglie. 1991. Transgenic Plants with Enhanced Resistance to the Fungal Pathogen *Rhizoctonia solani*. *Science* 254 (5035):1194-1197.

Cantrell, R. P., and T. G. Reeves. 2002. The rice genome. The cereal of the world's poor takes center stage. *Science* 296 (5565):53.

Chisholm, S. T., G. Coaker, B. Day, and B. J. Staskawicz. 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124 (4):803-14.

Collinge, D. B., K. M. Kragh, J. D. Mikkelsen, K. K. Nielsen, U. Rasmussen, and K. Vad. 1993. Plant chitinases. *Plant J* 3 (1):31-40.

Datta, K., J. Tu, N. Oliva, I. I. Ona, R. Velazhahan, T. W. Mew, S. Muthukrishnan, and S. K. Datta. 2001. Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. *Plant Sci* 160 (3):405-414.

Ebbole, D. J. 2007. Magnaporthe as a model for understanding host-pathogen interactions. *Annu Rev Phytopathol* 45:437-56.

Eizenga, G. C. 2002. Screening *Oryza* Species Plants for Sheath Blight Resistance. *Plant Disease* 86 (7):808-812.

EPPO, CABI and. 1990. Xanthomonas oryzae Data Sheets on Quarantine Pests.

Evenson, R. E., R. W. Herdt, and M. Hossain. 1998. Rice research in Asia: Progress and Priorities. *CAB International*:-418.

Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol* 43:205-27.

Gnanamanickam, S. S., V. B. Priyadarisini, N. N. Narayanan, P. Vasudevan, and S. Kavitha. 1999. An overview of bacterial blight disease of rice and strategies for its management. *Current Science* 77 (11).

Hannon, G. J. 2002. RNA interference. *Nature* 418 (6894):244-51.

Horton, P., K. J. Park, T. Obayashi, N. Fujita, H. Harada, C. J. Adams-Collier, and K. Nakai. 2007. WoLF PSORT: protein localization predictor. *Nucleic Acids Res* 35 (Web Server issue):W585-7.

Huckelhoven, R. 2007. Cell wall-associated mechanisms of disease resistance and susceptibility. *Annu Rev Phytopathol* 45:101-27.

IRRI. 2007. *Why an international research center for rice?* Monday, December 03, 2007 1:52:59 PM 2007 [cited December 3, 2007 2007]. Available from http://www.irri.org/about/international.asp.

IRRI, Rice Doctor -. 2007. *Sheath Blight Fact Sheet* Monday, November 26, 2007 1:02:22 AM 2003 [cited 11/26/2007 8:40 AM 2007]. Available from http://www.knowledgebank.irri.org/RiceDoctor/default.htm.

Jones, J. D., and J. L. Dangl. 2006. The plant immune system. *Nature* 444 (7117):323-9.

Kaku, H., Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio, E. Minami, and N. Shibuya. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc Natl Acad Sci U S A* 103 (29):11086-91.

Kankanala, P., K. Czymmek, and B. Valent. 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* 19 (2):706-24.

Kasprezewska, Anna. 2003. Plant Chitinases - Regulation and Function. *Cellular and Molecular Biology Letters* 8:809-824.

Kim, S. T., Y. H. Kang, Y. Wang, J. Wu, Z. Y. Park, R. Rakwal, G. K. Agrawal, S. Y. Lee, and K. Y. Kang. 2009. Secretome analysis of differentially induced proteins in rice suspension-cultured cells triggered by rice blast fungus and elicitor. *Proteomics* 9 (5):1302-13.

Lee, Joohyun, Terry M. Bricker, Michael Lefevre, Shannon R. M. Pinson, and James H. Oard. 2006. Proteomic and genetic approaches to identifying defence-related proteins in rice challenged with the fungal pathogen Rhizoctonia solani. *Molecular Plant Pathology* 7 (5):405-416.

Li, Z. K., S. R. M. Pinson, M. A. Marchetti, J. W. Stansel, and W. D. Park. 1995. Characterization of Quantitative Trait Loci (QTLS) in Cultivated Rice Contributing to Field-Resistance to Sheath Blight (*Rhizoctonia solani*). *Theoretical and Applied Genetics* 91 (2):382-388.

Lin, Willie, C. S. Anuratha, Karabi Datta, Ingo Potrykus, S. Muthukrishnan, and Swapan K. Datta. 1995. Genetic engineering of Rice for Resistance to Sheath Blight. *Nature Biotechnology* 13:686-691.

Liu, B., S.H. Zhang, X.Y. Zhu, Q. Yang, S. Wu, M. Mei, R. Mauleon, J.E. Leach, T. Mew, and H. Leung. 2004. Candidate defense genes as predictors of quantitative disease resistance in rice. *Molecular Plant-Microbe Interactions* 17:1146-1152.

Ma, W., and G. A. Berkowitz. 2007. The grateful dead: calcium and cell death in plant innate immunity. *Cell Microbiol* 9 (11):2571-85.

Ma, W., A. Smigel, R. Verma, and G. A. Berkowitz. 2009. Cyclic nucleotide gated channels and related signaling components in plant innate immunity. *Plant Signal Behav* 4 (4):277-82.

Majeau, Nathalie, Jean Trudel, and Alain Asselin. 1990. Diversity of Cucumber Chitinase Isoforms and Characterization of One Seed Basic Chitinase with Lysozyme Activity. *Plant Science* 68:9-16.

Mauch, F., B. Mauch-Mani, and T. Boller. 1988. Antifungal Hydrolases in Pea Tissue : II. Inhibition of Fungal Growth by Combinations of Chitinase and beta-1,3-Glucanase. *Plant Physiol* 88 (3):936-942.

McDonald, B. A., and C. Linde. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu Rev Phytopathol* 40:349-79.

Meister, G., and T. Tuschl. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* 431 (7006):343-9.

Mitani, N., J. F. Ma, and T. Iwashita. 2005. Identification of the silicon form in xylem sap of rice (Oryza sativa L.). *Plant Cell Physiol* 46 (2):279-83.

Miya, A., P. Albert, T. Shinya, Y. Desaki, K. Ichimura, K. Shirasu, Y. Narusaka, N. Kawakami, H. Kaku, and N. Shibuya. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc Natl Acad Sci U S A* 104 (49):19613-8.

Nakai, K., and P. Horton. 2007. Computational prediction of subcellular localization. *Methods Mol Biol* 390:429-66.

Nakazaki, T., T. Tsukiyama, Y. Okumoto, D. Kageyama, K. Naito, K. Inouye, and T. Tanisaka. 2006. Distribution, structure, organ-specific expression, and phylogenic analysis of the pathogenesis-related protein-3 chitinase gene family in rice (Oryza sativa L.). *Genome* 49 (6):619-30.

Neuhaus, J. M. 1999. Plant Chitinases (PR-3, PR-4, PR-8, PR-11). In *Pathogenisis-Related Proteins In Plants*, edited by S. K. Datta and S. Muthukrishnan: CRC Press LLC.

Nielsen, K. K., J. D. Mikkelsen, K. M. Kragh, and K. Bojsen. 1993. An acidic class III chitinase in sugar beet: induction by Cercospora beticola, characterization, and expression in transgenic tobacco plants. *Mol Plant Microbe Interact* 6 (4):495-506.

Nishizawa, Y., Z. Nishio, K. Nakazono, M. Soma, E. Nakajima, M. Ugaki, and T. Hibi. 1999. Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. *Theor Appl Genet* 99:383-390.

Nürnberger, Thorsten, Frederic Brunner, Birgit Kemmerling, and Lizelle Piater. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* 198:249-266.

Panstruga, R., J. E. Parker, and P. Schulze-Lefert. 2009. SnapShot: Plant immune response pathways. *Cell* 136 (5):978 e1-3.

Passarinho, Paul, A., and Sacco de Vries, C. 2009. Arabidopsis Chitinases: a Genomic Survey. In *The Arabidopsis Book*: The American Society of Plant Biologists.

Perry, Laura G., Elan R. Alford, Junichiro Horiuchi, Mark W. Paschke, and Jorge M. Vivanco. 2007. Chemical Signals in the Rhizosphere: Root-Root and Root-Microbe Communication. In *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*, edited by R. Pinton, Z. Varanini and P. Nannipieri. Boca Raton, FL: CRC Press.

Pinson, Shannon R. M., Fabian M. Capdevielle, and James H. Oard. 2005. Confirming QTLs and Finding Additional Loci Conditioning Sheath Blight Resistance in Rice Using Recombinant Inbred Lines. *Crop Science* 45:503-510.

Ramalingam, J., C. M. Vera Cruz, K. Kukreja, J. M. Chittoor, J. L. Wu, S. W. Lee, M. Baraoidan, M. L. George, M. B. Cohen, S. H. Hulbert, J. E. Leach, and H. Leung. 2003. Candidate defense genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. *Mol Plant Microbe Interact* 16 (1):14-24.

Ramalingam, J., C.M. Vera Cruz, K. Kukreja, J.M. Chittoor, J.L. Wu, S.W. Lee, M.R. Baraoidan, M.L. George, M. Cohen, S. Hulbert, J.E. Leach, and H. Leung. 2003. Candidate resistance genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. *Mol Plant-Microbe Interact* 16:14-24.

Ridley, B. L., M. A. O'Neill, and D. Mohnen. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57 (6):929-67.

Robinson, S. P., A. K. Jacobs, and I. B. Dry. 1997. A class IV chitinase is highly expressed in grape berries during ripening. *Plant Physiol* 114 (3):771-8.

Shepherd, R. W., and G. J. Wagner. 2007. Phylloplane proteins: emerging defenses at the aerial frontline? *Trends Plant Sci* 12 (2):51-6.

Shrestha, C. L. , I. I. Ona, S. Muthukrishnan, and T. W. Mew. 2008. Chitinase levels in rice cultivars correlate with resistance to the sheath blight pathogen Rhizoctonia solani. *European Jounal of Plant Pathology* 120:69-77.

Sontheimer, E. J. 2005. Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol* 6 (2):127-38.

Takeuchi, Yuichi, Masaru Tohbaru, and Atsushi Sato. 1994. Polysaccharides in Primary Cell Walls of Rice Cells in Suspension Culture. *Phytochemistry* 35 (2):361-363.

Tokunaga, T., and M. Esaka. 2007. Induction of a novel XIP-type xylanase inhibitor by external ascorbic acid treatment and differential expression of XIP-family genes in rice. *Plant Cell Physiol* 48 (5):700-14.

Tokunaga, T., Y. Miyata, Y. Fujikawa, and M. Esaka. 2008. RNAi-mediated knockdown of the XIP-type endoxylanase inhibitor gene, OsXIP, has no effect on grain development and germination in rice. *Plant Cell Physiol* 49 (7):1122-7.

Vierheilig, Horst, Monica Alt, Jean-Marc Neuhaus, Thomas Boller, and Andres Wiemken. 1993. Colonization of Transgenic *Nicotiana sylvestris* Plant, Expressing Different Forms of *Nicotiana tabacum* Chitinase, by the Root Pathogen *Rhizoctonia solani* and by the Mycorrhizal Symbiont *Glomus mosseae*. *MPMI* 6 (2):261-264.

Wan, J., X. C. Zhang, D. Neece, K. M. Ramonell, S. Clough, S. Y. Kim, M. G. Stacey, and G. Stacey. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. *Plant Cell* 20 (2):471-81.

Wilson, R. A., and N. J. Talbot. 2009. Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. *Nat Rev Microbiol* 7 (3):185-95.

Wisser, R. J., Q. Sun, S. H. Hulbert, S. Kresovich, and R. J. Nelson. 2005. Identification and characterization of regions of the rice genome associated with broad-spectrum, quantitative disease resistance. *Genetics* 169 (4):2277-93.

Young, D.H, and G. F. Pegg. 1982. The action of tomato and Verticillium albo-atrum glycosidases on the hyphal wall of V. albo-atrum. *Physiological Plant Pathology* 21:411-423.

Zhao, K. J., and M. L. Chye. 1999. Methyl jasmonate induces expression of a novel Brassica juncea chitinase with two chitin-binding domains. *Plant Mol Biol* 40 (6):1009-18.

Zhao, S., and X. Qi. 2008. Signaling in plant disease resistance and symbiosis. *J Integr Plant Biol* 50 (7):799-807.

Zhu, Qingsong, Yasuyuki Arakane, Richard W. Beeman, Karl J. Kramer, and Subbaratnam Muthukrishnan. 2009. Functional specialization among insect chitinase family genes revealed by RNA interference. *PNAS* 105 (18):6650-6655.

Zhu, Qun, Eileen A. Maher, Sameer Masoud, Richard A. Dixon, and Chris J. Lamb. 1994. Enhanced Protection Against Fungal Attack by Constitutive Co-expression fo Chitinase and Glucanase Genes in Transgenic Tobacco. *Nature Biotechnology* 12:807-812.

Zou, J. H., X. B. Pan, Z. X. Chen, J. Y. Xu, J. F. Lu, W. X. Zhai, and L. H. Zhu. 2000. Mapping quantitative trait loci controlling sheath blight resistance in two rice cultivars (Oryza sativa L.). *Theoretical and Applied Genetics* 101 (4):569-573.

Chapter II. Role of Chitinases in Basal Resistance to Rice Diseases

INTRODUCTION

Close to half the population of the world consumes rice (*Oryza sativa*) on a daily basis (Cantrell and Reeves 2002; IRRI 2007; Li et al. 1995), making the grain extremely important economically, nutritionally, and scientifically. Meeting consumption demands requires efficiency in all aspects of rice production, including pest and pathogen management. Yield losses of up to 5% annually are attributed to rice pathogens alone (Evenson, Herdt, and Hossain 1998). Classical breeding programs have effectively incorporated disease resistance into commercial rice varieties for decades; unfortunately, pathogen populations change to overcome the incorporated resistance (Peever et al. 2000). To combat the erosion of disease resistance in rice, efforts to find durable, broad-spectrum resistance have increased.

The molecular bases for durable, broad-spectrum resistances are not yet well understood. Defense response proteins, including enzymes that can directly act on pathogen components, have been linked to basal resistance (Huckelhoven 2007) and resistance governed by quantitative traits (Liu et al. 2004; Ramalingam et al. 2003), both of which are associated with broad-spectrum resistance. Chitinases are an example of defense response enzymes that have been linked to basal resistance.

Chitinases are a large group of family 18 and 19 glycosyl hydrolases, which hydrolyze the β-1,4-linkage between *N*-acetylglucosamine residues of chitinoligosaccharides. Chitinoligosaccharides make up chitin, an essential component to the cell walls of many fungi, as well as a structural component to insect exoskeletons (Neuhaus 1999; Zhu et al. 2009). Chitinase nomenclature classification is based on sequence homology in their catalytic domain and by the presence or absence of a chitin binding domain (Neuhaus 1999). Bioinformatic analysis of the *Oryza sativa* cv. Nipponbare genome, in this study, identified 16 family 19 glycosyl hydrolase chitinases or chitinase-like genes of class I, II, IV, and VII,and 35 family 19 glycosyl hydrolase chitinase, or chitinase-like genes of class III and V, that conform to the current chitinase classification system (Table 1). *OsChia1, 2, 4,* and *7* gene designations are given to their respective family 19 class, whereas *OsChib1* and *OsChic1* gene designations correspond with class III and V family 18 chitinases respectively.

Much of the early work in chitinase research was done in dicotyledonous plant species. Extensive research in monocotyledonous plant species suggests chitinase function is conserved with dicotyledonous species. Given the high conservation of chitinase structure and function between species, a wealth of information can be inferred from the early dicot research to a corresponding role in a monocot system. Ultrastructural studies that examined interactions between *Pisum sativum* and *Trichoderma longibrachiatum* show gold labeled antibodies of *P. sativum* chitinases accumulating at the synthesis sites of expanding hyphae (Arlorio et al. 1992). *In vitro* assays of tomato, pea, and bean chitinase enzymes,

in crude protein extracts, or in purified form, inhibited fungal growth (Boller et al. 1983; Mauch, Mauch-Mani, and Boller 1988; Schlumbaum et al. 1986; Young and Pegg 1982). Plant chitinases have also been shown to exhibit activity on structurally related lipochitooligosaccharides generated by symbiotic *Rhizobium* spp. (Schultze et al. 1998). Chitinases and their relatives show a high degree of substrate specificity and processing efficiency as a result of variance in the conformation of their substrate binding site and catalytic domain (Mizuno et al. 2008; Park et al. 2002).

While chitinases are commonly associated with fungal growth inhibition, not all chitinases have this activity (Sela-Buurlage et al. 1993). Different chitinase classes have been associated with numerous roles in plant physiological function. Expression studies involving class IV chitinases have associated heightened chitinase expression prior to leaf senescence with programmed cell death in *Brassica Napus* and *Arabidipsis thaliana* (Hanfrey, Fife, and Buchanan-Wollaston 1996; Passarinho et al. 2001). Speculative roles for chitinases in these studies revolved around the processing and activation of endogenous oligosaccharide signal molecules. A similar role for class IV chitinases of endogenous oligosaccharide signal processing has also been proposed for embryo, seedling, germination, and root growth, as well as pollen-stigma interactions (for review see (Passarinho and de Vries 2009).

Some chitinases, particularly those sharing sequence homology to the *Hevea brasiliensi* endochitinase hevamine, exhibit both chitinase and lysozyme activity (Jekel, Hartmann, and Beintema 1991; Majeau, Trudel, and Asselin 1990;

Terwisscha van Scheltinga et al. 1994). These chitinases hydrolyze bacterial peptidoglycan, suggesting that such chitinases with lysozyme activity may also provide defense against bacterial pathogens.

