

DISSECTION OF QUANTITATIVE RESISTANCE TO RICE DISEASES

by

PATRICIA M. MANOSALVA

B.S., Universidad Ricardo Palma, Lima, Peru, 1997
M.S., Universidad Peruana Cayetano Heredia, Lima, Peru, 2001

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College of Agriculture

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Abstract

Because it is predicted to be durable and broad spectrum, quantitative trait loci (QTL)-based resistance is an important option for rice disease control. However, manipulation of this type of resistance requires knowledge of the contributing genes. This study demonstrates the contribution of two of three defense response (DR) genes to QTL-governed resistance, and identifies a third gene that negatively regulates resistance.

The contribution to QTL-governed resistance of one of nine rice *OsPAL* genes, which encode phenylalanine ammonia-lyase, was determined using reverse genetics. Mutant *ospal4* contains a 750 bp deletion in *OsPAL4* and was identified using a PCR-pooling strategy. *OsPAL4* underlies a QTL on chromosome 2, and is located in cluster with three other *OsPAL* members. Rice lines mutated in *OsPAL4* are more susceptible to a virulent strain of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) than lines with the wild type allele. RNAi suppression was used to evaluate the contributions of genes encoding oxalate oxidase-like proteins (*OsOXL*) and a 14-3-3 protein (*GF14-e*) to disease resistance. Silencing of 12 *OsOXL* genes clustered on chromosome 8, varied from suppression of a few gene members to silencing of all expressed family members. Screening of transgenic lines by challenge with *Magnaporthe grisea* (*Mg*), the rice blast pathogen, revealed that the more chromosome 8 *OsOXL* genes suppressed, the more susceptible the plants were to *Mg*. *GF14-e* co-localizes with a disease resistance QTL on chromosome 2. Specific suppression of *GF14-e* by RNAi silencing did not result in enhanced susceptibility to *Mg*. Instead, the lines exhibited spontaneous HR-type lesions. The presence of this lesion mimic phenotype correlated with enhanced resistance to a virulent strain of *Xoo*, suggesting that the *GF14-e* encoded 14-3-3 protein functions as a negative regulator of plant cell death and bacterial resistance in rice. This study supports the hypothesis that DR genes, such as *OsOXL* and *OsPAL4* contribute to disease resistance governed by QTL. The role of *GF14-e* is less clear, however its down regulation may contribute to QTL-governed resistance. Thus, incorporation of regions harboring the effective DR gene alleles into rice will enhance broad spectrum and durable resistance.

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Approved by:

Major Professor
Jan E. Leach

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Table of Contents

List of Figures	viii
List of Tables	x
Acknowledgements	xi
Dedication	xii
CHAPTER 1 - LITERATURE REVIEW: DISSECTION OF QUANTITATIVE RESISTANCE TO RICE DISEASES	1
RICE AND ITS MOST IMPORTANT DISEASES	2
MECHANISM OF DEFENSE RESPONSE AND ITS MOLECULAR BASES	4
QUALITATIVE RESISTANCE	7
QUANTITATIVE RESISTANCE	8
QTL MAPPING AND CLONING	10
CANDIDATE GENE APPROACH	11
CANDIDATE GENES AS PREDICTORS FOR QUANTITATIVE RESISTANCE IN RICE	15
Roles of DR gene candidates:	19
Phenylalanine ammonium-lyase	19
Oxalate oxidase and oxalate oxidase-like genes	22
P14-3-3	25
FORWARD AND REVERSE GENETICS FOR CANDIDATE GENE VALIDATION	27
GENE SILENCING FOR CANDIDATE GENE VALIDATION	30
Figures and Tables Chapter 1	34
REFERENCES	36
CHAPTER 2 - Mutation of a rice <i>phenylalanine ammonia lyase</i> gene is associated with susceptibility to bacterial blight	50
Summary	51
Introduction	52
Results	56

Discussion.....	62
Experimental Procedures	69
Figures and Tables Chapter 2	73
REFERENCES	89
CHAPTER 3 - <i>Oxalate Oxidase-Like</i> Genes Contribute to Rice Blast Disease Resistance	
Governed By QTL	95
Summary.....	96
Introduction.....	97
Results.....	101
Discussion.....	106
Methods	112
Construct for RNAi Silencing and Agrobacterium Mediated-Transformation ..	112
DNA and RNA Analysis.....	113
Plant and Fungal Growth and Inoculation Methods	113
Figures and Tables Chapter 3	115
REFERENCES	126
CHAPTER 4 - <i>GF14e</i> is a negative regulator of plant cell death and resistance against virulent strain of <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	
Summary.....	130
Introduction.....	131
Material and Methods	135
Results.....	138
Discussion.....	140
Tables and Figures Chapter 4	145
REFERENCES	154
CONCLUSIONS AND REMARKS	157

List of Figures

Figure 1.1 Candidate genes co-localized with QTLs against rice diseases	35
Figure 2.1 Reconstruction experiments using a natural 135 bp deletion in the Xa21 gene of rice line IR24.....	74
Figure 2.2 Pooling and screening strategy	75
Figure 2.3 Gene prediction and structures of rice <i>phenylalanine ammonia-lyase</i> (<i>PAL</i>) gene family	76
Figure 2.4 Identification of a deletion in an <i>OsPAL</i> gene family member detected in rice DEB mutants	77
Figure 2.5 Characterization of the <i>OsPAL</i> deletion	78
Figure 2.6 Confirmation of the mutation using nested PCR.....	79
Figure 2.7 PCR confirmation of the mutated <i>OsPAL4</i> on chromosome 2.....	80
Figure 2.8 The <i>OsPAL4</i> deletion is correlated with disease susceptibility to the virulent strain of <i>Xanthomonas oryzae</i> pv <i>oryzae</i> (<i>Xoo</i>).	81
Figure 2.9 <i>ospal4</i> mutant line cross with Kitaake transmit the <i>ospal4</i> deletion in the progeny ...	82
Figure 2.10 SSR amplification to identify F ₁ hybrids between Kitaake and <i>ospal4</i> mutant line.	83
Figure 2.11 Rice inflorescence phenotype between wild type and <i>PAL</i> mutants.....	84
Figure 3.1 Dendogram of the GLP proteins from rice and barley.	116
Figure 3.2 Multiple sequence alignment of the Chr. 8 <i>OsOXL</i> gene members and the closest related members on chr. 12.	117
Figure 3.3 RNAi silencing vector pTSi-1 map.	118
Figure 3.4 RNAi silencing of the 12 chr. 8 <i>OsOXL</i> genes in independant T ₀ Kitaake transgenic plants varied among the different T ₀ plants.	119
Figure 3.5 <i>In vitro</i> spot inoculation of rice detached leaves with <i>Magnaporthe grisea</i> (<i>Mg</i>). ...	120
Figure 3.6 Suppression of multiple <i>OsOXL</i> gene in T ₀ Kitaake lines correlates with increased susceptibility to <i>M. grisea</i>	121
Figure 3.7 T ₁ Kitaake transgenic lines exhibited various levels of silencing of the chr. 8 <i>OsOXL</i> genes.	122

Figure 3.8 Suppression of multiple <i>OsOXL</i> gene in T ₁ Kitaake lines correlates with increased susceptibility to <i>M. grisea</i> Che86061.	123
Figure 4.1 <i>GF14-e</i> selected to RNAi is a member of a family of eight gene members.	146
Figure 4.2 Multiple sequence alignment of the GF14-e sequence that was used as an RNAi trigger with the sequences corresponding to the other GF14 gene members	147
Figure 4.3 RNAi silencing of <i>GF14-e</i> , <i>c</i> , and <i>a</i> genes in T ₀ Kitaake transgenic lines was detected using RT-PCR with gene specific primers for each family member.	148
Figure 4.4 Lesion mimics (LM) phenotype in T ₀ and T ₁ <i>GF14-e</i> suppressed lines.	149
Figure 4.5 Intensity of lesion mimic phenotype in T ₀ transgenic lines correlates with the level <i>GF14-e</i> silencing.....	150
Figure 4.6 Lesion mimic phenotype in T ₁ <i>GF14-e</i> suppressed segregant lines.....	150
Figure 4.7 Bacterial blight lesions between lesion mimics and no lesion mimic T ₁ segregants.	151
Figure 4.8 <i>GF14-e</i> gene is a negative regulator of resistance against bacterial blight.	152
Figure 4.9 <i>GF14-e</i> suppression slow down the growth of <i>Xanthomonas oryzae</i> pv. <i>oryzae</i>	153

List of Tables

Table 2-1 Primers for candidate genes used to screen the rice mutant collection.	85
Table 3-1 Nucleotide sequence homology between the RNAi trigger region and the GLPs predicted on chromosome 8 and 12.	124
Table 3-2 Gene specific primers for <i>OsOXL</i> genes on Chr.2 and control RT-PCR primers.	125

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Dedication

**To God, my parents José and Yolanda, my sister Iveth (You are with me!!),
Raheem, Uncle Jorge, my best friends (You know Who You Are!!), and my TATA (Always
remember you).....**

CHAPTER 1 - LITERATURE REVIEW: DISSECTION OF QUANTITATIVE RESISTANCE TO RICE DISEASES

RICE AND ITS MOST IMPORTANT DISEASES

Rice (*Oryza sativa*), a member of the grass family, is the most important grain in the world. Three billion people in the developing world depend on it as the major component of their subsistence diet (Cantrell and Reeves, 2002). The nutritional value of rice is directly reflected in their quality of life.

Rice culture began in Asia and it now is cultivated in 113 countries corresponding to all continents except Antarctica. It is grown in a large range of soil wetness regimes, from deep flood to dryland, and in diverse soil conditions (<http://www.fao.org/rice2004/en/concept.html>). Two of 23 species from the genus *Oryza* are cultivated. *Oryza sativa*, which originated in the humid tropics of Asia, is also the more widely used. There are three major ecotypes of the cultivated species, *Oryza sativa: japonica*, *javanica*, and *indica* (Yano and Sasaki, 1997).

Among the cereal crops, such as maize, millet and sorghum, rice has several attributes that qualify it as a model for monocot plants. Rice has a small genome size (~430 Mb) which is about three times the size of the *Arabidopsis thaliana* genome. The small genome of rice includes a large percentage of single-copy DNA (75%) (McCouch, 1998). A vast reservoir of germplasm (>200,000 accessions), including both cultivated and wild rice, is available for genetics and breeding programs at the International Rice Research Institute (IRRI, Los Baños, Phillipines). In the last decade, two high-density molecular linkage maps of rice containing about 3,000 markers have been developed in the US and Japan, making the marker density in the rice genome, on average, one marker per cM (200-300 kb) (Causse et al., 1994; Harushima et al., 1998). A recently initiated project to a large fraction of the single nucleotide polymorphisms (SNPs) present in cultivated rice through whole-genome comparisons of 20 rice genomes, including cultivars, germplasm lines, and landraces (McNally et al., 2006). The SNP data will be entirely public (www.oryzasnp.org), and will provide needed data for whole genome scans. Rice has proven to be the most readily transformable cereal crop (Hiei et al., 1994).