In addition to direct action on pathogen cell walls, indirect roles for chitinases have been predicted in basal disease resistance (Boller, 1995). In these cases, the activity of the chitinases on pathogen cell walls may generate elicitors of the defense responses, including chitin oligosaccharides or lipids (Chisholm et al. 2006; Huckelhoven 2007; Nürnberger et al. 2004). Global expression studies in rice tissue culture cells, treated with bacterial lipopolysaccharide (LPS), and chitin oligosaccharide elicitors, showed similar downstream defense response gene expression patterns, as well as shared upstream expression of signaling components associated with induced cellular responses (Desaki et al. 2006). The chitin oligosaccharide elicitors are bound by plant membrane-anchored plasma membrane receptors, and this association activates downstream defense responses. In rice, the chitin oligosaccharides are bound by a high-affinity binding protein CeBiP (Kaku et al. 2006). In *Arabidopsis*, a LysM receptor-like kinase LysM RLK (also known as CERK1), which possesses an intercellular kinase domain, acts in chitin oligosaccharide perception and signaling (Miya et al. 2007; Wan et al. 2008).

In rice, several lines of evidence implicate chitinases as contributors to disease resistance. Rice chitinase expression and enzyme activity are linked to disease resistance to fungal pathogens. Analysis of the secretome of rice calli and leaves treated with *Magnaporthe oryzae*, the rice blast pathogen, or an

extracted elicitor revealed differential induction of some chitinases (Kim et al. 2009). When overexpressed in rice, genes for type I chitinases CHI11 (homologous to Os06g51050) (Lin et al. 1995) and RC7 (homologous to Os05g33130) (Datta et al. 2001) increased resistance to *Rhizoctonia solani,* the sheath blight pathogen. Several rice chitinases have been enyzmatically characterized for their substrate binding specificity, catalytic efficiency, and activity as a fungal or bacterial inhibitor. Purified rice basic class III chitinase Os01g64110, expressed in *Pichia pastoris*, was shown to be an effective lytic agent of *Micrococcus lysodeikticus* at pH 3-5, but a weak fungal inhibitor of *Trichoderma reesei* (Park et al. 2002). In a study characterizing class I (Os06g51050) and class VII (Os04g41620) chitinases in rice, it was found that both class I and class IV type catalytic domains had similar *N*-acetyl-chitin-oligosaccharide degradation efficiencies, but very different processing products (Truong et al. 2003). Each enzyme had antifungal activity against *Trichoderma reesei* purified from a *Pichia pastoris* expression system (Truong et al. 2003). Interestingly, the same study also demonstrated that the chitin binding region of Os06g51050 allowed for a 5 fold increase in colloidal chitin processing efficiency (Truong et al. 2003). These data support the hypothesis that rice chitinases play an important role in interactions with pathogens.

One rice chitinase, Os02g39330, which is induced by *M. oryzae* (Kim et al., 2009), co-localizes with blast disease resistance QTL in several mapping studies (Carillo 2009; Liu et al. 2004; Wu et al. 2004). A related class VII chitinase, Os04g41620, efficiently degrades several forms of N-

acetylchitooligosaccharides, and is a likely candidate for direct defense response interactions (Truong et al. 2003). In a study examining a class IV chitinase (*OgChitIVa*) isolated from *Oryza grandiglumis*, which shares 96% protein homology with *O. sativa* class IV chitinase Os04g41680, transgenic overexpression lines were developed in *A. thaliana*, and were shown to increase resistance against necrotrophic pathogen *Botrytis cinera*. Also in this study, *OgChitIVa* was demonstrated to act as an ectopic inducer of PR-1 (mild induction) and PR-2 (strong induction) proteins, suggesting a putative role in a defense-related signal transduction pathway (Pak et al. 2009).

The goal of my work was to determine if rice class IV and VII chitinases from the glycosyl hydrolase family 19 contributed to basal disease resistance to three different rice pathogens, *M. oryzae*, *R. solani,* and *X. oryzae* pv. *oryzae*, causal agents of rice blast, sheath blight and bacterial blight diseases. Using bioinformatic approaches, I identified 51 putative chitinases in the rice genome. Sixteen of the 51 are family 19 glycosyl hydrolases, and three of these are class IV and VII chitinases. Several of the 51 glycosyl hydrolases co-localize with rice disease resistance QTL, including the three class IV and VII chitinases. A phylogenetic tree of the rice family 19 glycosyl hydrolases shows that class IV and VII chitinases are distinct from other family 19 members. Based on analysis of publicly available MPSS data (Nakano et al. 2006), many family 18 and 19 members are induced during interactions with pathogens. Using a gene silencing approach, I demonstrate that altered expression of a subset of chitinase genes

renders rice more susceptible to disease. Overall, these results are consistent with a role for chitinases in broad-spectrum disease resistance.

MATERIALS AND METHODS

2.1 Association of family 18 and 19 rice chitinases with disease resistance QTL.

QTL analysis and mapping were generated using an in-house QTL database which maps known physical coordinates for genetic markers to corresponding rice chromosomal locations (Davidson et al., unpublished). Rice QTL papers were hand checked against QTL marker loci determined by the on-line Gramene QTL database (http://www.gramene.org/qtl/index.html). Individual family 18 and 19 rice chitinase gene coordinates were included as a separate category for inclusion with the in-house QTL mapping tool.

Phylogenetic analysis and characterization of rice chitinases.

Fifty-one putative chitinases were identified in rice using published information (Silverstein et al. 2007; Xu, Fan, and He 2007), and included those identified by chitinase or glycosyl hydrolase family 18 and 19 function (MSU/TIGR v6.1 Putative Function Search Tool), and nucleotide or peptide sequence searches against Os02g39330 using blastn, and blastp (MSU/TIGR v6.1, http://rice.plantbiology.msu.edu/blast) (NCBI GenBank 173.0 release, http://ncbi.nlm.nih.gov/BLAST/) (Altschul et al. 1990; Benson et al. 2008; Ouyang et al. 2007) . In this study, OsXXgXXXX refer to TIGR locus identifiers with the initial identifier LOC_ removed. To select for genes that potentially had chitinase activity, a secondary screen of the sequences was performed. That screen

included a search for conserved molecular patterns relating to the chitin binding domain (PROSITE PS00026), family 18 glycosyl hydrolase active site (PROSITE PS01095), and family 19 glycosyl hydrolase signatures (PROSITE PS00773, PS00774) (http://expasy.ch/prosite/) (Bairoch 1992). Family 19 chitinases were further characterized for potential activity by alignment, and identification of residues within the catalytic region that are important for substrate binding and enzyme activity, as outlined previously for class I chitinases (Garcia-Casado et al. 1998; Passarinho and de Vries 2009). The chitinase nomenclature used herein conforms to chitinase PR-protein classification outlined by Neuhaus (1999) (Table 1). Chitinases are given their glycosyl hydrolase family name and class designation corresponding to presence, abundance, and variance in chitin binding domain, linker peptide (Class VI only), and variation in glycoside domain (Neuhaus 1999; Neuhaus et al. 1996). Protein sequences were further analyzed for domain architecture and signal peptide presence using the comprehensive Simple Modular Architecture Research Tool (SMART ver. 6) (http:// smart.embl-heidelberg.de/) (Letunic, Doerks, and Bork 2009). Lastly, all protein sequences were examined for subcellular localization using the online WoLF PSORT prediction program (http://wolfpsort.org/) (Horton et al. 2007; Nakai and Horton 2007).

The sixteen family 19 chitinases were selected for phylogenetic analysis of their relationship with one another, and to model species *A. thaliana* and *Populus trichocarpa*, as well as representatives from fellow monocot species *Sorghum bicolor, Triticum aestivum,* and *Oryza grandiglumis*. Sequences were obtained

using the online UniprotKB database search against prosite identifier, PS00773, for family 19 chitinases (http://uniprot.org) (The UniProt 2009), as well as sequences obtained by a general function search for “chitinase” using The Arabidopsis Information Resource (TAIR v.9) (http://arabidopsis.org) (Swarbreck et al. 2008). Inferred amino acid sequences from 61 family 19 chitinase genes were aligned using ClustalX version 1.83 (Thompson et al. 1997). Ambiguous regions were identified manually and removed. A total of 277 amino acid characters were analyzed (data set available as supplementary material). A gene tree was reconstructed using Bayesian MCMC analysis (Figure 2) (Ronquist and Huelsenbeck 2003). A mixed amino acid model ("aamodelpr=mixed") was specified for the prior distribution, and four chains were allowed to run for 7.5 X 10^6 generations. The analysis was repeated twice to ensure convergence onto a stationary posterior distribution of trees. The burn-in period used was 5 X 10^6 generations, after which 2500 trees were sampled from each run. The resulting 2500 sampled trees were used to determine the final consensus tree, and posterior probabilities for each clade. For display purposes, the consensus tree was arbitrarily rooted at one of three long internal nodes.

2.2 Analysis of rice chitinase expression patterns in defense response interaction.

The Massively Parellel Signature Sequencing database for rice (http://**mpss**.udel.edu/**rice**/) (Nakano et al. 2006) was used to predict rice chitinase expression patterns in rice leaves during resistant and susceptible interactions with *X. oryzae* pv. *oryzae* and *M. oryzae.* Using corresponding TIGR

locus ID's, a bulk query analysis of the 51 putative rice chitinase and chitinase-like genes were investigated for induction after infection. Signatures used for analysis occurred within 500 bp of the 3' end of the annotated gene, or within the annotated gene itself. Only signatures with one perfect match within the genome were selected for analysis. On the rare occurrence of no single matches, signatures were used only when the corresponding match was a chitinase in close physical proximity to the target gene e.g. Os05g33130 and Os05g33140, which have high sequence homology. Signature hits correspond with mRNA transcripts per million. Differential expression values were estimated by normalization to mock 0 h constitutive expression in both *M. oryzae* and *X. oryzae* pv. *oryzae* libraries. Statistical comparisons were not used to determine expression differences.

2.3 RNAi silencing

Primers used in this study are shown in Table 2. A 239 bp antisense fragment based on the glycosyl hydrolase domain and 3' UTR of Os02g39330 was used for gene silencing (Figure 3A). The antisense fragment was amplified from rice cultivar Kitaake genomic DNA using primers ChitinaseF5 (5'-CACCATCCGCGCCATCAACG-3') and Chi3UTRR5 (5'-CTCCTATGCCGCAAACAACG), which correspond to the last 127 bp of the second Os02g39330 exon and subsequent 113 bp of the 3'UTR. The PCR product was subcloned into entry vector TOPO pENTER (Cat. No. K2400-20, Invitrogen); the clone was transformed into *E. coli* and colonies containing cloned DNA were selected on 50 µg/ml Kanamycin (Km). The entry vector was then

recombined with the destination pANDA vector using the Gateway LR Clonase Enzyme Mix (Cat. No. 11791-019, Invitrogen). The vector contains a maize ubiquitin promoter which drives the hairpin dsRNAi silencing complex. After amplification in *E. coli* strain DB3.1 with 50 µg/ml Km for selection, the clone was transferred to *Agrobacterium tumefaciens* strain EHA105 for plant transformation (Miki and Shimamoto 2004). Transformants were screened for presence of the transgene in $T_{0,}$, T_1, and T_2 generation plants by PCR amplification of the hygromycin selection gene (Hyg F&R). Transcript abundance of chitinase Os02g39330 relative to wild type Kitaake was analyzed in T_0 plants for advancement to T_1 silenced lines for further study. Reduced relative mRNA transcript abundance of target genes, presence of hygromycin, and severity of disease phenotype were used as criteria for advancement to T_2 for assessment of disease response.

For initial screening of T_1 transgenic lines, Platinum High Fidelity DNA Taq Polymerase (Invitrogen cat# 11304-102) was used to allow amplification of low transcript target genes. For T_2 transgenic lines, a primer set was developed for use with a standard *Taq* DNA Polymerase (New England Biolabs cat# M0273L). In addition to the *OsChia4* and *7* chitinases predicted to be silenced by homology (Os02g39330, Os04g41620 and Os04g41680), expression of a subset of chitinases from both Family 18 and 19 glycosyl hydrolases, e.g. representatives of *OsChia1,OsChia2, OsChia4, OsChia7, OsChib1*, chitinase-like, and chitinase-like xylanase inhibitors, were surveyed for altered expression levels as a result of silencing *OsChia4* and *OsChia7* chitinases. A total of 11 rice chitinase/chitinase-

like genes were screened for expression in this study (Table 1). All 11 corresponding primer sets were developed in regions spanning the catalytic domain and 3' UTR for gene specificity. All expression data collected for T_1 and T_2 chitinase silenced lines was normalized to the constitutively expressing 18s reference gene for relative sample mRNA transcript abundance.

2.4 Plant Materials and Growth Conditions

Rice cultivar Kitaake was used for development of RNAi silenced lines. Cultivars Jasmine85 and Lemont were used respectively as resistant and susceptible controls for *R. solani* disease development, and cultivars Sanhuangzhan 2 (SHZ-2) and Lijiangxintuanheigu (LTH) were used as resistant and susceptible controls, respectively, for *M. oryzae*. *X. oryzae* pv. *oryzae* experiments included only wild type Kitaake plants and transgenic plants.

Rice seed were pre-germinated in fungicide Maxim XL (Syngenta, Product # A10013A) (Active Ingredients: 21.0% Fludioxonil and 8.4% Mefenoxam) for 3 days prior to planting in potting mixture (4:4:1, peat: Pro-Mix BX: sand). For rice blast and bacterial blight experiments, plants were grown in a growth chamber with a photoperiod of 16 h light/ 8 h dark, with a photon flux of 135 µmol $m^{-2}s^{-1}$ and day/night temperatures of 28ºC/26ºC and were fertilized at 10 days after transplanting to soil with 20 µM Ammonium Sulfate. Greenhouse-grown rice for sheath blight experiments were fertilized after one month. Microchamber conditions used during sheath blight experiments were 90-100% relative humidity and 34ºC/26ºC day/night temperatures. Supplementary lighting was applied to maintain a 16 h light/ 8 h dark photoperiods.

2.5 Sheath blight disease evaluation

R. solani isolate RM01401 was grown on Difco potato dextrose agar (Becton Dickinson, Franklin Lakes, NJ) at 26°C; cultures were stored on pre-sterilized barley seeds inoculated by *R. solani* sclerotia. To assess sheath blight disease, 14-day-old Kitaake (transgenic parent/moderately resistant), Jasmine85 (R), Lemont (S) and transgenic, silenced lines were inoculated with *R. solani* isolate RM01401 using the microchamber method (Jia et al. 2007). Plant leaf tissue was collected at 12 days. For most plants, the first fully emerged leaf was sampled, but in cases where transgenic plants were stunted, portions of still emerging leaves were collected. Two days after harvesting leaves for RNA extraction, plants were inoculated as described (Jia et al. 2007). Sheath blight symptoms were scored using a quantitative disease index (DI) = (lesion length/plant height*9) modified from (Groth, Rush, and Lindberg 1990), and a visual index (VI) = (0-9 scale). Disease was measured after susceptible control plants showed severe lesions moving beyond stem tissue (14 days after inoculation).

2.6 Rice blast disease evaluation

M. oryzae isolates P06-6 and KV-1 (Kankanala, Czymmek, and Valent 2007) were grown on oatmeal agar in continuous light at 26ºC and stored on sterile filter paper at -20ºC (Valent, Farrall, and Chumley 1991). To evaluate effect of silencing on rice blast responses, 15-day-old Kitaake (transgenic parent/moderately resistant control) lines of transgenic plants, SHZ-2 (R), LTH (S), and Kitaake plants transformed with an empty vector were inoculated with *M.*

oryzae isolate P06-6 in the T_1 generation, and *M. oryzae* isolate KV-1 in the T_2 generation, using a detached leaf spot method (Jia 2003). Optimal inoculation conditions for the assay were determined with Kitaake wild type plants using a spore suspension concentration graidient for each *M. oryzae* isolate. The second youngest leaves were collected at 12 days and spotted with 5 µl drops of $1x10^5$ spore/ml suspension. Pictures were taken 7 days post inoculation for phenotypic evaluation. 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*.

2.7 Bacterial blight disease evaluation

X. oryzae pv. *oryzae* strain KXO212 were grown on nutrient agar media, at 28ºC, for 2 days, then suspended in sterile distilled water at 10^8 cfu/ml. To evaluate the effect of chitinase silencing on bacterial blight interactions, 7-week-old transgenic and wild type Kitaake plants were inoculated by leaf clip inoculation, as described (Kauffman et al. 1973). Inoculations were performed

on the three youngest leaves of the plant. Inoculated plants were scored for lesion length after two weeks.

RESULTS

2.8 Phylogenetic analysis of OsChia genes.

Sixteen PR-3 plant chitinase family 19 glycosyl hydrolase family members of Class I, II, IV, and VII, as well as a newly emerging class of chitinase-like genes were predicted from the rice genome based on NCBI and TIGR/MSU blastp searches against Os02g39330. The predicted rice chitinase family members were further designated by class using protein sequence alignment and analysis of all rice family 19 glycosyl hydrolases as outlined in Neuhaus (1999) and Passarhino and de Vries (2009). Nakasaki et al. (2006) identified 12 of these genes and documented their allelic diversity, phylogenetic relationship, and organ-specific expression profiles in rice. I detected two additional Class II chitinases (Os05g04690 and Os10g39700) which differ by the deletion of a loop structure corresponding to the protein's sugar binding region (Mizuno et al. 2008; Neuhaus 1999). Two markedly different putative chitinases (Os08g41100, Os09g32080) were identified with truncated half chitin binding domains that weakly resemble the binding domain of a Class VI chitinase isolated from *Beta vulgaris* (Berglund et al. 1995), but lack the extended proline-rich linker sequence associated with Class VI chitinases. Both the putative truncated chitin binding domain and the family 19 catalytic region of these chitinases show homology to *A. thaliana* chitinase-like proteins (At01g05850, known to encode POM1, ERH2,

ELP1, CTL1, ELP, HOT2, ATCTL1), which have been implicated in drought, salt, heat, and developmental physiological responses (Kwon et al. 2007; Zhong et al. 2002). Blastp sequence query against Os08g41100 and Os09g32080 resulted in several matches to other species of both monocots and dicots, suggesting this type of chitinase-like gene is common to both monocot and dicot species. Studies thus far have not proven chitinase activity for the products of these genes, and the high degree of sequence divergence in the catalytic domain suggests that they may not have chitin degrading activity.

A phylogenetic tree (Fig. 2) was constructed from the sixteen family 19 rice chitinases, as well as a large representation of 45 other selected family 19 chitinases from *A. thaliana, Populus trichocarpa*, *Sorghum bicolor, Triticum aestivum,* and *Oryza grandiglumis*. The tree clearly reflects the class relationships associated with chitinase nomenclature. Differences between monocot and dicot chitinases are also very apparent, and divergence within dicot class I and class IV chitinases can be seen between diverse genera such as *P. trichocarpa* and *A. thaliana.* Class IV chitinases are unmistakably divergent from class I and II chitinases of both monocot and dicot sequences.

The *O. sativa* cv. Nipponbare genome has markedly few class IV chitinases relative to class I chitinases. In contrast, *A. thaliana's* genome contains a much greater abundance of class IV chitinases than class I chitinases. Of the surveyed family 19 chitinases, *S. bicolor* seems to have the highest resemblance to *O. sativa* chitinases, which is not surprising considering their common ancestry, similar ploidy (*O. sativa* cv. Nipponbare 2n=24, *S. bicolor* 2n=20) number.

Another cereal with common ancestry, *T. aestivum*, is significantly different with respect to genome size and ploidy, but does not have a fully sequenced genome to reflect its complete chitinase diversity. Representative wheat chitinase sequences show homology with other cereal chitinases in the phylogeny. Although not represented in phylogeny, wheat does have single corresponding class IV and class VII chitinases with high sequence similarity to the other cereal class IV chitinases in the phylogeny.

The phylogram shows that family 19 chitinases are well represented in both monocot and dicot species. Close relationships between genera less evolutionarily divergent can be seen within the monocot chitinases. Rice chitinases that have been identified as important in defense responses are good candidate genes for examination of orthologous chitinases in other species. Closely related chitinases within genera are also good targets for examination under different stress (pathogen/pest/wounding/physiological) conditions to determine the functional relationships of all chitinase genes within a genome.

2.9 Expression of *OsChia* genes during disease and defense responses in MPSS studies.

The rice MPSS expression signatures (Nakano et al. 2006) for predicted rice chitinases were compared to detect variations in expression of individual chitinases in response to infection with *M. oryzae* and *X. oryzae* pv. *oryzae* during resistant and susceptible interactions. Of the 53 putative chitinases in rice, 31 genes were differentially expressed among all treatments. Thirteen of the sixteen family 19 glycosyl hydrolases were differentially expressed during

resistant interactions with the two pathogens (Table 3B). No family 19 glycosyl hydrolases were specific to bacterial interactions, but one acidic class I chitinase (Os03g30470) was specifically expressed only in the *M. oryzae* resistant interaction. One acidic and one basic class I chitinase (Os05g33140 and Os05g33130, respectively) were specifically expressed only in resistant and susceptible *M. oryzae* interactions. These two genes share common expression signatures, thus either or both genes could be responsible for the generation of the transcript. Os06g51050, an acidic class 1 chitinase, increased expression in both *M. oryzae* interactions, but to a lesser degree in the *X. oryzae* pv. *oryzae* resistant interaction.

Among the 35 family 18 glycosyl hydrolases surveyed (Table 3A), 17 showed differential expression in response to *X. oryzae* pv. *oryzae* and *M. oryzae* inoculation, in both resistant and susceptible interactions, within the first 48 h. Nine of these were expressed in all treatments, and three were specific to the *X. oryzae* pv. *oryzae* resistant interaction (Os01g64100, Os04g30770, and Os11g47570). Six were specific to the *M. oryzae* interactions (Os07g01770, Os08g40690, Os10g28120, Os11g47500, Os11g47560, and Os11g47600). The differentially expressed family 18 glycosyl hydrolases grouped into three subclasses: (1) those most homologous to archetypal class III chitinase/lysozyme hevamine, (2) those with homology to the putative seed storage protein narbonin, and (3) those with homology to wheat xylanase inhibitor protein (XIP), which constituted the most abundant group. Aside from

the highly expressing Os05g15770, transcripts in all family 18 chitinase genes rarely rose above 400 transcripts/million.

2.10 Silencing of two OsChia genes Os02g39330, Os04g41620 genes in rice.

We focused on three family 19 glycosyl hydrolase genes (Os02g39330, Os04g41620, and Os04g1680**)** for gene silencing studies because they co-localize with disease resistance QTL in mapping studies, and because they were differentially expressed during susceptible and resistant interactions in the MPSS data. Of the three chitinase genes predicted to be silenced by the construct OsChia4SIL (Figure 3), only two (Os02g39330 and Os04g41620) were expressed in Kitaake, the japonica rice cultivar used for silencing experiments (data not shown). Kitaake does not exhibit typical *R*-gene mediated resistance responses against *M. oryzae* isolates KV-1 or P06-6 (data not shown), but exhibits a moderate level of resistance against these isolates and also exhibits moderate resistance to *R. solani*. Silencing could not be performed in cultivars where the chromosome 2 rice blast QTL co-localizing with the Os02g39330 chitinase was originally identified (Liu et al. 2004), because these indica cultivars are recalcitrant to transformation.

The genome insertion of the silencing transgene was confirmed in T_0, T_1, and T_2 lines by PCR using primers specific to the vector and transgene (e.g., Figure 4). In the T_0 generation, Os02g39330 expression was reduced relative to wild type in all lines examined, but the relative amounts of silencing varied from line to line (Figure 4a). Examination of mRNA expression of the silencing targets

Os02g39330 and Os04g41620, in three T_1 transgenic lines 28.8, 28.10, and 28.12, derived from the same T_0 parent (line 28) (Figure 4b), showed variation in silencing of both genes, but a much greater and more consistent reduction in expression of Os04g41620. Similar results were observed in the T_2 population, that is, Os02g39330 showed variable silencing, and Os04g41620 expression was consistently silenced in all individuals among all selected lines (Figure 4c).

2.11 Silenced T_1 and T_2 lines exhibit enhanced susceptibility to bacterial blight, sheath blight, and blast diseases.

To assess the impact of silencing on disease interactions, populations of transgenic plants from the T_1 and T_2 generations were inoculated with three different pathogens, *R. solani (Rs), M. oryzae (Mo),* and *X. oryzae* pv. *oryzae (Xoo)* to determine the impact of silencing on disease interactions. The T_1 lines were derived from T_0 plants that exhibited silencing of Os02g39330, and the T_2 lines used for inoculation were selected from T_1 parents that exhibited higher levels of sheath blight and blast disease than wild type plants.

Populations of transgenic plants inoculated with *R. solani* exhibited a wide range of disease symptoms measured as Disease Index (DI, Figure 5). For example, inoculation of 153 T_1 individuals, from eight different T_0 lines showed a range of disease index from wild type levels (DI from 1-3) up to high DI (>7-8) (Figure 6A). In the T_2 populations, the notable skew in the distribution of transgenic plants to higher DI is likely because T_1 lines advanced to T_2 were selected for DI greater than wild type plants. The increased number of plants

with DI > 4, in both T_1 and T_2 populations, clearly show a relationship between transgenic lines and increased sheath blight disease.

Spot inoculation of 86 T_1 leaves with *M. oryzae* isolate PO6-6 resulted in disease scores (DS) ranging 0-7 (Figure 6a), with 16% of inoculated leaves (for all individuals) resulting in disease scores considered moderate (DS = 4-5) to highly susceptible (DS = 6-7). Line Chi33 showed the highest number of susceptible phenotypes, with 25% of individuals exhibiting moderate (DS = 4-5) to high disease (DS = 6-7). The selected T_2 progeny of silenced lines Chi28.8, Chi28.10, and Chi28.12 were inoculated with a different *M. oryzae* strain KV-1. There was no significant difference in disease score between the transgenic lines and wild type Kitaake for this isolate (Figure 6d). In summary, our data suggest that transgenic plants are more susceptible to *M. oryzae* isolate PO6-6, an isolate for which Kitaake is known to exhibit quantitative resistance. In contrast, the transgenic lines are not more susceptible to isolate KV-1; resistance of Kitaake to this isolate has not been demonstrated previously to be controlled by quantitative traits.

Susceptibility to *Xoo* KXO212, which is weakly virulent to Kitaake, was measured after scissor clip inoculation of 149 individual plants grown from the seed of nine different T_0 lines that exhibited silencing of Os02g39330 (Fig. 6e). This population trend shows that the chitinase silencing may have an effect on the disease interaction between *Xoo* and Kitaake chitinases.

Population analysis of disease susceptibility in T1 and T2 transgenic plants simply shows that there a number of individuals exhibiting higher disease

measurements than the wild type populations. The distribution of disease scores was visually skewed towards more disease in a significant number of the transgenic individuals versus wild type individuals for all interactions except the one involving *M. oryzae* isolate KV-1 (Figure 6d). These data suggest that silencing of Os02g39330 and Os04g41620 rice chitinases may affect disease resistance responses in all three pathogen interactions.

2.12 Relationship of Os02g39330 and Os04g41620 expression and sheath blight disease.

A subset of 12 individual T_2 transgenic plants from three Chi28 lines were analyzed for expression of the target genes Os02g39330 and Os04g41620 and for disease response to *R. solani* (Fig. 7). Relative expression of Os02g39330 and Os04g41620 varied among the three lines as well as among individual progeny of the lines. Expression of Os04g41620 was reduced in all plants, while Os02g39330 relative expression was reduced in most plants. Also, Os04g41620 expression was reduced more relative to wild type than was Os02g39330.

Among the T_2 transgenic plants, DI scores ranged from wild type levels (2) to very high (9). Of eleven sampled transgenic individuals, eight showed evidence of both Os04g41620 silencing, and high DI phenotype. Os02g39330 showed evidence of silencing coupled with high DI phenotype in fewer of the same transgenic individual samples. While DI was often correlated with silencing, the pattern was not consistent for a few individuals, i.e., in one case Chi28.8.12, where little silencing of Os02g39330 and Os04g41620 was observed, a high DI value was observed, and in another case, Chi28.10.29,

where both were genes were silenced, a low DI score was recorded. Linear regression analysis of Os02g39330 and Os04g41620 expression in sampled transgenic plants could not correlated with high DI phenotype (Figure 8 A & B).

2.13 Silencing of Os02g39330 and Os04g41620 results in altered expression of non-target chitinases.

To determine if the lack of correlation of DI to target gene silencing could result from alterations in expression of other non-target chitinases, the expression of five other chitinases was assessed in the T_2 plants (Fig. 7). Primers were designed for eight additional family 18 and 19 glycosyl hydrolase chitinase genes that were selected based on their differential expression patterns in the MPSS data (Tables 3A and 3B). Based on lack of any sequence homology, these chitinases were not likely to be silenced by the silencing construct (Figure 3B). Three genes (Os06g51060, Os10g39680, and Os11g47500) were either not expressed or were weakly expressed in the wild type population, or were not expressed at all in the transgenic populations (data not shown), and were excluded from the analysis. Expression of the five remaining genes varied among the transgenic lines, but in general, band intensities were noticeably reduced in transgenic relative to wild type controls (Figure 7). Thus, as a consequence of silencing Os02g39330 and Os04g41620, the expression of at least five non-target chitinase genes was altered.

To determine if altered expression of these genes correlated with disease index, linear regressions were performed for the same transgenic individuals used in the previous correlations for sheath blight disease index and gene

silencing of Os02g39330 and Os04g41620. The five non-target genes tested showed reduced expression relative to wild type, but the decreased expression did not show a linear correlation with disease interactions (data not shown). Overall, these data suggest that the increased disease susceptibility that results from the silencing of Os02g39330 and Os04g41620 may partially be due to altered expression of non-target chitinases.

2.14 Reduction in plant height correlates with increased disease in transgenic lines

The transgenic plants, in general, appeared shorter than the wild type plants. Several chitinases co-localize with plant height QTL (Figure 1) and plant chitinase, or chitinase-like genes putatively have a role in endogenous cell wall function (Zhong et al. 2002). To address if reduction in height correlated with amount of disease, plant height was assessed in the T_1 and T_2 populations and compared to visual disease index for each of the plants. Visual index ranks disease from 0-9 based on a visual assessment, and does not involve physical measurements of lesion length and plant height, which are used to calculate disease index. Plant height is correlated with visual index in both the T_1 and T_2 transgenic generations (Figure 9). A correlation of plant height and gene silencing was not performed.

DISCUSSION AND CONCLUSIONS

Activity and location of chitinase enzymes as well as expression profiles of chitinase genes have been correlated with plant defense responses in many plant/pathogen interactions (for review (Collinge et al. 1993; Kasprezewska 2003;

Passarinho and de Vries 2009). In rice, the co-localization of particular chitinase genes with disease resistance QTL was inferred as evidence that these chitinase genes might contribute to broad-spectrum disease resistance. In this study, we show that transgenic plants silenced for two family 19 rice chitinases, Os02g39330 and Os04g41620 exhibited increased susceptibility to three rice pathogens. However, the increased susceptibility, although a consequence of gene silencing, is not entirely caused by reduced expression of the two targeted chitinases. The increase in disease could be at least partially due to the changes in expression of non-targeted chitinases that occurred as a consequence of silencing of Os02g39330 and Os04g41620.

Os02g39330 and Os04g41620 were selected for silencing based on co-localization with disease resistance QTL (Alam and Cohen 1998; Liu et al. 2004; Liu et al. 2009; Pinson, Capdevielle, and Oard 2005) and Fig. 1.), phylogenetic relationships (Fig. 2), and gene expression trends after pathogen inoculation (Tables 3A and 3B). Co-localization of Os02g39330 and Os04g41620, as well as other chitinase family members, to disease resistance QTL, imply that chitinases are regularly associated with defense response gene-rich genomic regions in rice; this association with QTL's, along with the large body of literature supporting chitinase's involvement in defense response interactions, makes these chitinases very attractive for putative basal defense responses. Phylogenetic analysis of all identified family 19 glycosyl hydrolases present in the *O. sativa* genome clearly separate the Os02g39330 and Os04g41620 chitinase genes (Class IV and VII) from other family 19 chitinase classes, but that they are

relatively closely related to one another. These relationships were confirmed during sequence alignment and selection of the region to be targeted for silencing (Fig. 3). Finally, examination of family 19 rice chitinases during disease and defense interactions with *M. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzae* in the rice MPSS database suggested that Os02g39330 and Os04g41620 are differentially expressed within the first 48 h of the disease interaction (Table 3B).

Screening of large populations of transgenic plants suggested that silencing of Os02g39330 and Os04g41620 increased disease to two distinct pathogens, *R. solani* and *X. oryzae* pv. *oryzae.* Individual transgenic lines harboring the silencing construct exhibited variation of silencing of Os02g39330 and Os04g41620 (Fig. 4). Transgenic overexpression lines developed in several species, although less commonly reported, have frequently exhibited a variable silencing phenomena attributed to numerous factors (James et al. 2004). Logically, evidence has pointed to the involvement of small RNA's as the silencing mechanism in chitinase overexpression lines both in tobacco and rice (Chareonpornwattana et al. 1999; Hart et al. 1992). However, in lines both homo- and hemi- zygous for the transgene, the location and intensity of these silencing phenotypes were influenced by environmental conditions, transcript abundance, and developmental regulation. Similar variation in silencing of gene family members has been demonstrated for other defense gene families. For example, in a recent study, rice plants silenced for a family of 12 rice germin-like protein genes showed silencing variation among closely linked family members using a highly conserved target sequence. The number of genes silenced, as

well as the effectiveness of transcriptional repression, correlated with increased disease severity to both *M. oryzae* and *R. solani* (Manosalva et al. 2009).

Genotyping of several *R. solani* inoculated T_2 transgenic lines, showing a range of increasingly severe phenotypes, did not show significant correlation with the susceptibility phenotype. Interestingly, in the T_1 and T_2 transgenic populations, plants were much shorter than wild type, and in *R. solani* inoculated individuals, disease severity was significantly greater in the shorter plants. Lastly, our research indicated that reducing expression of genes, Os02g39330 (*Chia4*) and Os04g41620 (*Chia7*), putatively resulted in a decrease in expression of other *OsChia* (Family 19) and *OsChib* (Family 18) genes. These cumulative data describe pieces of the overall basal defense response puzzle and the role that chitinase genes may play in that response. Overexpression of class I rice chitinases in previous studies have demonstrated increased resistance to pathogens, but approaches to examine chitinase's endogenous role in basal defense cannot be determined by overexpression.

Functional redundancy of gene family members has been observed in defense related oxalate oxidase gene family members (Manosalva et al. 2009). Proteomic and secretome analyses in rice interactions have shown different chitinases are induced upon pathogen invasion, and in some cases suites of chitinase/chitinase-like genes are induced (Kim et al. 2009; Lee et al. 2006). MPSS expression analysis of differentially expressed chitinases suggests that different chitinases may be functionally active throughout the progression of the observed time course within the first 48h of infection; this is in agreement with the

concept of QTL governed resistance, where small contributions of multiple genes contribute to the overall response. The nature of these QTL interactions, and the putative dual role that chitinase may play in plant physiological function, make a very difficult picture to interpret. Analyses of resistant cultivars in several plant species, have shown that chitinases exhibit higher enzyme, protein, and transcript activity, at early stages of pathogen inoculation (Collinge et al. 1993; Shrestha et al. 2008). The age of the plant, both at inoculation and tissue harvest, in this experiment, captures only a moment of chitinase activity *in planta*. Constitutive chitinase activity has been demonstrated for some chitinases to increase as the plant ages (Kasprezewska 2003). Our experiments could not have fully encompassed the complexity of chitinase interactions, or the importance of chitinases as the plant ages, but does add information to the overall picture of chitinase activity in rice plants.