A high quality, finished sequence of the *japonica* subspecies (var. Nipponbare) was recently published by the International Rice Genome Sequencing Project (2005) and a draft sequence (~6X sequence coverage) of the *indica* subspecies (var. 93-11) (Yu et al., 2005) generated by the Beijing Genomics Institute is also available (Sasaki and Burr, 2000; Barry,

2001; Goff et al., 2002; Yu et al., 2005). In addition, high quality, uniform annotation of the rice genome is ongoing at the structural and functional level (Yuan et al., 2005). Furthermore, rice functional genomic resources for assessing gene function on a genome-wide scale are well established or on-going in rice. At the transcript level, serial analysis of gene expression (SAGE) projects is underway for rice (Matsumura et al., 1999; Gowda et al., 2004). SAGE data, coupled with the ~32,000 full-length cDNAs (Kikuchi et al., 2003) and ~400,000 *Expressed Sequence Tags* in Genbank, provide a rich resource for transcript structure and expression patterns in rice. In addition, a large, public Massively Parallel Signature Sequencing project has commenced in rice (<http://mpss.udel.edu/rice/>) to more deeply sample the transcriptome. A collection of microarray platforms are available for genome-wide expression studies including a publicly available long oligonucleotide array (www.ricearray.org), an Affymetrix expression array (<http://www.affymetrix.com/products/arrays/specific/rice.affx>), and an Agilent expression array (<http://www.chem.agilent.com/Scripts/PDS.asp?lPage=12133>). These are complemented by a project to develop an atlas of expression in a panel of rice tissues throughout development (<http://plantgenomics.biology.yale.edu/riceatlas>). Collections of tagged lines are available in rice using *Tos17*, *Ac/Ds*, and T-DNA (Hirochika et al., 2004; <http://orygenesdb.cirad.fr/>) and provide induced variation. The goal of the post-sequencing era is to assign gene function to the approximately 35,000 to 40,000 predicted rice genes which will advance both breeding and basic science discovery.

Diseases are among the most important limiting factors that affect rice production, causing annual yield loss conservatively estimated at 5%. More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been reported on rice (Ou, 1985), among which rice blast, caused by the fungus *Magnaporthe grisea* (*Mg*), bacterial leaf blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), and sheath blight, caused by the fungus *Rhizoctonia solani* are the most serious constraints to high productivity (Ou, 1985). Resistant cultivars and application of pesticides have been used for disease control. However, the useful life-span of many cultivars containing these resistance genes is only a few years, due to the breakdown of the resistance in the face of high pathogenic variability of the pathogen population. Use of pesticides is costly and can damage the environment or human health. New strategies need to be developed to increase durable resistance, giving protection for a long time and over a broad geographic area. Among

such new strategies, systemic acquired resistance (SAR) and quantitative resistance are examples of a defense mechanism offering long-lasting disease resistance against a broad-spectrum of pathogens, and are promising for sustainable rice production in the future (Song and Goodman, 2001).

MECHANISM OF DEFENSE RESPONSE AND ITS MOLECULAR BASES

Plants are exploited as a source of food and shelter by a wide range of parasites, including viruses, bacteria, fungi, nematodes, insects and even other plants. Plants lack a circulating adaptive immune system to protect themselves against pathogens. They have evolved other mechanisms of antimicrobial defense, which are either constitutive or inducible (Ođjakova and Hadjiivanova, 2001). Over time, pathogens have adapted numerous ways to overcome this host immune system while the host responds with new defenses.

In general, the exterior surfaces of a plant have waxy cuticles and preformed antimicrobial (mostly phenolic) compounds to prevent the entry of many pathogens. Cell walls provide an effective secondary barrier to any invaders that are able to gain access to interior spaces. Any invaders that overcome both barriers must still overcome the plant immune response. Plant immunity can be described as two components operating on different time scales. The basal response to pathogen-associated molecular patterns (PAMPs) that appears early in pathogen interactions, and the gene-for gene response specific to a pathogen that operates on the time scale of hours (Navarro et al., 2004). On the pathogen side, the pathogens encode for a range of functions that are required for the successful infection of the plant such associating with the cell wall, formation of penetration structures, degradation of host chemical and physical barriers, production of toxins to weaken the host, and the inactivation of the plant defense response mechanisms (Dixon and Lamb, 1990).

The evolution of the plant immune system necessitated the ability to distinguish between “self” and “non-self” to ensure that an immune response is not mounted against the its own tissues. This recognition is partially achieved by the recognition of the invader’s chemical motifs

by host surface receptors. Among the different plant-pathogen interactions we can distinguish: non-host interactions, compatible, and incompatibles interactions.

Nonhost interaction

In this interaction the putative pathogen is unable to reproduce and the plant is not affected by such infection, mainly because the plant does not satisfy the requirements for a successful colonization of the pathogen. The majority of the interactions among plants and pathogens are this kind. Considering that most plants are exposed to all kind of pathogens including bacteria, virus, fungi, nematodes and insects in their environments, these plants have some levels of these non-host resistance. Nonhost resistance is an operational definition, which refers to situations where an entire plant species is resistant against all strains of a given pathogen that are able to infect other plants (Heath, 1991; Somerville, 2004). Nonhost resistance can be further classified as preformed nonhost resistance and inducible nonhost resistance, both of which can be effective against potential parasites in a non-specific manner.

Incompatible interaction

Incompatible interactions are the interaction between a resistant host and an avirulent pathogen. The race-specific recognition of the pathogen is produced as a result of the genetic incompatibility between the pathogen and the plant. This incompatibility is produced by the direct or indirect interaction of the resistance gene product encoded for the R-gene in the plant with a product encoded by the avirulence gene (*Avr*) product in the pathogen (Keen, 1990). This interaction results in a severe reduction of the ability of the pathogen to grow or multiply and spread, and often includes a hypersensitive reaction (HR) (Agrois, 1988). The HR is characterized by localized cell and tissue death at the site of infection (Van Loon, 1997). As a result the pathogen remains confined to necrotic lesions near the site of infection. A ring of cells surrounding necrotic lesions become inhibitory to subsequent infection, and this is known as localized acquired resistance (Hammon-Kosack and Jones, 1996; Baker et al., 1997; Fritig et al., 1998). These local responses often trigger nonspecific resistance throughout the plant, known as systemic acquired resistance, which provides durable protection against challenge infection by a broad range of pathogens (Ryals et al. 1996; Sticher et al., 1997; Van Loon, 1997; Fritig et al. 1998). The metabolic alterations in localized acquired resistance include: cell wall reinforcement by deposition and crosslinking of polysaccharides, proteins, glycoproteins and insoluble

phenolics; stimulation of secondary metabolic pathways, some of which yield small compounds with antibiotic activity (the phytoalexins) but also defense regulators such as salicylic acid, ethylene and lipid-derived metabolites; accumulation of broad range of defense-related proteins and peptides (Hahn, 1996; Fritig et al., 1998).

Compatible interaction

The compatible interaction is the interaction that occurs between a susceptible or moderate resistance host and a virulent pathogen. There is not a specific gene interaction in compatible interactions. Since there is not specific pathogen recognition, genetic incompatibility does not occur and the pathogen is not recognized by the plant and can successfully colonize the plant. As a result of this colonization the plant may activate a defense response mechanism mainly induced by PAMPs to limit the pathogen growth (partial resistance). The defense response genes that are induced after this interaction modify the degree of susceptibility of the host and most likely are correlated with quantitative resistance.

The earliest defense reactions of plant cells include changes in plasma membrane permeability leading to calcium and proton influx and potassium and chloride efflux (McDowell and Dangl, 2000). Ion fluxes subsequently induce extracellular production of reactive oxygen intermediates, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl free radical ($OH\bullet$), catalyzed by a plasma membrane-located NADPH oxidase and/or apoplastic-localized peroxidases (Somssich and Hahlbrock, 1998). The initial transient reactions are, at least in part, prerequisites for further signal transduction events resulting in a complex, highly integrated signaling network that triggers the overall defensive response. The role of calcium is shown in experiments with calcium channel inhibitors, where preventing increases of cytosolic calcium concentrations delayed the development of the hypersensitive response. Heterotrimeric GTP-binding proteins and protein phosphorylation/dephosphorylation processes are probably involved in transferring signals from the receptor to calcium channels that activate downstream reactions (Legendre et al., 1992). The changes in ion fluxes trigger localized production of reactive oxygen intermediates and nitric oxide, which act as second messengers for hypersensitive response induction and defense gene expression (Piffanelli et al., 1999). Synergistic interactions between

reactive oxygen intermediates, nitric oxide and salicylic acid have been postulated (McDowell and Dangl, 2000).

Other components of the signal network are specifically induced phospholipases, which act on lipid-bound unsaturated fatty acids within the membrane, resulting in the release of linolenic acid, which serves as a substrate for the production of jasmonate, methyl jasmonate and related molecules via a series of enzymatic steps. The oxidative burst is a central component of plants' defense machinery (Lamb and Dixon, 1997; Alvarez et al., 1998). The burst of H₂O₂ production at the plant cell surface drives rapid peroxidase-mediated oxidative cross-linking of structural proteins in the cell wall, thereby reinforcing this physical barrier against pathogen ingress (Scheel, 1998). Additionally, low doses of reactive oxygen metabolites act as signals for the induction of detoxification mechanisms involving superoxide dismutases and glutathione-S-transferase, and activation of other defense reactions in neighbouring cells. Signal pathways involving one or more of the three regulators jasmonate, ethylene and salicylic acid regulate most of the inducible, defense-related genes (Van Wees et al., 2000; Devadas et al., 2002). The exact role of ethylene as a defense regulator is not clear but it has been shown that this hormone preferentially induces basic pathogenesis-related proteins.

Activation of the signal transduction network after pathogen recognition results in reprogramming of cellular metabolism, and involves large changes in gene activity. Plants contain many defense related proteins. In addition to resistance R genes and genes encoding signal transduction proteins, they possess downstream defense genes, such as pathogenesis-related proteins (PRs), enzymes involved in the generation of phytoalexins, the enzymes of oxidative stress protection, tissue repair, lignification, and others. Many of these defense induced genes are involved in secondary metabolism, such as shikimate and phenylpropanoid pathways (Somssich and Hahlbrock, 1998).

QUALITATIVE RESISTANCE

Host resistance often refers to gene-for-gene type of specific resistance, although other types of resistance like quantitative resistance and systemic acquired resistance are also very common in nature (Flor, 1971). The gene-for-gene type is also called host resistance, qualitative resistance and race specific resistance, and describes a situation where a set of disease resistance

(*R*) genes in a given plant species can specifically recognize the corresponding set of Avirulence (*Avr*) genes expressed by pathogens, which will result in disease resistance. Either the lack of *R* genes or the absence of the corresponding *Avr* genes will result in disease susceptibility (Flor, 1971).