Expression patterns from the MPSS data suggest that differential expression patterns of thirteen family 19 chitinases correlate with responses to pathogen invasion. These data, coupled with the phylogenetic analysis of all rice family 19 chitinases, clearly show that all represented classes of chitinase may be important to pathogen response. Orthologous members of the class I chitinases have been largely focused on in classical research relating to family 19 chitinases as defense response genes; rice chitinase overexpression lines exhibiting resistance to blast (Nishizawa et al. 1999) and sheath blight (Datta et al. 2001; Lin et al. 1995) have used solely class I chitinases. Our study examined a related class I chitinase, Os06g51060, which was recently identified in the

secretome of *O. sativa* cv. Jinheung in response to *M. oryzae* and its elicitors (Kim et al. 2009). In agreement with *O. sativa* cv. Nipponbare expression data in the MPSS database, this gene is not constitutively active in healthy Kitaake plants, indicating that expression may require induction. MPSS data suggests that class I chitinases are not likely expressed constitutively, and conversely, are part of the downstream basal defense responses activated after signal recognition.

Interestingly, the class IIa and IIb chitinases, which lack a chitin-binding domain and have a catalytic domain very similar to that of class I chitinases, largely differ by a deletion in the second loop of their tertiary structure. This divergence can be seen in our phylogenetic tree. Class IIa, having the loop deletion, is thought to be the form of the enzyme that is induced in pathogen response (Neuhaus 1999); in a recent deletion study of class I rice chitinase Os05g33130, the corresponding loop II deletion was seen to significantly enhance hydrolytic activity as well as broaden substrate specificity (Mizuno et al. 2008), increasing the likelihood that class II chitinases containing the same deletion would have an enhanced relationship in fungal pathogen interactions. Os10g39680, an orthologous counterpart of *Rcht2*, a chitinase protein that requires dephosphorylation for elicitor induction, in *O. sativa indica* cv. Cheongcheongbyeo, has recurrently been shown to be responsive to *M. oryzae* elicitors (Kim et al. 1998; Kim et al. 2009). Our study examined two class IIa chitinases, Os03g04060 and Os10g39680. Of the two, only Os03g04060 was constitutively expressed in the MPSS database, and this was reflected in our

expression studies, as Os10g39680 was not consistently or highly expressed in wild type plants. However, in a related study (data not shown), 3 days after *R. solani* inoculation, Os10g39680 exhibited signs of expression induction in wild type Kitaake. Os03g04060 expression was seen to be significantly reduced in silenced transgenic lines showing highest susceptibility to *R. solani*. Accumulated data on rice class IIa chitinases would strongly suggest that reduced expression of either of the genes we examined could have an impact on defense response interactions with *R. solani* and would likely contribute to basal defense response.

Our study also examined a number of family 18 glycosyl hydrolase chitinases in rice. One, Os01g64110 most resembles the archetypal family 18 bi-functional chitinase, hevamine, which exhibits both chitinase and lysozyme activity (Jekel, Hartmann, and Beintema 1991). Os01g64110 did not show significant reduction in expression, although this may have been due to outliers in the wild type expression, because a number of lines with the extreme susceptibility phenotype had very low expression values for the gene.

Two pututative xylanse inhibitors, Os05g15770 and Os11g47500, which share regions of sequence homology to hevamine, but have a very distinct substitution in the chitinase active site that changes a critical glutamic acid residue to aspartic acid that likely abolishes chitinase activity (Payan et al. 2003), were selected because they appear to be differentially expressed in the pathogen responsive MPSS library. Like chitinase, these enzymes may have a dual function within the plant. Our expression data corroborate the MPSS data

wherein Os05g15770 is expressed highly and constitutively, and Os11g47500 is not expressed in healthy tissues. In related studies (data not shown), Os11g47500 was shown to be highly induced at 3 days post *R. solani* inoculation in wild type Kitaake plants, making it a potential candidate for defense response. Sequence variation in xylanase-interacting regions likely plays an important part in the substrate binding ability to different xylanases (Durand et al. 2005). As the hemicellulose component of rice cell walls is largely of arabinoxylan (Takeuchi, Tohbaru, and Sato 1994), these xylanase inhibitors may be necessary in plant growth and development processes. Because of its constitutive activity, Os05g15770 would be a likely candidate for developmental activity. A recent proteomic study also showed that Os05g15770 accumulates to higher levels in resistant rice cultivars inoculated with *R. solani* (Lee et al. 2006). It has been suggested, in a study of protein abundance profiles of xylanase inhibitor families in several wheat cultivars, that chitinases and xylanase inhibitors may be co-regulated as has been shown for β-1,3-glucanse and chitinase (Croes et al. 2009). Where β-1,3-glucanse and chitinase are likely interacting directly with the fungal cell wall glucans and chitin oligosaccarides, xylanase inhibitors would directly inhibit fungal secreted, plant cell wall degrading xylanases as another component to the multigenic defense response. This observation suggests that a plant needs several gene products, which are similar, but specialized, to combat the wide variety of pathogen elicitors produced during host infiltration. Our data show that Os051770 expression is reduced significantly in silenced lines, this suggests that class IV or VII chitinases may have a broad regulatory effect on

unrelated chitinase, or chitinase-like genes, and also strengthens the idea that chitinases and xylanase inhibitors may be co-regulated.

Os10g28050, a putative chitinase class III homolog, with decidedly different sequence structure than the traditional hevamine-like, or xylanase inhibitor-like class III chitinases, was also shown to be differentially expressed across all pathogen interactions in the MPSS database. While Os10g28050 may be sequentially divergent, the core glutamic acid residues required for chitinase activity are present. Our data shows that Os10g28050 expression was reduced in silenced lines, indicating that it may also be affected by reduced expression of the class IV and VII chitinases.

Our phylogenetic tree and bioinformatic analysis indicate that class IV chitinases in rice are a small group of the overall chitinases present in the *O. sativa* cv. Nipponbare genome. Rice MPSS data suggest that of the three identified class IV and VII chitinases, only Os02g39330 is constitutively active in leaf tissue, and also shows differential expression within 3 h post-pathogen inoculation. MPSS data also imply that Os04g41620 and Os04g41680 are not constitutively active in leaf tissue, and show no activity until at least 12 h after inoculation. These data suggest that Os02g39330 may be on the frontline of pathogen interactions. While these chitinases share sequence homology in their catalytic domains, there is divergence, indicating that they may have different function within the plant. Promoter analysis would likely identify more specific interactions for these genes. Nakazaki noted no Os02g39330 amino acid sequence variation among 41 varietes of *indica* and *japonica* rice subspecies

(Nakazaki et al. 2006). This conservation among subspecies suggests that the structure of the catalytic domain for this gene may be functionally very important. Os04g41620 and Os04g41680 also showed high conservation in their catalytic domain among rice subspecies, again implicating the domain as structurally important to its function in rice. The low copy number and conserved domain of these class IV and VII chitinases suggests an important, albeit unknown, role in rice physiology or defense-response.

Given the speculative role chitinases play in plant growth and development, one can only guess at the effect of silencing chitinase family members integral to signaling or developmental pathways on plant vigor, or defense element regulation. The genomic profile of individual progeny, among the silenced lines, naturally alters in response to crossing-over events occurring during sexual reproduction, introducing variability to trans-gene expression and effectiveness. Also, the possibility of the plant becoming aware of foreign material in the genome, and subsequently methylating the transgene, would change the chitinase expression profile. The examined Chi28.xx T2 progeny are segregants from a shared protogenic silencing event; the variation in the population of segregants reflects the independent changes likely occurring in the genetically similar, but divergent, transgenic population.

An interesting study in barley recently described an enigma surrounding the virus-induced gene silencing (VIGS), an alternative method of gene silencing similar to RNAi, of a cell wall cellulose synthase gene. Not only was the target gene silenced, but several closely related cellulose synthase genes, as well as

several distantly relate glycosyl transferase genes were down regulated by the silencing of the single gene (Held et al. 2008). The proposed method of regulation was by naturally occurring siRNA derived from *cis*-antisense pairs, which putatively mediate common regulatory control points of the cellulose synthase and glycosyl transferase genes (Held et al. 2008). This evidence makes a very attractive model for our chitinase silencing story, where several closely and distantly related chitinases showed decreased transcription in transgenic individuals. If siRNAs do indeed regulate suites of genes in the cell wall, it seems likely that such a mechanism could be relevant for glycosyl hydrolase family genes, another putative component to cell wall function.

Our research shows that class IV and VII chitinases likely play a role in rice basal defenses. Transgenic individuals, inoculated by several pathogens, recurrently showed signs of increased susceptibility, as well as decreased expression of chitinases present within the genome. Unfortunately, the parameters of our experiment, and the complexity of chitinase activity, did not allow for us to tease apart the role of individual class IV and VII chitinases in rice. Limitations, presented by the RNAi transformation process, such as non-target silencing due to the transgenic siRNA transcript attaching to host mRNA with slightly imperfect sequence homology, or random insertion of the transgene into developmental or regulatory elements, require an alternative strategy for future work. An additional caveat to the introduction of exogenous silencing transcripts to the plant cell, observed in recent research, is the possibility that siRNAs may play a role as a regulator of gene expression not only for mRNA degradation, but

also in translational inhibition, or heterochromatin regulation, and thus transgenic siRNAs may act on non target gene expression or some aspect of developmental regulation (Jackson and Linsley 2004). Observations on the detrimental effects of multiple insertion events, or somaclonal variation, in transformed rice RNAi silenced lines, have been seen to significantly affect the defense response (Delteil et al. 2010). Two potential alternatives are available to address the significant issues. The first, silencing of genes using artificial micro RNAs is now available to the rice researcher (http://weigelworld.org) (Warthmann et al. 2008). This system does not address random insertion, but does address specificity issues that are apparent using the RNAi silencing technique. By utilizing only a 21mer sequence that is highly specific to the target gene, and stronger promoters to drive the silencing construct, this second generation technology has been shown to significantly increase the silencing efficiency, as well as significantly decrease the side effects associated with the first generation technology (Warthmann et al. 2008). The second alternative utilizes openly available gene-knockout t-DNA insertion mutant lines of rice developed in various countries. These lines completely inhibit transcription of the target gene, and, when characterized for single insertion, provide a putatively unbiased portrait of the target gene's effect on plant interactions.

Complications in our research were not solely due to the transformation process. Variable expression of the18s internal control in the lines represented in Figure 7 suggests that the cDNA present in the sample may have been damaged, or concurrently, mechanical error in pipetting may have led to unequal

amounts of template cDNA. Either of these causes could attribute to an inaccurate representation of the true expression profile of the plant. Unfortunately, this makes the accrued expression data for the surveyed T2 population unreliable, and will require further examination to verify the expression profile associated with all of the surveyed chitinases genes. Supplies of mRNA extracted during experimentation, for examination in other T1 transgenic individuals, remain for analysis. These samples may be of better quality for further chitinases analysis. Or conversely, examination of T3 individuals from highly susceptible T2 parents remains to be pursued. If chitinases do have a role in developmental processes, slight changes in the abundance of transcriptional information could have very large effects on the defense response signal transduction pathway. More precise measurements of chitinase activity must be made to confirm Os02g39330 and Os04g41620 silencing, reduced expression levels in other chitinases, and any further rice chitinase relationships with disease resistance.

In 113 cultivars of rice, plants having higher constitutive expression of chitinases, as well as earlier and greater induction of chitinases downstream of perception, were associated with increased resistance to *R. solani* inoculation (Shrestha et al. 2008). To capture the true diversity occurring *in planta* for the class IV and VII chitinases, and their effect on other rice chitinases, a system with much finer accuracy, encompassing a broader time course would be necessary. RT-PCR, while semi-quantitative, is not nearly as sensitive to transcript abundance as real time quantitative PCR (qPCR). An ideal experiment

would utilize qPCR to first examine expression levels of Os02g39330, Os04g41620, and Os04g1680 in mutant lines, with each of the individual genes silenced, to give an idea of any potential compensation made by any of the immediately closest gene family members (those with the class IV catalytic domain). Tissues of several different ages would ideally be collected from leaf, sheath, and root for analysis. Once this initial research was completed, pathogen inoculation studies could be done to examine the class IV and VII chitinase expression activities in response to pathogen presence, as well as again examining the representatives from each of the other chitinase classes, in the mutant and corresponding wild type plants, over a time course post-inoculation. A time course post-inoculation is an integral component to further research, as it may reflect which chitinases are actually important to the interaction. These types of experiments would allow for a much improved picture of chitinase activity in basal defense response in rice.

Table 1. Chitinase genes used for nomenclature in this study.

Class	Glycosyl Hydrolase Family	Chitin Binding Domain	Catalytic Domain	Rice Gene Nomenclature	PR Class	Predicted Chitinase Genes in Nipponbare Genome[d]
I[c]	19	1	I	*OsChia1*	PR-3	6
II[c]	19	-	I/II[b]	*OsChia2*	PR-3	5
IV[c]	19	1	IV	*OsChia4*	PR-3	2
V	19	2	Class I Homology	*OsChia5*	PR-3	0
VI	19	1/2 + Proline Linker	Class I Homology	*OsChia6*	PR-3	0
VII[c]	19	-	IV	*OsChia7*	PR-3	1
I[c]	18	-	Hevamine-like	*OsChic1*	PR-11	2
III[c]	18	-	Hevamine-like	*OsChib1*	PR-8	31

[a] Nomenclature based on Neuhaus (1999); three additional chitinase-like genes were identified in Nipponbare, but did not conform to the nomenclature.

[b] Deletion in catalytic domain forms subclass [c] Chitinase class observed in this study [d] TIGR/MSU Pseudomolecule release 6.1

Table 2. Primers used for analysis in this study.

TIGR Locus Id	Primer Name	T_a	Amplicon (bp)	Primer Sequence ('5-3')
LOC_Os01g64110	1.64110F	61	305	GTACGGAGGGATCATGCTGT
	1.64110R			TGCCACATGCGAAAATAGAA
LOC_Os02g39330	T2-2.39330F	60	130	CAGCAACCTCACCTGCTAAT
	T2-2.39330R			ACTCCTATGCCGCAAACAAC
LOC_Os02g39330	T0-T1 2.39330F	57	239	CCCCGTGCTGGCGTTCC
	T0-T1 2.39330R			TAGCATATGTCAATCCCTGTAAT
LOC_Os03g04060	3.04060F	57.5	343	CGGGTCGGCTACTACAAGAG
	3.04060R			GGCATCGTTCCACCAGTAAT
LOC_Os04g41620	4.41620F	58	550	CAAGAGCAACAAACAGTGGC
	4.41620R			CGCCCTAAGATAGAGTAACATCG
LOC_Os04g41680*	4.41680F	59	750	TAACGCTGCCCACTCCTACT
	4.41680R			TCCGTACCAAACTCTTGACG
LOC_Os05g15770	5.15770F	56.3	287	TGGTAATGAGCCTGGTCTCC
	5.15770R			AAGAAAGTAAGCCTGCCCGT
LOC_Os06g51060*	6.51060F [1]	61	404	CATCGGCTCCAACCTGCT
	6.51060R [1]			ATTCATCGATGTAAAGCATCCG
LOC_Os08g41100	8.41100F	55.3	279	CCTGGCTTTGGTGCTACAAT
	8.41100R			ACGCTTTTCTTTGTTTGGGA
LOC_Os10g28050	10.28050F	62	411	TGAGACAGAATCGCAAGCAC
	10.28050R			TCACCACATCCACAGAACTGA
LOC_Os10g39680*	10.39680F [1]	61	402	GTATGGCGTCATCACCAACATC
	10.39680R [1]			ACTTCATGCATGTGTTCAAACG
LOC_Os11g47500*	11.47500F [1]	61	410	TAATTACGGCGTCATGTGTTTG
	11.47500R [1]			GGACACATCGATCACACATGC
LOC_Os09g00999/ NCBI AC# X00755	18sF [2]	54-56	169	ATGATAACTCGACGGATCGC
	18sR [2]			CTTGGATGTGGTAGCCGTTT
LOC_Os02g39330 Construct Primers	Chitinase F5	55-57	240	CACCATCCGCGCCATCAACG
	Chi3UTR R5			CTCCTATGCCGCAAACAACG
	GUS-linkF			CATGAAGATGCGGACTTACG
	GUS-linkR	60	636	ATCCACGCCGTATTCGG
	HygroF			GAGCCTGACCTATTGCATCTCC
	HygroR	54	456	GGCCTCCAGAAGAAGATGTTGG
LOC_Os03g08050	EF1aF			AGCCTCGTTCAAATGGTGGT
	EF1aR	55-61	126	TAGTGCACATTGCGAGCAGA

[1] Kim et al. 2009 [2] Kim et al. 2003 * Inconsistent or no expression in 12 day old Kitaake leaves

Table 3. Expression trends of family 18 (A) and 19 (B) glycosyl hydrolases in response to *M. oryzae and X. oryzae pv. oryzae* in susceptible and resistant interactions. Data are summarized from MPSS (Nakano et al. 2006) database (http://**mpss**.udel.edu/**rice**/). ***LOC_OsXXgXXXXX*** in bold and italic were examined in this study.