Host specific resistance is normally conditioned by single or few genetic loci, and is therefore easily selected by plant breeders. Therefore, modern crop cultivars often carry an assortment of useful alleles at these disease resistance gene loci due to many generations of selection for resistance. In most cases, the host specific resistance is very effective. In the past 10 years, significant progress has been made in deciphering host specific resistance. More than 40 race-cultivar specific resistance genes have been cloned (Hulbert et al., 2001; Martin et al., 2003). Most of them encode receptor-like proteins, which are consistent with the gene-for-gene model for predicting the functions of host resistance proteins. Many downstream signal transduction components involved in the specific *R/Avr* gene interactions have also been isolated and characterized. Many of the cloned host resistance genes have been used to improve the crop resistance (Campbell et al., 2002; Michelmore, 2003; Pedley and Martin, 2003). However, the host specific resistance genes are often only effective against specific strains of pathogens and tend to lose their effectiveness due to shifts in the pathogen population (McDonald and Linde, 2002). To achieve the goal of durable resistance, more attention has been put on other resistance mechanisms, like quantitative resistance.

QUANTITATIVE RESISTANCE

Genetic variation in nature often takes the form of a quantitative phenotypic range, with an approximately normal distribution, rather than of qualitative phenotypes that fall into discrete categories. Phenotypic variation for quantitative traits results from segregation at multiple quantitative trait loci (QTL), the effects of which are modified by the internal and external environments (Paran and Zamir, 2003). Quantitative traits are governed by several genes with additive effects, and have a polygenic inheritance.

Classical quantitative geneticists only analyze quantitative traits by describing the nature of the loci involved in the phenotype. Before the development of the QTL-mapping concept, quantitative resistance and its variation were studied by using statistical analysis using progenies from two parental lines that show different resistant phenotype. Using this strategy, it was

possible to calculate inheritance, number of loci involved, degree of dominance, and gene x gene and gene x environment interactions (Salvi and Tuberosa, 2005). The concept for detecting QTL using linked major genes was developed by Sax in 1923, when he associated the size of the seed (polygenic trait) and the color of the seed coat (monogenic trait) in *Phaseolus* genotypes. This study demonstrated that quantitative traits were not that different from simple Mendelian traits and it was expected that the quantitative traits will exhibit similar segregation and recombination properties. These findings opened the possibility to localize individual QTL by linkage with morphological markers. However, it was difficult to put this concept in practice using conventional morphological markers.

Advances in molecular biology made possible the development of co-dominant DNA markers that are used to develop saturated genetic maps, which are a very valuable resource to localize QTL controlling important traits, including plant disease resistance (Yano and Sasaki, 1997; Paran and Zamir, 2003). Paterson et al. (1988) first used a linkage map to dissect quantitative traits into Mendelian factors in tomato, introducing the idea of QTL mapping using molecular DNA markers. They developed statistical methods (maximum likelihood, and LOD score) to map QTL intervals affecting the soluble solid concentration and pH in tomato fruits. They were able to determine the QTL chromosome location and its phenotypic contribution. After this study, many QTL have been clarified using DNA markers in several crops plants, including tomato (Paterson et al., 1991) and maize (Stuber et al., 1992).

Quantitative resistance to plant pathogens is also known as non-race specific, polygenic, or field resistance. The understanding of the molecular basis of quantitative resistance, which is controlled by multiple genes with smaller effects, has made little progress in the past decades. Even in cases where a few genes exert strong effects, genetic analysis to determine the numbers of genes and their precise chromosomal location are difficult because of the lack of discrete phenotypic segregation in the progeny (Yano and Sasaki, 1997). QTL-based disease resistance is predicted to be broad spectrum because it is not apparently strain specific, or pathogen specific. Moreover, quantitative resistance is predicted to be durable because it is difficult for the pathogen to breakdown a resistance governed by several genes. To break down QTL-based resistance, the pathogen would need to mutate or modify several genes at the same time. Quantitative resistance is predicted to be stable because its capacity to maintain a balance among all the races present in a specific location.

The presumed durability has made quantitative resistance a popular target for plant breeders, even though QTL are more difficult to assay and manipulate in breeding programs. Despite the importance of quantitative disease resistance in plants, the genetic basis of this trait or the mechanism of action of the genes controlling it remains unclear. The elucidation of these aspects of quantitative resistance will be valuable for understanding host-pathogen interactions as well as for improving crop production. Without knowledge of the genes that contribute to disease resistance QTL, plant breeders cannot develop the molecular markers needed to track and select for the genes in their crop improvement programs.

QTL MAPPING AND CLONING

Current QTL mapping is a standard procedure in quantitative genetics. QTL mapping usually begins with the collection of genotypic (based on molecular markers) and phenotypic data from a segregating population, followed by statistical analysis to reveal all possible marker loci where allelic variation correlates with the phenotype. Because this procedure only allows for an approximate mapping of the QTL, it is usually referred to as primary QTL mapping. In this stage, F₂ population or recombinant inbred lines (RILs) can be used as a primary mapping population. After this primary mapping, the QTL is positioned within a chromosomal interval of ~10-30 centimorgans (cM) which usually includes several hundred genes (Salvi and Tuberosa, 2005).

More recent technical progress in the area of molecular biology and genomics has made the cloning of QTL (identification of the gene or genes responsible for QTL) possible. Among the QTL isolated from plants to date, the majority have been cloned via positional map-based cloning. To date no resistance QTL have been cloned in any plant species. Positional map-based cloning involves several steps to enable assignment of a QTL to the shortest possible genetic interval (QTL fine mapping) and to identify the corresponding interval on the DNA sequence (QTL physical mapping) where candidate genes are selected for evaluation (Salvi and Tuberosa, 2005). For QTL fine mapping experiments, a new experimental population is produced by crossing nearly isogenic lines (NILs) that differ only in the allelic constitution harboring the QTL (QTL-NILs). In such populations, because of the absence of other segregant QTL, the target QTL becomes the major genetic source of variation, and the phenotypic means of the QTL genotypic classes can be statistically differentiated. Under these conditions, the QTL is

considered ‘Mendelized’, and cM distances between a QTL and the nearby molecular markers can be estimated more precisely (Salvi and Tuberosa, 2005). In this fine mapping step increasing the population size is required to minimize the size of the candidate gene region.