A TIGR LOCUS ID	*Mo* Resistant	*Mo* Susceptible	*Xoo* Resistant	*Xoo* Susceptible
LOC_Os01g19750	-	-	-	-
LOC_Os01g43220	▲	▲	▲	▼
LOC_Os01g47070	▲	▲	▲	▲
LOC_Os01g49320	▲	▲	▲	▲
LOC_Os01g64100	-	-	▲ /-	-
LOC_Os01g64110	▲	▲	▲	▲
LOC_Os04g27980	-	-	-	-
LOC_Os04g30770	-	-	▲	-
LOC_Os05g15770	▲	▲	▲	▲
LOC_Os05g15850	-	-	-	-
LOC_Os05g15880	-	-	-	-
LOC_Os05g15920	-	-	-	-
LOC_Os06g25010	▲	▲	▲ /-	▲
LOC_Os07g01770	▲ /-	-	-	-
LOC_Os07g19040	-	-	-	-
LOC_Os07g23850	-	-	-	-
LOC_Os07g43820	-	-	-	-
LOC_Os08g40680	-	-	-	-
LOC_Os08g40690	▲	▲	-	-
LOC_Os08g40740	-	-	-	-
LOC_Os10g28050	▲	▲	▲	▲
LOC_Os10g28080	▲	▲	▲	▲
LOC_Os10g28120	▲	▲	▲	▲
LOC_Os11g27400	-	-	-	-
LOC_Os11g47500	▲	▲	-	-
LOC_Os11g47520	-	-	-	-
LOC_Os11g47530	-	-	-	-
LOC_Os11g47550	-	-	-	-
LOC_Os11g47560	▲	▲	-	-
LOC_Os11g47570	-	-	▲	-
LOC_Os11g47580	-	-	-	-
LOC_Os11g47590	-	-	-	-
LOC_Os11g47600	▲	▲	-	-
LOC_Os11g47610	-	-	-	-

▲ Increased expression trend relative to 0hr treatment
▼ Decreased expression trend relative to Ohr treatment
▲ /- Increased expression but at less than 20 transcripts per million
▼ /- Decreased expression, but at less than 20 transcripts per million
- No expression

B. LOCUS ID	Mo Resistant	Mo Susceptible	Xoo Resistant	Xoo Susceptible
LOC_Os01g18400	▲	▲ /-	▲ /-	▼ /-
LOC_Os02g39330	▲	▲	▲	▲
LOC_Os03g04060	▲	▲	▼	▼
LOC_Os03g30470	▲	-	-	-
LOC_Os04g41620	▲	▲ /-	▲	▲
LOC_Os04g41680	-	▲ /-	▲ /-	▲ /-
LOC_Os05g04690	-	-	-	-
LOC_Os05g33130/ LOC_Os05g33140	Δ	Δ	-	-
LOC_Os05g33150	-	-	-	-
LOC_Os06g51050	▲	▲	▲ /-	-
LOC_Os06g51060	▲	▲	▲	▲ /-
LOC_Os08g41100	▼	▼	▲	▲
LOC_Os09g32080	▲	▲	▼	▼
LOC_Os10g39680	▲	▲	▲	-
LOC_Os10g39700	-	-	-	-

▲ Increased expression trend relative to 0hr treatment

▼ Decreased expression trend relative to 0hr treatment

▲/- Increased expression but at less than 20 transcripts per million

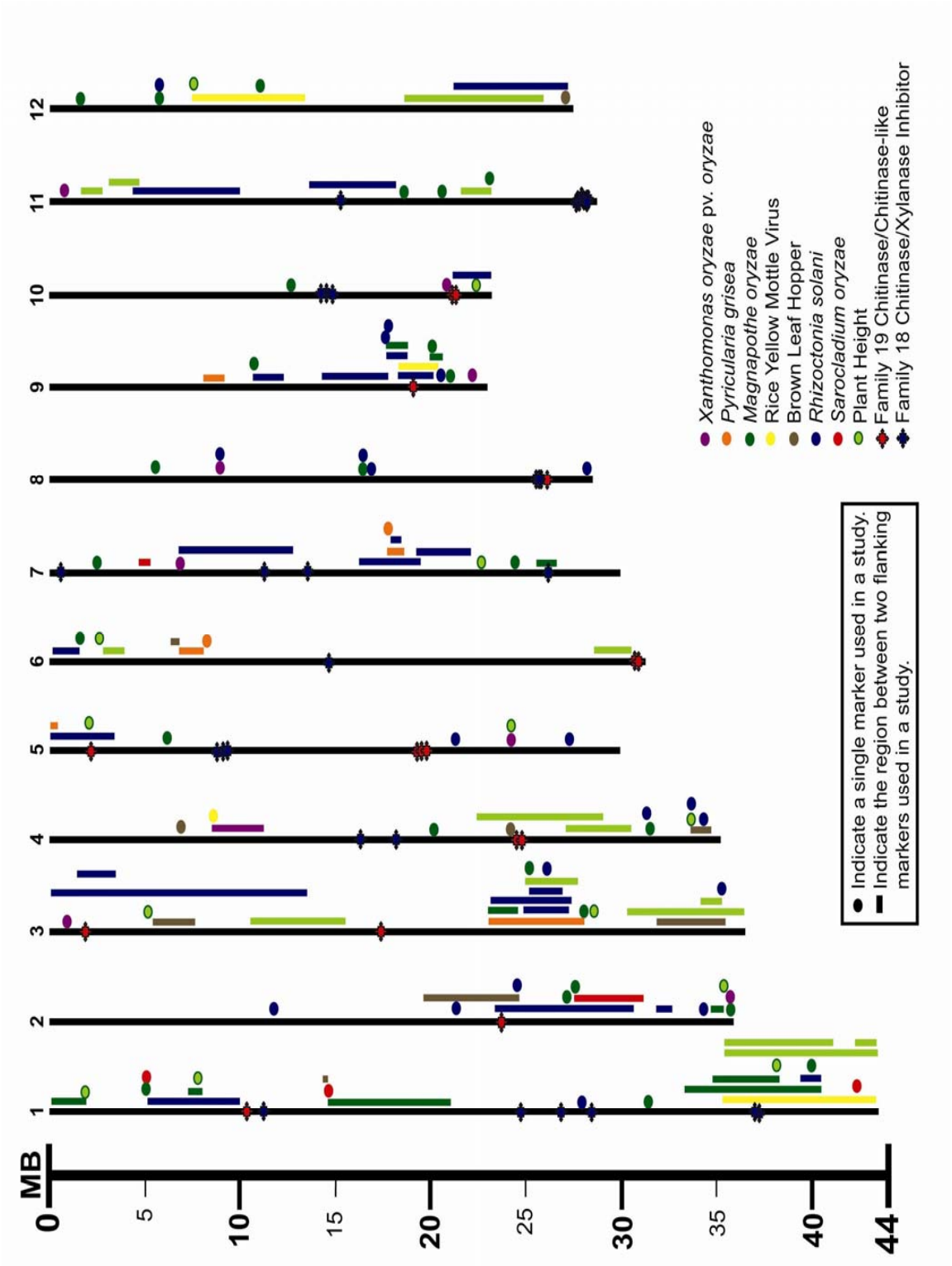
▼/- Decreased expression, but at less than 20 transcripts per million

Δ Shared expression signature

- No expression

Figures

Figure 1. Estimated location of rice defense response and plant height QTL in 27 mapping studies (Alam and Cohen 1998; Albar et al. 1998; Chen et al. 2003; Courtois et al. 2003; Cui et al. 2008; Fukuoka and Okuno 2001; Han et al. 2002; Hemamalini, Shashidhar, and Hittalmani 2000; Hittalmani et al. 2002; Huang et al. 2001; Li et al. 1995; Li et al. 1999; Liu et al. 2004; Liu et al. 2009; MacMillan et al. 2006; Maheswaran et al. 2000; Miyamoto, Yano, and Hirasawa 2001; Pinson, Capdevielle, and Oard 2005; Ramalingam et al. 2003; Sirithunya et al. 2002; Srinivaschary et al. 2002; Tabien et al. 2002; Wang et al. 1994; Wu et al. 2004; Xu et al. 2004; Zenbayashi et al. 2002; Zou et al. 2000). Glycosyl hydrolase family 18 and 19 genes with chitinase or chitinase-like structures often show co-localization with regions of the genome strongly linked to defense response and plant height QTL.


MB
0
5
10
15
20
25
30
35
40
44
1
2
3
4
5
6
7
8
9
10
11
12
Xanthomonas oryzae pv. oryzae
Pyricularia grisea
Magnapothe oryzae
Rice Yellow Mottle Virus
Brown Leaf Hopper
Rhizoctonia solani
Sarocladium oryzae
Plant Height
Family 19 Chitinase/Chitinase-like
Family 18 Chitinase/Xylanase Inhibitor
Indicate a single marker used in a study.
Indicate the region between two flanking markers used in a study.

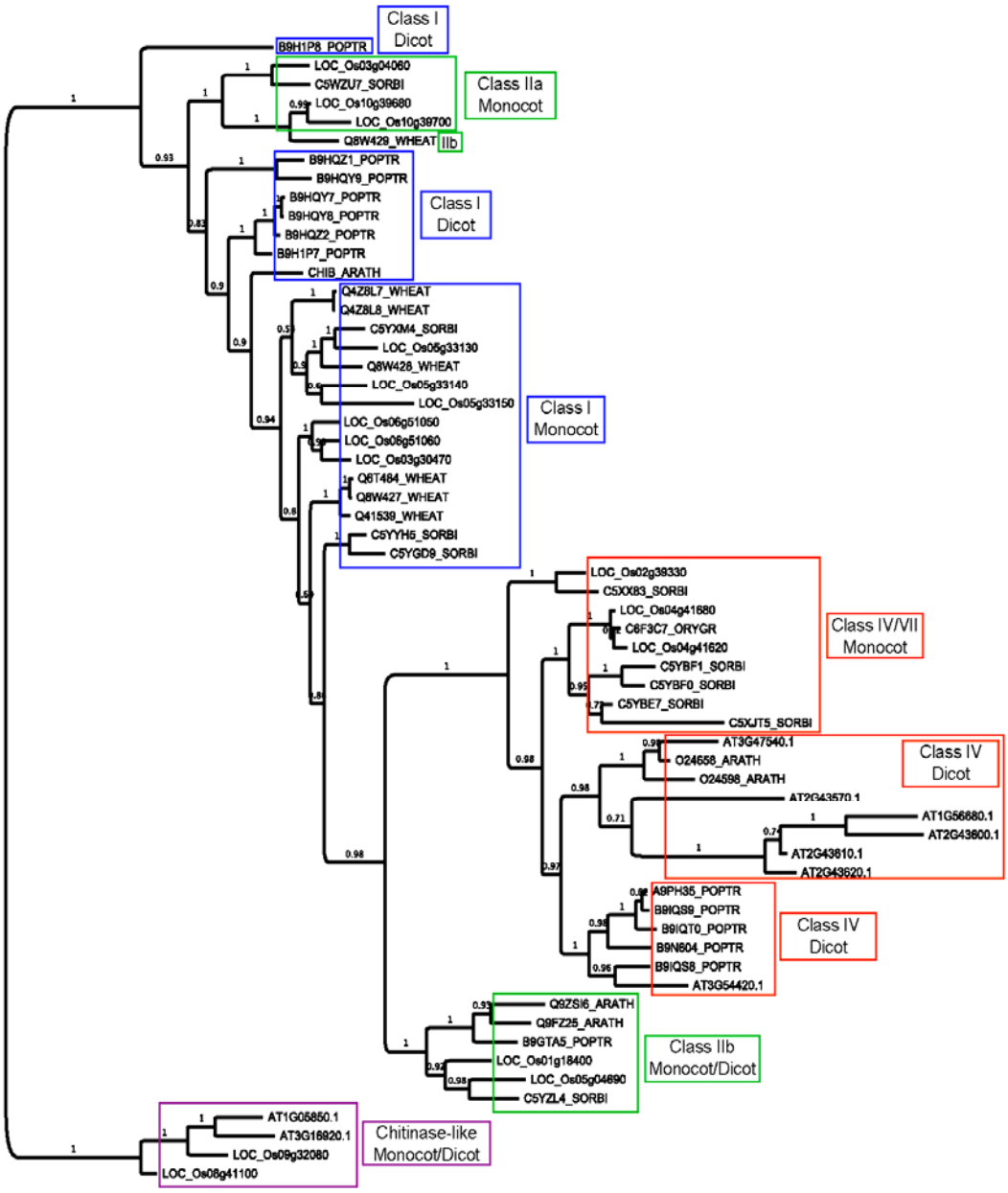


Figure 2. Relationships among Family 19 chitinase genes from *Oryza sativa* (LOC_OsXXgXXXXXX), *A. thaliana* (ATXGXXXXX, ARATH), *S. bicolor* (SORBI), *T. aestivum* (WHEAT), *P. trichocarpa* (POPTR), and *O. grandiglumis* (ORYGR) reconstructed using Bayesian analysis of amino acid sequences. Posterior probabilities (scaled to 100) are indicated at nodes.

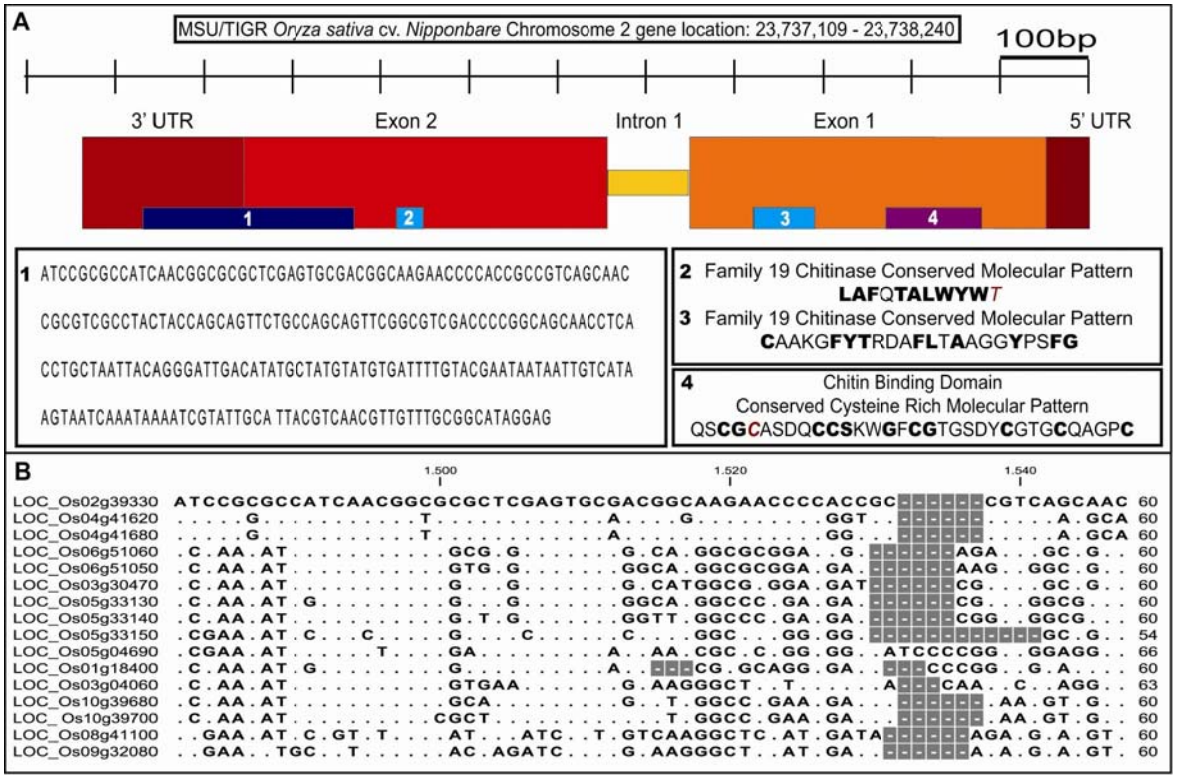


Figure 3. (A) Os02g39330 gene structure. Silencing construct location and sequence (1), chitinase catalytic domain molecular patterns (2, Prosite-PS00774) (3, PS00773), and chitin binding molecular pattern (4, PS00026). Amino acid residues in bold are highly conserved in the chitin binding domain or catalytic domain. Residues in *red italic* show sequence variations from the molecular pattern standard. (B) Alignment of family 19 glycosyl hydrolase chitinases over the most conserved 60 bp of the 239 bp region selected for silencing. Dots represent consensus sequence to Os0239330; grey areas represent gaps in the alignment.

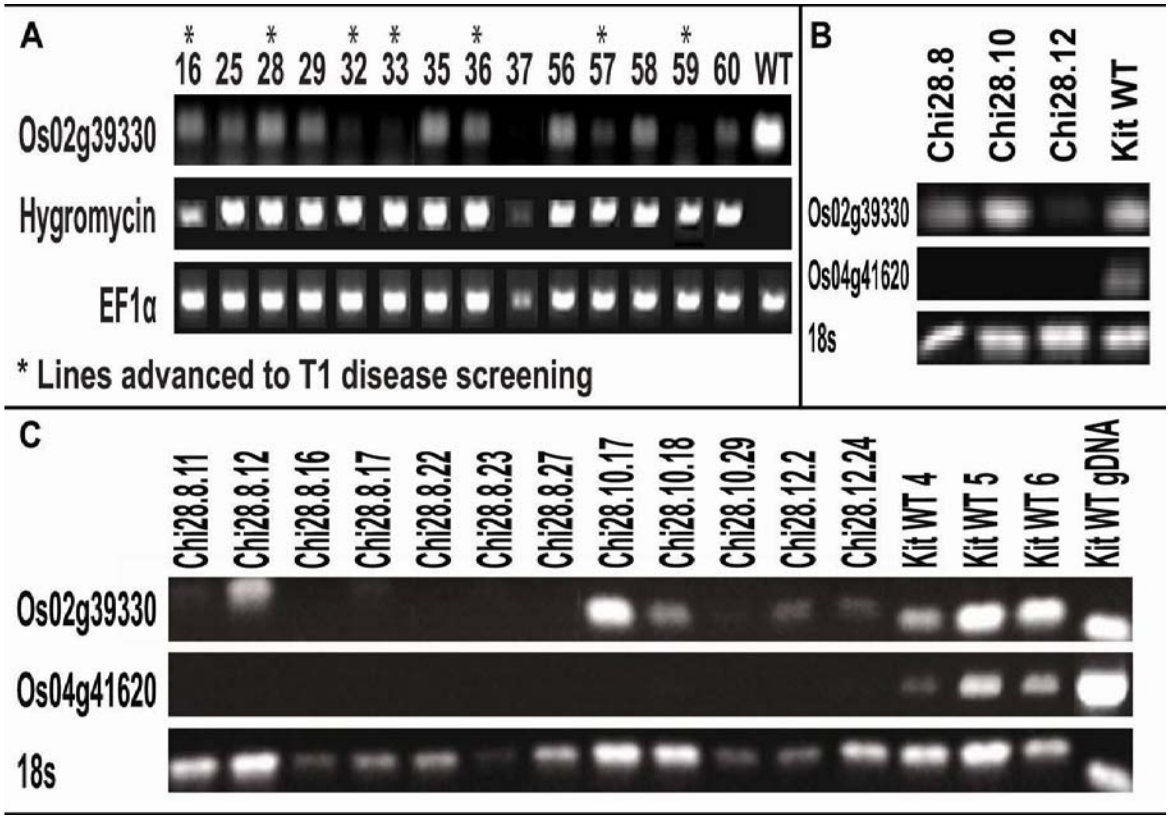


Figure 4. Silencing of Os02g39330 compared to wild type control Kitaake (Kit) in T0 (A) lines, and silencing of Os02g39330 and Os04g41620 compared to wild type control Kitaake (Kit) in T1 (B) and T2 (C) lines.