By combining the analysis of a large segregating population and the use of chromosome-region-specific molecular markers, it is possible to define a candidate genomic region of less than 50 kilobases (kb) (Yano, 2001). After the candidate genomic region has been defined, the markers closest to the QTL are used for anchoring the genetic map to the physical map (i.e. the genomic sequence or a BAC contig covering the QTL region). In this phase, bioinformatics provides an important contribution in terms of gene predictions of putative candidates in that region (Salvi and Tuberosa, 2005). Once the putative candidate gene or genes in the region co-segregating with the QTL have been established, validation of a candidate gene or genes need to be done by expression profiling, genetic complementation and gene suppression strategies of the candidate gene or genes (Yano et al., 2001).

Recently, examples of the molecular identification of genes at QTL in *Arabidopsis*, maize, tomato, and rice have been reported. However, none of these QTL controlled disease resistance traits. In *Arabidopsis*, QTLs controlling flowering time (*ED1* and *FLW*), glucosinolate structure (*GS-elong*), and root morphology (*BRX*) were cloned (El-Din, et al., 2001; Werner et al., 2005; Kroymann et al., 2003; Mouchel et al., 2004). In maize a gene encoded by a transcription factor involved in plant architecture was cloned and proved to control the *Tb1* QTL (Doebley, et al., 1995). In tomato, three QTL have been cloned (*Brix9-2-5*, *Ovate*, *fw2.2*) controlling fruit sugar content, fruit shape, and fruit weight, respectively (Fridman et al., 2004; Liu et al., 2002; Frary et al., 2000). In rice, three QTL controlling heading time (flowering) have been identified (*Hd1*, *Hd3a*, and *Hd6*) and cloned (Doi et al., 2004; Yano et al., 2000; Takahashi et al., 2001). All these QTL have been cloned by map-based positional cloning except one in maize that was cloned by transposon tagging (Salvi and Tuberosa, 2005).

CANDIDATE GENE APPROACH

The small effects of many QTL make precise mapping difficult, and therefore, the use of map-based cloning to identify the genes contributing to these QTL would be difficult (Yano, 2001; Salvi and Tuberosa, 2005). An alternative approach is a candidate gene approach where a systematic analysis of candidate plant defense genes in linkage disequilibrium with resistance,

and associated with QTL, would allow the more precise identification and localization of genes conditioning quantitative resistance and may identify suitable targets for marker-assisted selection, cloning, or both (Liu et al., 2003; Yano, 2001). The candidate gene approach implies that the major part of the quantitative genetic variation of a specific trait is caused by the functional variation of the gene or genes directly involved in the development and physiology of the trait (candidate gene) (Soller, 1998).

The associations between candidate genes and the phenotype of interest can be studied by comparison of allelic frequencies of these candidate genes among individuals having contrasting phenotypic values (Rothschild and Soller, 1997). Markers corresponding to genes potentially involved in the biochemical pathway leading to trait expression can be localized in the genetic map of the population in study, and this localization will be compared with the chromosomal location of QTL for the trait of interest. For instance, completion of genome sequences and improved bioinformatics should facilitate in silico cross-matching of candidate sequences with QTL in positional cloning programs or association mapping. The creation of more powerful bioinformatic tools for gene annotation should facilitate the choice of functional candidates among and outside the positional candidate genes (Salvi and Tuberosa, 2005). The candidate gene approach has emerged as a promising method of merging QTL analysis with the extensive data available on the cloning and characterization of genes involved in plant defenses.

Using the candidate gene approach, several map locations of QTLs and their candidate genes have been reported. In maize, the candidate gene approach was used to co-localize genes encoding enzymes from sugar metabolism with QTL controlling growing processes (Causse et al., 1995). Goldman et al. (1993) co-localized a gene from the starch metabolism with a QTL controlling starch concentration. The strategy of candidate genes has been also applied to co-localizing candidate genes with disease resistance QTL in several pathosystems (Yu et al., 1996; Byrne et al., 1996; Leonard-Shippers et al., 1994; Faris et al., 1999; Geffroy et al., 2000; Trognitz et al., 2002; Ramalingam et al., 2003; Wu et al., 2004; Liu et al., 2004).

Byrne et al. (1996) first published the most compelling case for linking candidate genes involved in the flavone synthesis pathway of maize with the host defense response phenotype associated with QTL resistance to corn earworm. In this study, they found that the *pl* locus,

coding for a transcriptional activator, together with three other candidate genes were responsible for 75.9% of the phenotypic variation for resistance to corn earworm. Candidate genes involved in defense responses can be classified as i) genes involved in the initial recognition of pest or pathogen (R-genes or major genes), and genes involved in the early events of signal transduction upon pathogen recognition, which relay the R gene signal to the nucleus, ii) defense response (DR) genes, which include the enzymes that function in some way to inhibit pathogen spread, and regulatory genes, which regulate the coordinate expression of the genes during plant defense responses.

A large number *R*-genes, which are involved in recognition of pathogen avirulence, have been cloned (Bent, 1996). These genes share common sequences motifs, reflecting related functions in signal transduction pathways (Martin et al., 1993; Jones et al., 1994; Staskawicz et al., 1995). Common motifs include encoding a protein kinase (e.i. *Pto* and *Xa21*) and gene products with a leucine rich repeat (LRR). Other conserved features include the nucleotide binding site (NBS) and coiled coil or TIR domains (Hulbert et al., 2001). While these recognition type resistance genes are most commonly associated with qualitative resistance, evidence suggests that some of them are also associated with quantitative resistance (Wang et al., 1994). Many of these loci are complex, and carry multiple homologous genes. Further mapping studies have shown that some putative QTL are localized in chromosomal regions that harbor these R genes (Faris et al., 1999; Wang et al., 1994), although it has not yet been determined whether the QTL are members of the R gene family.

The second class of candidate genes contains those involved in plant defense responses (DR genes). DR genes are frequently associated with resistance by their increased expression and enzymatic activity after pathogen challenge. Some DR genes are thought to act downstream from *R*-gene recognition and are probably regulated by one or more signal-transduction pathways, while others are constitutively expressed or pathogen-inducible. Proteins encoded by these genes include: 1) structural proteins that are incorporated into the extracellular matrix and participate in the confinement of the pathogen at the penetration sites. In this class we can also consider those proteins involved in the cross-linking of cell wall such as, peroxidases, oxidases, among others, 2) enzymes of secondary metabolism, for instance, members of the phenylpropanoid pathway important in the synthesis of antimicrobial phenolic compounds such phytoalexins, flavonoids

and lignin, 3) enzymes which are implicated to be directly involved in the defense responses, including the pathogenesis related proteins such PR1, chitinases, glucanases, and proteins that inactivate fungal ribosomes and bind chitin (Stintzi et al., 1993). Overexpression of some of these DR genes in several crops has shown increased resistance of these transgenic plants against pathogens (Wu et al., 1995; Kachroo et al., 2003; Livingstone et al., 2005; Hu et al., 2003), and suppression of these genes by epigenetic suppression, virus induced-gene silencing, and RNAi interference enhance disease susceptibility (Pallas et al., 1996; Zimmermann et al., 2006; Christensen et al., 2004). However, overexpression or suppression of these DR genes does not always increase resistance or enhanced susceptibility respectively, suggesting that the contribution of these genes to the resistance phenotype may be quantitative and that, when studied individually, their effects on resistance may not be significant. Moreover, since most of the DR genes in plants are members of multigene families and they usually shown functional redundancy (Zimmerman et al., 2006, Federico et al., 2006), it is difficult to determine the contribution of individual members in quantitative disease resistance. In the last decade, the use of candidate genes approach to map QTLs have been increasing and it has been successfully applied in several pathosystems.

Faris et al. (1999) reports the application of candidate gene approach for mapping disease resistance QTL in a population of wheat recombinant inbred lines (RILs). They mapped over 50 loci representing several DR genes to QTLs against several diseases including tan spot, leaf rust, Karnal bunt, and steam rust. Their analysis revealed QTL with larger effects in regions of putative *R*-genes. Several DR genes such *oxalate oxidase*, *peroxidase*, *superoxide dismutase*, *chitinase*, *P14-3-3*, and *thaumatin*, mapped within previously identified QTL and explained a greater amount of the phenotypic variation. They found that some of these DR genes are found in cluster arrangement on the QTL chromosomal region. Geffroy et al. (2000) found genes corresponding to *phenylalanine ammonia lyase (PAL)* and *hydroxyproline-rich glycoprotein (HRGP)* co-localized with resistance QTL against *Colletotrichum lindemuthianum* in bean. In potato, QTLs for resistance against *Phytophthora infestans* have been co-localized with genes from the phenylpropanoid pathway (*PAL*, *chalcone synthase*, *chalcone isomerase*), *cytchrome P450*, *osmotin (PR-5)*, and the *WRKY* gene transcription factor (Trognitz et al., 2002). In this study they also found that most of these DR genes are clustered on the QTL region.

CANDIDATE GENES AS PREDICTORS FOR QUANTITATIVE RESISTANCE IN RICE

In the last decade the candidate gene approach has been used in rice to demonstrate the association of several candidate DR-genes with quantitative resistance against several diseases including rice blast, bacterial blight (BB), and sheath blight (SB), and also against pests such brown plant-hopper (BHP) (Wang et al., 1994; Prashanth et al., 1998; Chen et al., 2001; Chen et al., 2003; Li et al., 1995; Li et al., 1999; Zou et al., 2000; Ramalingan et al., 2003). Resistance to the bacterial blight pathogen, *Xoo*, has been reported to have both qualitative and quantitative components (Li et al., 2001). One quantitative resistance system that has been well characterized in rice is resistance against rice blast (Wang et al., 1994, Prashanth et al., 1998; Sallaud et al., 2003; Talukder et al., 2005; Chen et al., 2003; Liu et al., 2004, Wu et al., 2004, Carrillo et al., 2005). In most of these studies, the association of major genes (*R*-genes), minor genes (DR-genes), environment x QTL interactions and the concept of durable resistance were all considered. The primary QTL mapping populations used in these studies were recombinant inbred lines (RILs), double haploid populations (DH), and backcrossing populations (BC3F3, and BC3F4).

Rice blast disease can be controlled by a combination of both qualitative and quantitative resistances. Wang et al. (1994) conducted the first comprehensive analysis of the quantitative resistance observed in *Moroberekan*, a traditional rice variety in Africa with a reputation for having durable resistance against rice blast. They used a mapping population of RILs derived from *Moroberekan/CO39* (a highly susceptible rice variety) to map QTL. Two important observations were made in this study. First, durable resistance is contributed by a combination of major genes and QTLs. Second, some QTLs with strong phenotypic effects map at regions containing major blast resistance genes (*Pi*), suggesting that some QTLs are possibly a manifestation of major defeated R-genes.