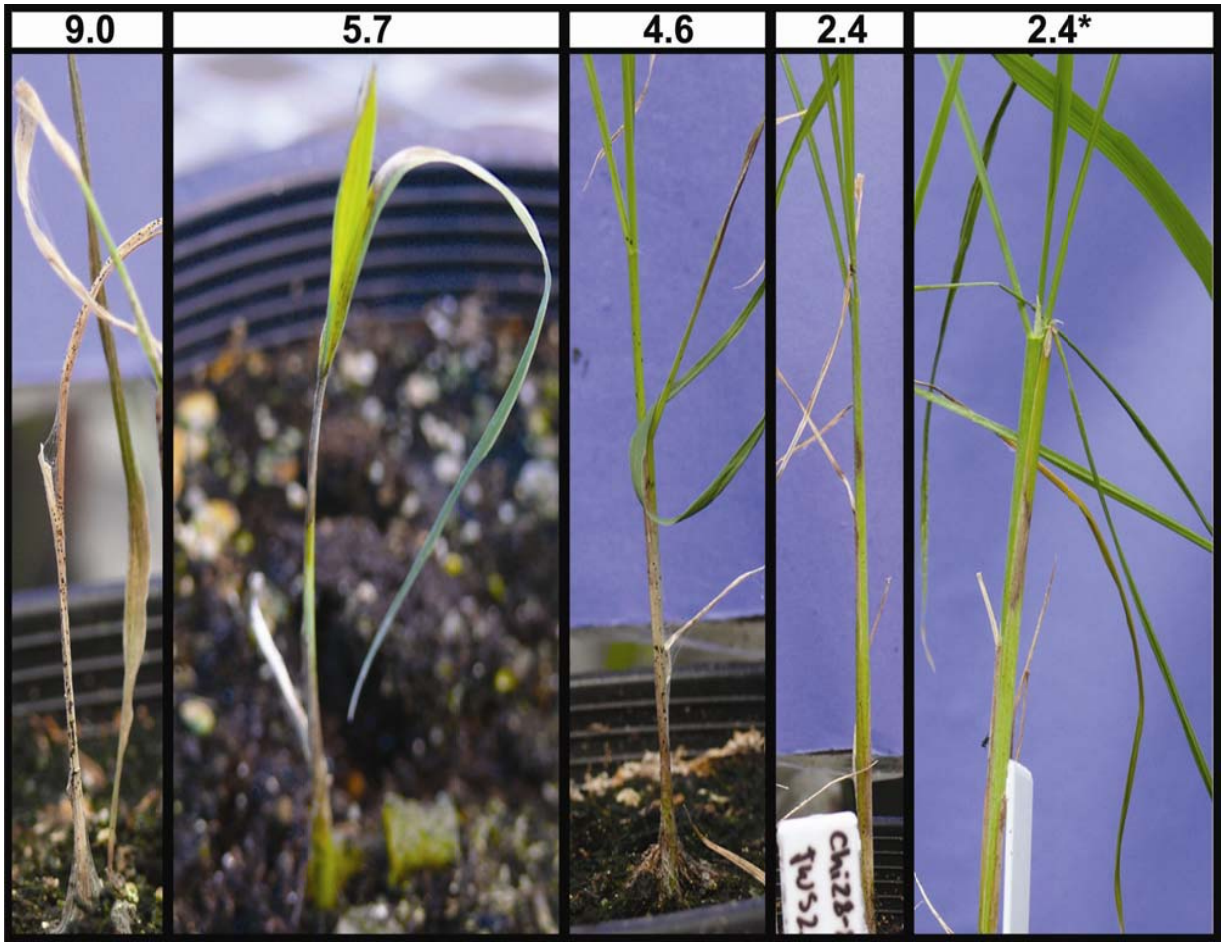


Figure 5. Representative photos of the range of disease phenotypes and corresponding numeric values, on a scale of 0-9, of disease indexes (DI = (lesion length/plant height)*9) in a subset of R. solani inoculated T2 chitinase transgenic and wild type plants. (*) indicates Kitaake wild type plant and DI.

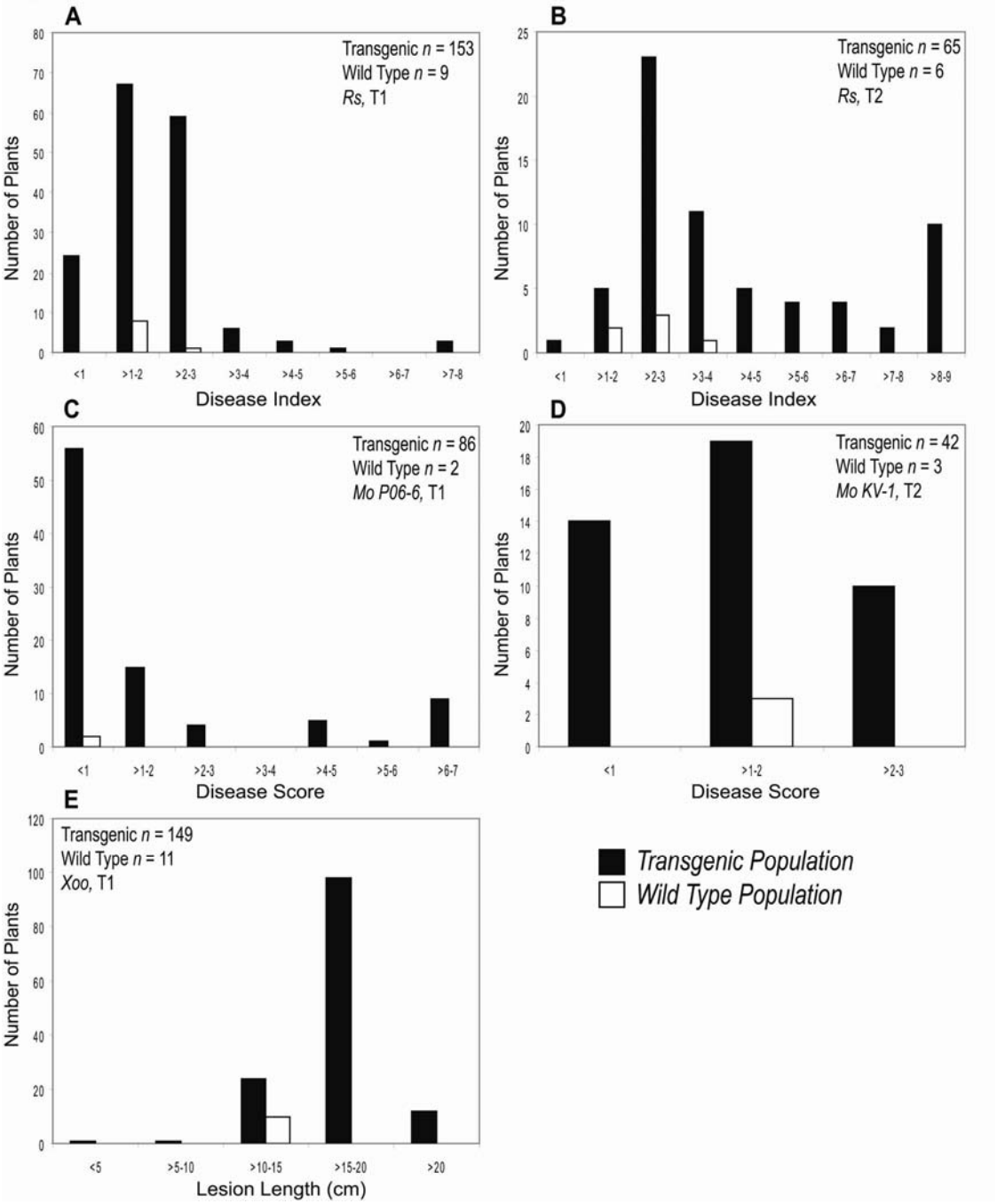


Figure 6. Population distribution of disease phenotypes in T1 (A, C, E) and T2 (B, D) lines relative to wild type control. Transgenic and wild type plants were inoculated with *R. solani* (A, B), *M. oryzae* isolate P06-6 (C), *M. oryzae* isolate KV-1 (D), and *X. oryzae* pv. *oryzae* (E).

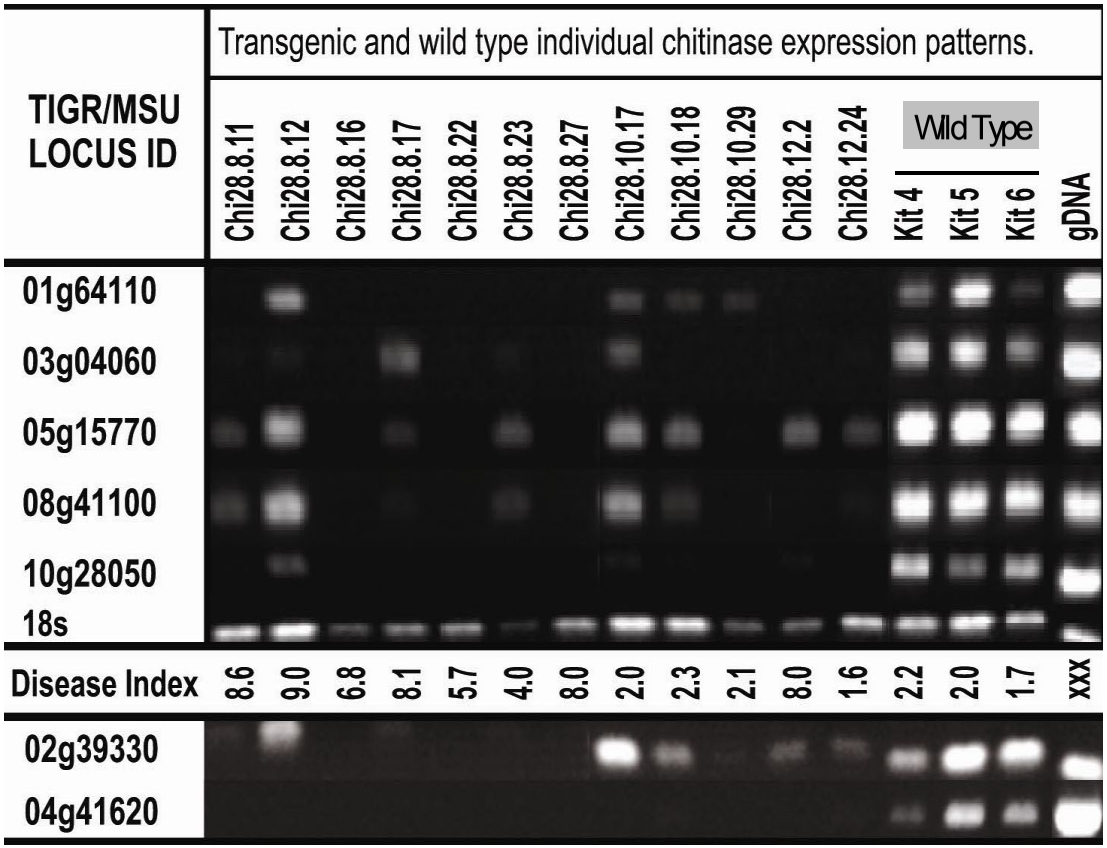


Figure 7. Reduced chitinase expression in selected T2 silenced and wild type individuals. Gel bands in each column show variable expression of other rice chitinase genes in the family 18 and 19 glycosyl hydrolases can been seen among transgenic individuals.

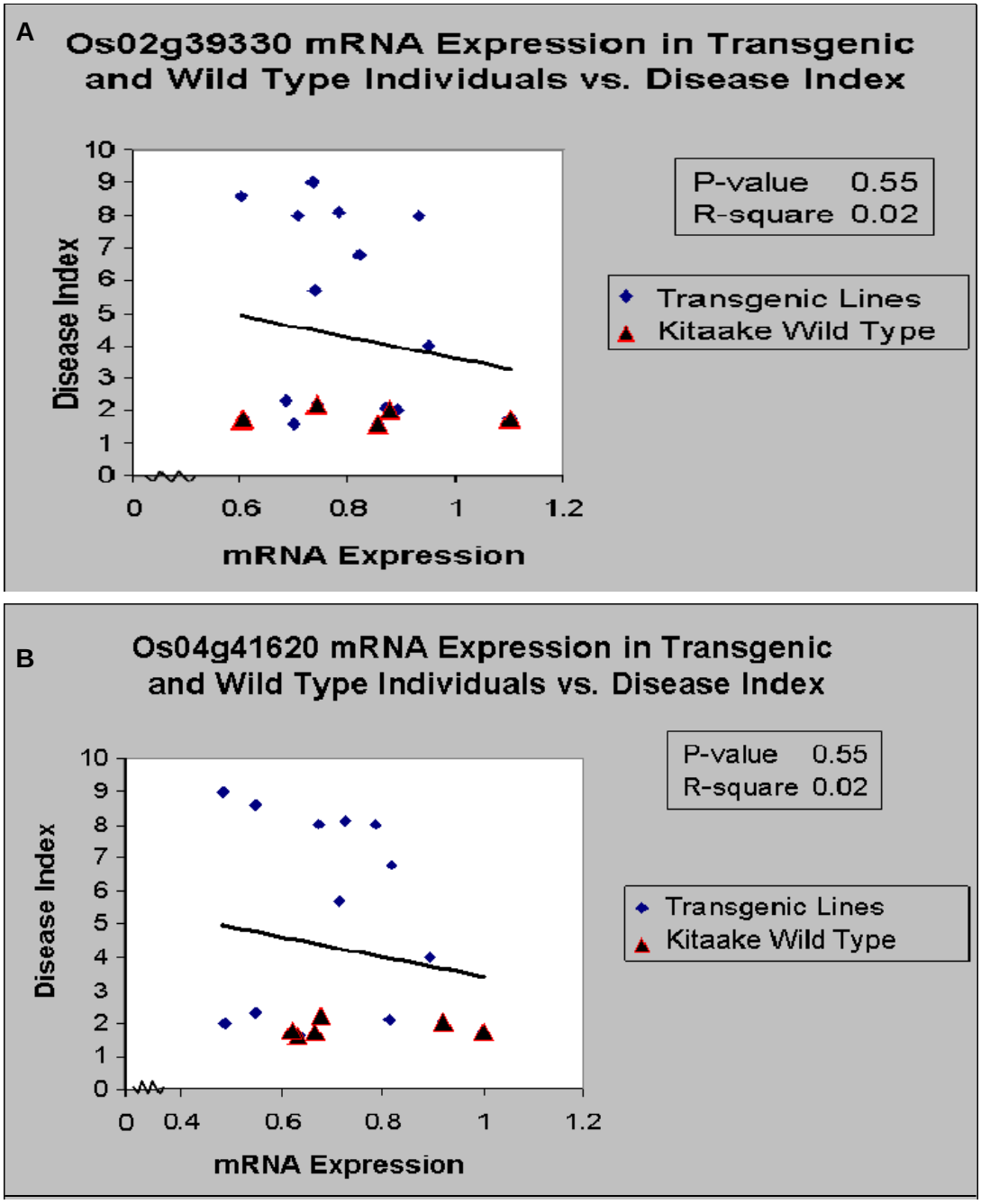


Figure 8. Linear regressions of Os02g39330 (A) and Os04g41620 (B) mRNA expression values versus *R. solani* disease index show that disease phenotype does not correlate with silencing in transgenic individuals.

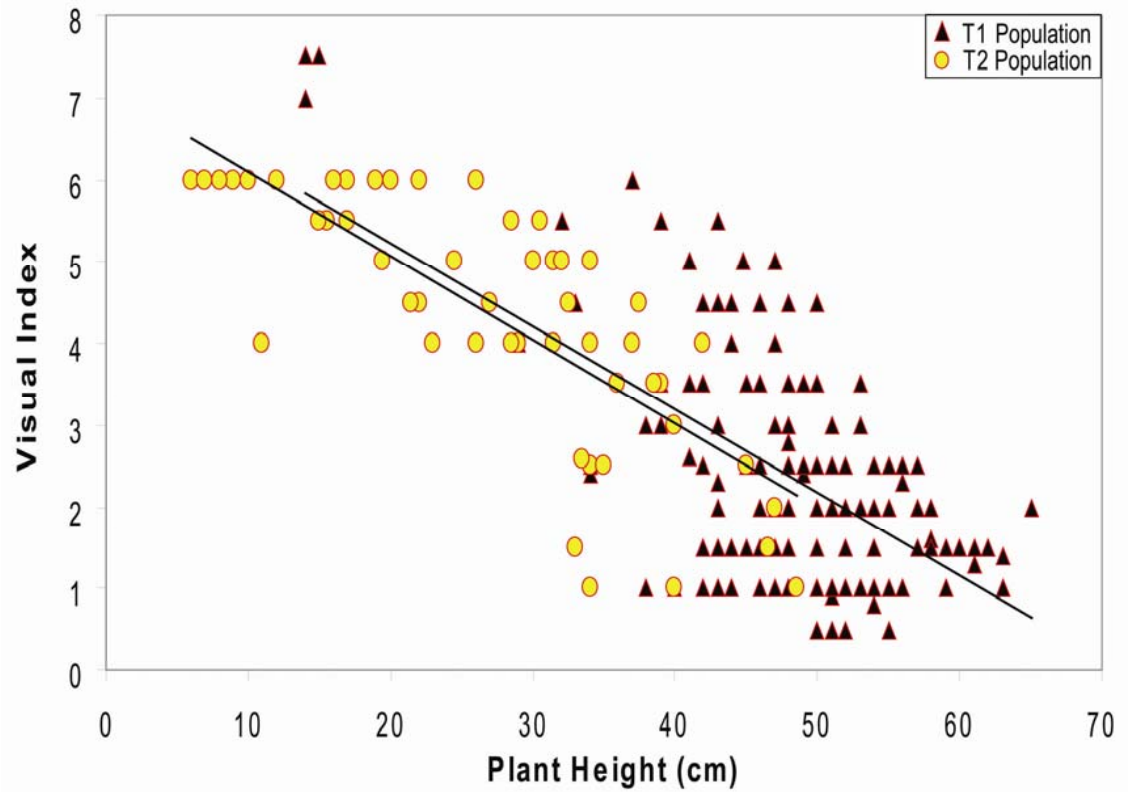


Figure 9. Sheath blight disease (visual index) and plant height (cm) are negatively correlated in T1 (n = 153) and T2 (n = 65) transgenic populations. As plant height increases in the T1 and T2 chitinase silenced lines, disease severity decreases.

References

Aki, T., M. Shigyo, R. Nakano, T. Yoneyama, and S. Yanagisawa. 2008. Nano scale proteomics revealed the presence of regulatory proteins including three FT-Like proteins in phloem and xylem saps from rice. *Plant Cell Physiol* 49 (5):767-90.

Alam, S. N., and M. B. Cohen. 1998. Detection and analysis of QTLs for resistance to the brown planthopper, Nilaparvata lugens, in a doubled-haploid rice population. *Theoretical and Applied Genetics* 97 (8):1370-1379.

Albar, L., M. Lorieux, N. Ahmadi, I. Rimbault, A. Pinel, A. A. Sy, D. Fargette, and A. Ghesquiere. 1998. Genetic basis and mapping of the resistance to rice yellow mottle virus. I. QTLs identification and relationship between resistance and plant morphology. *Theoretical and Applied Genetics* 97 (7):1145-1154.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J Mol Biol* 215 (3):403-10.

Arlorio, M., A. Ludwif, T. Boller, and P. Bonfante. 1992. Inhibition of fungal growth by plant chtinases and β-1,3-glucanases. *Protoplasma* 171:34-43.

Bairoch, Amos. 1992. Prosite: a dictionary of sites and patterns in proteins. *Nucl. Acids Res.* 20 (suppl):2013-2018.

Baulcombe, D. 2004. RNA silencing in plants. *Nature* 431 (7006):356-63.

Benson, Dennis A., Ilene Karsch-Mizrachi, David J. Lipman, James Ostell, and David L. Wheeler. 2008. GenBank. *Nucl. Acids Res.* 36 (suppl_1):D25-30.

Berglund, L., J. Brunstedt, K. K. Nielsen, Z. Chen, J. D. Mikkelsen, and K. A. Marcker. 1995. A proline-rich chitinase from Beta vulgaris. *Plant Mol Biol* 27 (1):211-6.

Boller, T. 1988. Ethylene and the regulation fo antifungal hydrolases in plants. *Oxford Surveys of Plant Molecular and Cell Biology* 5:145-174.

Boller, T., and S. Y. He. 2009. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science* 324 (5928):742-4.

Boller, T., A. Gehri, F. Mauch, and U. Vogeli. 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* 157:22-31.

Broglie, Karen, Ilan Chet, Mark Holliday, Robert Cressman, Phyllis Biddle, Susan Knowlton, Jeffry C. Mauvais, and Richard Broglie. 1991. Transgenic Plants with Enhanced Resistance to the Fungal Pathogen *Rhizoctonia solani*. *Science* 254 (5035):1194-1197.

Cantrell, R. P., and T. G. Reeves. 2002. The rice genome. The cereal of the world's poor takes center stage. *Science* 296 (5565):53.

Carillo, Maria Gay C. 2009. Genomic analyses of rice oxalate oxidases and their association with quantitative resistance to rice blast., Molecular Biology and Biotechnology, UPLB, Los Banos, Laguna.

Chareonpornwattana, S., K. V. Thara, L. Wang, S. K. Datta, W. Panbangred, and S. Muthukrishnan. 1999. Inheritance, expression, and silencing of a chtitinase transgene in rice. *Theor Appl Genet* 98:371-378.

Chen, H., S. P. Wang, Y. Z. Xing, C. G. Xu, P. M. Hayes, and Q. F. Zhang. 2003. Comparative analyses of genomic locations and race specificities of loci for quantitative resistance to Pyricularia grisea in rice and barley. *Proceedings of the National Academy of Sciences of the United States of America* 100 (5):2544-2549.

Chisholm, S. T., G. Coaker, B. Day, and B. J. Staskawicz. 2006. Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124 (4):803-14.

Collinge, D. B., K. M. Kragh, J. D. Mikkelsen, K. K. Nielsen, U. Rasmussen, and K. Vad. 1993. Plant chitinases. *Plant J* 3 (1):31-40.

Courtois, B., L. Shen, W. Petalcorin, S. Carandang, R. Mauleon, and Z. Li. 2003. Locating QTLs controlling constitutive root traits in the rice population IAC 165 x Co39. *Euphytica* 134 (3):335-345.

Croes, E., K. Gebruers, S. Carpentier, R. Swennen, J. Robben, K. Laukens, E. Witters, J. A. Delcour, and C. M. Courtin. 2009. A quantitative portrait of three xylanase inhibiting protein families in different wheat cultivars using 2D-DIGE and multivariate statistical tools. *J Proteomics* 72 (3):484-500.

Cui, K. H., J. L. Huang, Y. Z. Xing, S. B. Yu, C. G. Xu, and S. B. Peng. 2008. Mapping QTLs for seedling characteristics under different water supply conditions in rice (Oryza sativa). *Physiologia Plantarum* 132 (1):53-68.

Datta, K., J. Tu, N. Oliva, I. I. Ona, R. Velazhahan, T. W. Mew, S. Muthukrishnan, and S. K. Datta. 2001. Enhanced resistance to sheath blight by constitutive expression of infection-related rice chitinase in transgenic elite indica rice cultivars. *Plant Sci* 160 (3):405-414.

Delteil, Amandine, Jie Zhang, Philippe Lessard, and Jean-Benoit Morel. 2010. Potential candidate genes for improving rice disease resistance. . *Rice.* ***In Review.***

Desaki, Y., A. Miya, B. Venkatesh, S. Tsuyumu, H. Yamane, H. Kaku, E. Minami, and N. Shibuya. 2006. Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells. *Plant Cell Physiol* 47 (11):1530-40.

Durand, A., R. Hughes, A. Roussel, R. Flatman, B. Henrissat, and N. Juge. 2005. Emergence of a subfamily of xylanase inhibitors within glycoside hydrolase family 18. *Febs J* 272 (7):1745-55.

Ebbole, D. J. 2007. Magnaporthe as a model for understanding host-pathogen interactions. *Annu Rev Phytopathol* 45:437-56.

Eizenga, G. C. 2002. Screening *Oryza* Species Plants for Sheath Blight Resistance. *Plant Disease* 86 (7):808-812.

EPPO, CABI and. 1990. Xanthomonas oryzae Data Sheets on Quarantine Pests.

Evenson, R. E., R. W. Herdt, and M. Hossain. 1998. Rice research in Asia: Progress and Priorities. *CAB International*:-418.

Fukuoka, S., and K. Okuno. 2001. QTL analysis and mapping of pi21, a recessive gene for field resistance to rice blast in Japanese upland rice. *Theoretical and Applied Genetics* 103 (2-3):185-190.

Garcia-Casado, G., C. Collada, I. Allona, R. Casado, L. F. Pacios, C. Aragoncillo, and L. Gomez. 1998. Site-directed mutagenesis of active site residues in a class I endochitinase from chestnut seeds. *Glycobiology* 8 (10):1021-8.

Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol* 43:205-27.

Gnanamanickam, S. S., V. B. Priyadarisini, N. N. Narayanan, P. Vasudevan, and S. Kavitha. 1999. An overview of bacterial blight disease of rice and strategies for its management. *Current Science* 77 (11).

Groth, D.E., M. C. Rush, and G. D. Lindberg. 1990. Foliar fungicides for control of rice diseases in the United States. In *Pest Management in Rice*, edited by B. T. Grayson, M. B. Green and L. G. Copping. London and New York: Elsevier Applied Science.

Han, YP, YZ Xing, ZX Chen, SL Gu, XB Pan, XL Chen, and QF Zhang. 2002. Mapping QTLs for horizontal resistance to sheath blight in an elite rice restorer line, Minghui 63. *Yi chuan xue bao= Acta genetica Sinica* 29 (7):622.

Hanfrey, C., M. Fife, and V. Buchanan-Wollaston. 1996. Leaf senescence in Brassica napus: expression of genes encoding pathogenesis-related proteins. *Plant Mol Biol* 30 (3):597-609.

Hannon, G. J. 2002. RNA interference. *Nature* 418 (6894):244-51.

Hart, C. M., B. Fischer, J. M. Neuhaus, and F. Meins, Jr. 1992. Regulated inactivation of homologous gene expression in transgenic Nicotiana sylvestris plants containing a defense-related tobacco chitinase gene. *Mol Gen Genet* 235 (2-3):179-88.

Held, M. A., B. Penning, A. S. Brandt, S. A. Kessans, W. Yong, S. R. Scofield, and N. C. Carpita. 2008. Small-interfering RNAs from natural antisense transcripts derived from a cellulose synthase gene modulate cell wall biosynthesis in barley. *Proc Natl Acad Sci U S A* 105 (51):20534-9.

Hemamalini, G. S., H. E. Shashidhar, and S. Hittalmani. 2000. Molecular marker assisted tagging of morphological and physiological traits under two contrasting moisture regimes at peak vegetative stage in rice (Oryza sativa L.). *Euphytica* 112 (1):69-78.

Hittalmani, S., H. E. Shashidhar, P. G. Bagali, N. Huang, J. S. Sidhu, V. P. Singh, and G. S. Khush. 2002. Molecular mapping of quantitative trait loci for plant growth, yield and yield related traits across three diverse locations in a doubled haploid rice population. *Euphytica* 125 (2):207-214.

Horton, P., K. J. Park, T. Obayashi, N. Fujita, H. Harada, C. J. Adams-Collier, and K. Nakai. 2007. WoLF PSORT: protein localization predictor. *Nucleic Acids Res* 35 (Web Server issue):W585-7.

Hu, K. M., D. Y. Qiu, X. L. Shen, X. H. Li, and S. P. Wang. 2008. Isolation and manipulation of quantitative trait loci for disease resistance in rice using a candidate gene approach. *Mol Plant* 1 (5):786-93.

Huang, Z, G He, L Shu, X Li, and Q Zhang. 2001. Identification and mapping of two brown planthopper resistance genes in rice. *TAG Theoretical and Applied Genetics* 102 (6):929-934.
Huckelhoven, R. 2007. Cell wall-associated mechanisms of disease resistance and susceptibility. *Annu Rev Phytopathol* 45:101-27.
IRRI. 2007. *Why an international research center for rice?* Monday, December 03, 2007 1:52:59 PM 2007 [cited December 3, 2007 2007]. Available from http://www.irri.org/about/international.asp.
IRRI, Rice Doctor -. 2007. *Sheath Blight Fact Sheet* Monday, November 26, 2007 1:02:22 AM 2003 [cited 11/26/2007 8:40 AM 2007]. Available from http://www.knowledgebank.irri.org/RiceDoctor/default.htm.
Jackson, A. L., and P. S. Linsley. 2004. Noise amidst the silence: off-target effects of siRNAs? *Trends Genet* 20 (11):521-4.
James, V. A., B. Worland, J. W. Snape, and P. Vain. 2004. Development of a standard operating procedure (SOP) for the precise quantification of transgene expression levels in rice plants. *Physiol Plant* 120 (4):650-656.
Jekel, P. A., B. H. Hartmann, and J. J. Beintema. 1991. The primary structure of hevamine, an enzyme with lysozyme/chitinase activity from Hevea brasiliensis latex. *Eur J Biochem* 200 (1):123-30.
Jia, Y., F. Correa-Victoria, A. McClung, L. Zhu, G. Liu, Y. Wamishe, J. Xie, M. A. Marchetti, S. R. M. Pinson, J. N. Rutger, and J. C. Correll. 2007. Rapid Determination of Rice Cultivar Responses to the Sheath Blight Pathogen *Rhizoctonia solani* Using a Micro-Chamber Screening Method. *Plant Disease* 91:485-489.
Jia, Y., Valent, B., Lee, F. N. . 2003. Determination of host responses to *Magnaporthe grisea* on detached rice leaves using a spot inoculation method. . *Plant Dis* 87:129-133.
Jones, J. D., and J. L. Dangl. 2006. The plant immune system. *Nature* 444 (7117):323-9.
Kaku, H., Y. Nishizawa, N. Ishii-Minami, C. Akimoto-Tomiyama, N. Dohmae, K. Takio, E. Minami, and N. Shibuya. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc Natl Acad Sci U S A* 103 (29):11086-91.
Kankanala, P., K. Czymmek, and B. Valent. 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* 19 (2):706-24.
Kasprezewska, Anna. 2003. Plant Chitinases - Regulation and Function. *Cellular and Molecular Biology Letters* 8:809-824.
Kauffman, H. E., A. P. K. Reddy, S. P. Y. Hsieh, and S. D. Merca. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Dis. Rep.* 57:537-541.
Kim, C. Y., S. W. Gal, M. S. Choe, S. Y. Jeong, S. I. Lee, Y. H. Cheong, S. H. Lee, Y. J. Choi, C. D. Han, K. Y. Kang, and M. J. Cho. 1998. A new class II rice chitinase, Rcht2, whose induction by fungal elicitor is abolished by protein phosphatase 1 and 2A inhibitor. *Plant Mol Biol* 37 (3):523-34.

Kim, S. T., Y. H. Kang, Y. Wang, J. Wu, Z. Y. Park, R. Rakwal, G. K. Agrawal, S. Y. Lee, and K. Y. Kang. 2009. Secretome analysis of differentially induced proteins in rice suspension-cultured cells triggered by rice blast fungus and elicitor. *Proteomics* 9 (5):1302-13.

Kwon, Y., S. H. Kim, M. S. Jung, M. S. Kim, J. E. Oh, H. W. Ju, K. I. Kim, E. Vierling, H. Lee, and S. W. Hong. 2007. Arabidopsis hot2 encodes an endochitinase-like protein that is essential for tolerance to heat, salt and drought stresses. *Plant J* 49 (2):184-93.

Lee, Joohyun, Terry M. Bricker, Michael Lefevre, Shannon R. M. Pinson, and James H. Oard. 2006. Proteomic and genetic approaches to identifying defence-related proteins in rice challenged with the fungal pathogen Rhizoctonia solani. *Molecular Plant Pathology* 7 (5):405-416.

Letunic, I., T. Doerks, and P. Bork. 2009. SMART 6: recent updates and new developments. *Nucleic Acids Res* 37 (Database issue):D229-32.

Li, Z. K., S. R. M. Pinson, M. A. Marchetti, J. W. Stansel, and W. D. Park. 1995. Characterization of Quantitative Trait Loci (QTLS) in Cultivated Rice Contributing to Field-Resistance to Sheath Blight (*Rhizoctonia solani*). *Theoretical and Applied Genetics* 91 (2):382-388.

Li, Z. K., L. J. Luo, H. W. Mei, A. H. Paterson, X. Z. Zhao, D. B. Zhong, Y. P. Wang, X. Q. Yu, L. Zhu, R. Tabien, J. W. Stansel, and C. S. Ying. 1999. A "defeated" rice resistance gene acts as a QTL against a virulent strain of Xanthomonas oryzae pv. oryzae. *Molecular and General Genetics* 261 (1):58-63.

Lin, Willie, C. S. Anuratha, Karabi Datta, Ingo Potrykus, S. Muthukrishnan, and Swapan K. Datta. 1995. Genetic engineering of Rice for Resistance to Sheath Blight. *Nature Biotechnology* 13:686-691.

Liu, B., S.H. Zhang, X.Y. Zhu, Q. Yang, S. Wu, M. Mei, R. Mauleon, J.E. Leach, T. Mew, and H. Leung. 2004. Candidate defense genes as predictors of quantitative disease resistance in rice. *Molecular Plant-Microbe Interactions* 17:1146-1152.

Liu, G., Y. Jia, F. J. Correa-Victoria, G. A. Prado, K. M. Yeater, A. McClung, and J. C. Correll. 2009. Mapping quantitative trait Loci responsible for resistance to sheath blight in rice. *Phytopathology* 99 (9):1078-84.

Ma, W., and G. A. Berkowitz. 2007. The grateful dead: calcium and cell death in plant innate immunity. *Cell Microbiol* 9 (11):2571-85.

Ma, W., A. Smigel, R. Verma, and G. A. Berkowitz. 2009. Cyclic nucleotide gated channels and related signaling components in plant innate immunity. *Plant Signal Behav* 4 (4):277-82.

MacMillan, K., K. Emrich, H. P. Piepho, C. E. Mullins, and A. H. Price. 2006. Assessing the importance of genotype x environment interaction for root traits in rice using a mapping population II: conventional QTL analysis. *Theoretical and Applied Genetics* 113 (5):953-964.

Maheswaran, M., Ning Huang, S.R. Sreerangasamy, and S. R. McCouch. 2000. Mapping quantitative trait loci associated with days to flowering and photoperiod sensitivity in rice (*Oryza sativa* L.). *Molecular Breeding* 6:145-155.

Majeau, Nathalie, Jean Trudel, and Alain Asselin. 1990. Diversity of Cucumber Chitinase Isoforms and Characterization of One Seed Basic Chitinase with Lysozyme Activity. *Plant Science* 68:9-16.

Manosalva, P. M., R. M. Davidson, B. Liu, X. Zhu, S. H. Hulbert, H. Leung, and J. E. Leach. 2009. A germin-like protein gene family functions as a complex quantitative trait locus conferring broad-spectrum disease resistance in rice. *Plant Physiol* 149 (1):286-96.

Mauch, F., B. Mauch-Mani, and T. Boller. 1988. Antifungal Hydrolases in Pea Tissue : II. Inhibition of Fungal Growth by Combinations of Chitinase and beta-1,3-Glucanase. *Plant Physiol* 88 (3):936-942.

McDonald, B. A., and C. Linde. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annu Rev Phytopathol* 40:349-79.

Meister, G., and T. Tuschl. 2004. Mechanisms of gene silencing by double-stranded RNA. *Nature* 431 (7006):343-9.

Miki, D., and K. Shimamoto. 2004. Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol* 45 (4):490-5.

Minic, Z. 2008. Physiological roles of plant glycoside hydrolases. *Planta* 227 (4):723-40.

Mitani, N., J. F. Ma, and T. Iwashita. 2005. Identification of the silicon form in xylem sap of rice (Oryza sativa L.). *Plant Cell Physiol* 46 (2):279-83.

Miya, A., P. Albert, T. Shinya, Y. Desaki, K. Ichimura, K. Shirasu, Y. Narusaka, N. Kawakami, H. Kaku, and N. Shibuya. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc Natl Acad Sci U S A* 104 (49):19613-8.

Miyamoto, M., M. Yano, and H. Hirasawa. 2001. Mapping of quantitative trait loci conferring blast field resistance in the Japanese upland rice variety Kahei. *Breeding Science* 51 (4):257-261.

Mizuno, R., T. Fukamizo, S. Sugiyama, Y. Nishizawa, Y. Kezuka, T. Nonaka, K. Suzuki, and T. Watanabe. 2008. Role of the loop structure of the catalytic domain in rice class I chitinase. *J Biochem* 143 (4):487-95.

Nakai, K., and P. Horton. 2007. Computational prediction of subcellular localization. *Methods Mol Biol* 390:429-66.

Nakano, M., K. Nobuta, K. Vemaraju, S. S. Tej, J. W. Skogen, and B. C. Meyers. 2006. Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Res* 34 (Database issue):D731-5.

Nakazaki, T., T. Tsukiyama, Y. Okumoto, D. Kageyama, K. Naito, K. Inouye, and T. Tanisaka. 2006. Distribution, structure, organ-specific expression, and phylogenic analysis of the pathogenesis-related protein-3 chitinase gene family in rice (Oryza sativa L.). *Genome* 49 (6):619-30.

Neuhaus, J. M. 1999. Plant Chitinases (PR-3, PR-4, PR-8, PR-11). In *Pathogenisis-Related Proteins In Plants*, edited by S. K. Datta and S. Muthukrishnan: CRC Press LLC.

Neuhaus, J. M., B. Fritig, H.J.M Linthorst, F. Meins, Jr., J. D. Mikkelsen, and J. Ryals. 1996. A Revised Nomenclature for Chitinase Genes. *Plant Molecular Biology Reporter* 14 (2):102-104.

Nielsen, K. K., J. D. Mikkelsen, K. M. Kragh, and K. Bojsen. 1993. An acidic class III chitinase in sugar beet: induction by Cercospora beticola, characterization, and expression in transgenic tobacco plants. *Mol Plant Microbe Interact* 6 (4):495-506.

Nishizawa, Y., Z. Nishio, K. Nakazono, M. Soma, E. Nakajima, M. Ugaki, and T. Hibi. 1999. Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. *Theor Appl Genet* 99:383-390.

Nürnberger, Thorsten, Frederic Brunner, Birgit Kemmerling, and Lizelle Piater. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. *Immunological Reviews* 198:249-266.

Ouyang, Shu, Wei Zhu, John Hamilton, Haining Lin, Matthew Campbell, Kevin Childs, Francoise Thibaud-Nissen, Renae L. Malek, Yuandan Lee, Li Zheng, Joshua Orvis, Brian Haas, Jennifer Wortman, and C. Robin Buell. 2007. The TIGR Rice Genome Annotation Resource: improvements and new features. *Nucl. Acids Res.* 35 (suppl_1):D883-887.

Pak, Jung-Hun, Eun-Sook Chung, Sang-Hyun Shin, Eun-Hee Jeon, Mi-Jin Kim, Hye-Young Lee, Ji-Ung Jeung, Nam-In Hyung, Jai-Heon Lee, and Young-Soo Chung. 2009. Enhanced fungal resistance in *Arabidopsis* expressing wild rice PR-3 (OgChitIVa) encoding chitinase class IV. *Plant Biotechnol Rep* 3:147-155.

Panstruga, R., J. E. Parker, and P. Schulze-Lefert. 2009. SnapShot: Plant immune response pathways. *Cell* 136 (5):978 e1-3.

Park, Seung-Moon, Dae-Hyuk Kim, Nam Hai Truong, and Yoshifumi Itoh. 2002. Heterologous expression and characterization of class III chitinases from rice (*Oryza sativa* L.). *Enzyme and Microbial Technology* 30:697-702.

Passarinho, P. A., A. J. Van Hengel, P. F. Fransz, and S. C. de Vries. 2001. Expression pattern of the Arabidopsis thaliana AtEP3/AtchitIV endochitinase gene. *Planta* 212 (4):556-67.

Passarinho, Paul, A., and Sacco de Vries, C. 2009. Arabidopsis Chitinases: a Genomic Survey. In *The Arabidopsis Book*: The American Society of Plant Biologists.

Payan, F., R. Flatman, S. Porciero, G. Williamson, N. Juge, and A. Roussel. 2003. Structural analysis of xylanase inhibitor protein I (XIP-I), a proteinaceous xylanase inhibitor from wheat (Triticum aestivum, var. Soisson). *Biochem J* 372 (Pt 2):399-405.

Peever, T.L., R. S. Zeigler, A. E. Dorrance, F. J. Correa-Victoria, and S. St. Martin. *Pathogen Population Genetics and Breeding for Disease Resistance.* 2000 [cited. Available from http://www.apsnet.org.

Perry, Laura G., Elan R. Alford, Junichiro Horiuchi, Mark W. Paschke, and Jorge M. Vivanco. 2007. Chemical Signals in the Rhizosphere: Root-Root and Root-Microbe Communication. In *The Rhizosphere: Biochemistry and Organic Substances at the Soil-Plant Interface*, edited by R. Pinton, Z. Varanini and P. Nannipieri. Boca Raton, FL: CRC Press.

Pinson, Shannon R. M., Fabian M. Capdevielle, and James H. Oard. 2005. Confirming QTLs and Finding Additional Loci Conditioning Sheath Blight

Resistance in Rice Using Recombinant Inbred Lines. *Crop Science* 45:503-510.
Postel, Sandra, and Birgit Kemmerling. 2009. Plant systems for recognition of pathogen-associated molecular patterns. *Seminars in Cell & Developmental Biology* 20 (9):1025-1031.
Ramalingam, J., C. M. Vera Cruz, K. Kukreja, J. M. Chittoor, J. L. Wu, S. W. Lee, M. Baraoidan, M. L. George, M. B. Cohen, S. H. Hulbert, J. E. Leach, and H. Leung. 2003. Candidate defense genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. *Mol Plant Microbe Interact* 16 (1):14-24.
Ramalingam, J., C.M. Vera Cruz, K. Kukreja, J.M. Chittoor, J.L. Wu, S.W. Lee, M.R. Baraoidan, M.L. George, M. Cohen, S. Hulbert, J.E. Leach, and H. Leung. 2003. Candidate resistance genes from rice, barley, and maize and their association with qualitative and quantitative resistance in rice. *Mol Plant-Microbe Interact* 16:14-24.
Ridley, B. L., M. A. O'Neill, and D. Mohnen. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry* 57 (6):929-67.
Robinson, S. P., A. K. Jacobs, and I. B. Dry. 1997. A class IV chitinase is highly expressed in grape berries during ripening. *Plant Physiol* 114 (3):771-8.
Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19 (12):1572-4.
Schlumbaum, Angela, F. Mauch, U. Vogeli, and T. Boller. 1986. Plant chtinases are potent inhibitors of fungal growth. *Nature* 324:365-367.
Schultze, Michael, Christian Staehelin, Frederic Brunner, Isabelle Genetet, Michel Legrand, Bernard Fritig, Eva Kondorosi, and Adam Kondorosi. 1998. Plant chitinase/lysozyme isoforms show distinct substrate specificity and cleavage site preference towards lipochitooligosaccharide Nod signals. *The Plant Journal* 16 (5):571-580.
Sela-Buurlage, M. B., A. S. Ponstein, S. A. Bres-Vloemans, L. S. Melchers, P. Van Den Elzen, and B. Cornelissen. 1993. Only Specific Tobacco (Nicotiana tabacum) Chitinases and [beta]-1,3-Glucanases Exhibit Antifungal Activity. *Plant Physiol* 101 (3):857-863.
Shepherd, R. W., and G. J. Wagner. 2007. Phylloplane proteins: emerging defenses at the aerial frontline? *Trends Plant Sci* 12 (2):51-6.
Shrestha, C. L. , I. I. Ona, S. Muthukrishnan, and T. W. Mew. 2008. Chitinase levels in rice cultivars correlate with resistance to the sheath blight pathogen Rhizoctonia solani. *European Jounal of Plant Pathology* 120:69-77.
Silverstein, K. A., W. A. Moskal, Jr., H. C. Wu, B. A. Underwood, M. A. Graham, C. D. Town, and K. A. VandenBosch. 2007. Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. *Plant J* 51 (2):262-80.
Sirithunya, P., S. Tragoonrung, A. Vanavichit, N. Pa-In, C. Vongsaprom, and T. Toojinda. 2002. Quantitative trait loci associated with leaf and neck blast

resistance in recombinant inbred line population of rice (Oryza sativa). *DNA Research* 9 (3):79-88.
Sontheimer, E. J. 2005. Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol* 6 (2):127-38.
Srinjvaschary, S. Hittalmani, K. G. Kumar, H. E. Shashidhar, and M. G. Vaishali. 2002. Identification of quantitative trait loci associated with sheath rot resistance (Sarocladium oryzae) and panicle exsertion in rice (Oryza sativa L.). *Current Science* 82 (2):133-135.
Swarbreck, David, Christopher Wilks, Philippe Lamesch, Tanya Z. Berardini, Margarita Garcia-Hernandez, Hartmut Foerster, Donghui Li, Tom Meyer, Robert Muller, Larry Ploetz, Amie Radenbaugh, Shanker Singh, Vanessa Swing, Christophe Tissier, Peifen Zhang, and Eva Huala. 2008. The Arabidopsis Information Resource (TAIR): gene structure and function annotation. *Nucl. Acids Res.* 36 (suppl_1):D1009-1014.
Tabien, R. E., Z. Li, A. H. Paterson, M. A. Marchetti, J. W. Stansel, and S. R. M. Pinson. 2002. Mapping QTLs for field resistance to the rice blast pathogen and evaluating their individual and combined utility in improved varieties. *Theoretical and Applied Genetics* 105 (2-3):313-324.
Takeuchi, Yuichi, Masaru Tohbaru, and Atsushi Sato. 1994. Polysaccharides in Primary Cell Walls of Rice Cells in Suspension Culture. *Phytochemistry* 35 (2):361-363.
Terwisscha van Scheltinga, A. C., K. H. Kalk, J. J. Beintema, and B. W. Dijkstra. 1994. Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. *Structure* 2 (12):1181-9.
The UniProt, Consortium. 2009. The Universal Protein Resource (UniProt) 2009. *Nucl. Acids Res.* 37 (suppl_1):D169-174.
Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25 (24):4876-82.
Tokunaga, T., and M. Esaka. 2007. Induction of a novel XIP-type xylanase inhibitor by external ascorbic acid treatment and differential expression of XIP-family genes in rice. *Plant Cell Physiol* 48 (5):700-14.
Tokunaga, T., Y. Miyata, Y. Fujikawa, and M. Esaka. 2008. RNAi-mediated knockdown of the XIP-type endoxylanase inhibitor gene, OsXIP, has no effect on grain development and germination in rice. *Plant Cell Physiol* 49 (7):1122-7.
Truong, N. H., S. M. Park, Y. Nishizawa, T. Watanabe, T. Sasaki, and Y. Itoh. 2003. Structure, heterologous expression, and properties of rice (Oryza sativa L.) family 19 chitinases. *Biosci Biotechnol Biochem* 67 (5):1063-70.
Valent, B., L. Farrall, and F.G Chumley. 1991. Magnaporthe grisea genes for pathogenicity and virulence identified through a series of backcrosses. *Genetics* 127:87-101.
Vierheilig, Horst, Monica Alt, Jean-Marc Neuhaus, Thomas Boller, and Andres Wiemken. 1993. Colonization of Transgenic *Nicotiana sylvestris* Plant,

Expressing Different Forms of *Nicotiana tabacum* Chitinase, by the Root Pathogen *Rhizoctonia solani* and by the Mycorrhizal Symbiont *Glomus mosseae*. *MPMI* 6 (2):261-264.

Wan, J., X. C. Zhang, D. Neece, K. M. Ramonell, S. Clough, S. Y. Kim, M. G. Stacey, and G. Stacey. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in Arabidopsis. *Plant Cell* 20 (2):471-81.

Wang, G. L., D. J. Mackill, J. M. Bonman, S. R. McCouch, M. C. Champoux, and R. J. Nelson. 1994. RFLP Mapping of Genes Conferring Complete and Partial Resistance to Blast in a Durably Resistant Rice Cultivar. *Genetics* 136 (4):1421-1434.

Warthmann, Norman, Hao Chen, Stephan Ossowski, Detlef Weigel, and Philippe Herve. 2008. Highly Specific Gene Silencing by Artificial miRNAs in Rice. *PLoS ONE* 3 (3):e1829.

Wilson, R. A., and N. J. Talbot. 2009. Under pressure: investigating the biology of plant infection by Magnaporthe oryzae. *Nat Rev Microbiol* 7 (3):185-95.

Wisser, R. J., Q. Sun, S. H. Hulbert, S. Kresovich, and R. J. Nelson. 2005. Identification and characterization of regions of the rice genome associated with broad-spectrum, quantitative disease resistance. *Genetics* 169 (4):2277-93.

Wu, J. L., P. K. Sinha, M. Variar, K. L. Zheng, J. E. Leach, B. Courtois, and H. Leung. 2004. Association between molecular markers and blast resistance in an advanced backcross population of rice. *Theor Appl Genet* 108 (6):1024-32.

Xu, C. G., X. Q. Li, Y. Xue, Y. W. Huang, J. Gao, and Y. Z. Xing. 2004. Comparison of quantitative trait loci controlling seedling characteristics at two seedling stages using rice recombinant inbred lines. *Theoretical and Applied Genetics* 109 (3):640-647.

Xu, F., C. Fan, and Y. He. 2007. Chitinases in Oryza sativa ssp. japonica and Arabidopsis thaliana. *J Genet Genomics* 34 (2):138-50.

Young, D.H, and G. F. Pegg. 1982. The action of tomato and Verticillium albo-atrum glycosidases on the hyphal wall of V. albo-atrum. *Physiological Plant Pathology* 21:411-423.

Zenbayashi, K., T. Ashizawa, T. Tani, and S. Koizumi. 2002. Mapping of the QTL (quantitative trait locus) conferring partial resistance to leaf blast in rice cultivar Chubu 32. *Theoretical and Applied Genetics* 104 (4):547-552.

Zhao, K. J., and M. L. Chye. 1999. Methyl jasmonate induces expression of a novel Brassica juncea chitinase with two chitin-binding domains. *Plant Mol Biol* 40 (6):1009-18.

Zhao, S., and X. Qi. 2008. Signaling in plant disease resistance and symbiosis. *J Integr Plant Biol* 50 (7):799-807.

Zhong, R., S. J. Kays, B. P. Schroeder, and Z. H. Ye. 2002. Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. *Plant Cell* 14 (1):165-79.

Zhu, Qingsong, Yasuyuki Arakane, Richard W. Beeman, Karl J. Kramer, and Subbaratnam Muthukrishnan. 2009. Functional specialization among

insect chitinase family genes revealed by RNA interference. *PNAS* 105 (18):6650-6655.

Zhu, Qun, Eileen A. Maher, Sameer Masoud, Richard A. Dixon, and Chris J. Lamb. 1994. Enhanced Protection Against Fungal Attack by Constitutive Co-expression fo Chitinase and Glucanase Genes in Transgenic Tobacco. *Nature Biotechnology* 12:807-812.

Zou, J. H., X. B. Pan, Z. X. Chen, J. Y. Xu, J. F. Lu, W. X. Zhai, and L. H. Zhu. 2000. Mapping quantitative trait loci controlling sheath blight resistance in two rice cultivars (Oryza sativa L.). *Theoretical and Applied Genetics* 101 (4):569-573.