Another study tested the specificity of QTL for partial resistance to blast disease by using isolates for which no major *R*-genes segregated in a mapping population (Talukder et al., 2004). Of the 18 QTL reported, eight were effective against only one isolate, seven against two, and only three against all the isolates tested. Fourteen QTLs mapped in regions where major *R*-genes were previously localized. They concluded that most of the QTLs detected are race specific and that quantitative resistance genes might be defeated major *R*-genes. Chen et al. (2003) use a RIL

mapping population derived from a cross between the rice varieties Zhenshan 97 and Minghui 63 to map QTLs for resistance against rice blast. In total, 12 QTLs were identified in this population. Two had effects on all the three blast isolates used in this study, and the other 10 had effects on only one or two isolates. This study concluded that some of the genes underlying the QTLs may be commonly involved in the defense response against a broad range of pathogens infections and others may be only involved in limited defense responses thus showing degrees of race specificity.

A valuable resource for analysis of the association of candidate genes with QTL has been a doubled haploid (DH) population created from a cross between IR64, an *indica* variety, and Azucena, a *japonica* variety (Huang et al., 1994). These parents are highly diverged, and exhibit contrasting phenotypes for a variety of agronomic traits. IR64, a popular *indica* variety grown in tropical Asia, has a large number of qualitative and quantitative resistance genes against major rice diseases and against the insect pest BPH. Azucena, a traditional *japonica* variety, has quality factors and tolerance to abiotic stresses. This DH population has been used for mapping diseases (Prashanth et al., 1998; Wang et al., 2001) and insect resistance (Alam and Cohen, 1998) as well as tolerance to drought (Courtois et al., 2000).

Prashanth et al. (1998) used the DH population of a cross between IR64, an *indica* variety and Azucena a *japonica* variety to map QTLs for leaf and neck blast resistance. The DH population, along with their parental lines, was screened for blast at IRRI, Philippines and at three locations in South India. The phenotypic screen demonstrated that IR64 has a high level of resistance to leaf blast across four locations. The Azucena parental line was moderately susceptible in all the locations. They identified several QTLs against both leaf and neck blast. Interestingly some of the QTLs were different from the previously reported QTLs, and were identified across these wide geographical locations. This indicated, first, the non-race specific effects of these QTLs and, second their stability for blast resistance.

In 2001 Wang et al. used the same DH mapping population from the cross between IR64 and Azucena to localize 109 expressed sequence tags (ESTs) showing highly similarity to diseases resistance genes or to defense response genes. In this study three EST clusters were mapped to the QTL regions on chromosome 1, 2, and 3. Seven ESTs map on chromosome 1 where a QTL for partial resistance against rice blast was identified. Three of those ESTs have

highly homology with the gene *NPR-1* from *Arabidopsis*. Two ESTs encoded LRR and receptor-like kinase domain. Six ESTs mapped in the region on chromosome 2 where a QTL for partial resistance to sheath blight is located (Li et al., 1995). These ESTs encode PAL, Pto-like kinase, stomal ascorbate peroxidase, PR protein, and thaumatin-like protein. Another interesting region is on chromosome 3 where QTLs for partial resistance to rice blast (Wang et al., 1994), Sheath blight (Li et al., 1995), and bacterial blight (Li et al., 1999) are located. Two ESTs that specify serine/threonine kinase domain homologs mapped to that region.

Ramalingam et al. (2003) placed 118 candidate gene markers on the existing RFLP map generated from the IR64 x Azucena DH population. Several candidate resistance gene analogs (RGAs) and DR gene markers were associated with QTLs from BB, rice blast, sheath blight, and BPH. Candidate genes such *dihydrofolate reductase thymidylate synthase*, *aldose reductase*, *oxalate oxidase*, *oxalate oxidase -like*, *JA-myb*, and *peroxidase* were associated with QTL for one or more pathogen or pest.

Using a set of recombinant inbred lines (RILs) derived from a cross between the blast resistant *indica* cultivar Sanhuangzhan 2 (SHZ-2) and the susceptible *japonica* cultivar Lijiangxin-tuan-heigu (LTH), Liu et al. (2004) tested for association of candidate genes with blast resistance QTL. First they evaluated the range of resistance of the SHZ-2 by artificial inoculation with 344 blast isolates from eight provinces in China and 50 isolates representing 14 genetic lineages in the Philippines (Chen et al., 1995). SHZ-2 expressed resistance to 96% of the isolates from China and 98% of the isolates from the Philippines, thus confirming the broad spectrum resistance of this cultivar. They evaluated the level of quantitative resistance of the RILs in greenhouse and in field conditions in China and in the Philippines. Different disease reactions of the RILs across the three field locations were observed, reflecting different blast pathogen population structures. To remove the masking effects of major genes, they analyzed 101 RILs that did not carry markers closely linked to the major gene conferring resistance to a Philippines isolate PO6-6 and showed susceptibility reactions to the same isolate in inoculation experiments. They showed a normal distribution in disease leaf area (DLA) among these 101 RILs. They identified by single-factor analysis of variance five putative candidate genes associated with resistance against rice blast in the RILs evaluated under greenhouse and field conditions in several locations. Genes encoding for oxalate oxidase-like proteins, dehydrin, PR-

1, chitinase 2a, and P14-3-3 proteins (*GF14-e*) accounted for 30%, 23%, 15.8%, 6.7% and 5.5% of disease leaf area (DLA) variation, respectively.

Finally, Wu et al. (2004) validated the association of some candidate genes by co-localization with QTLs against rice blast in an advanced backcross population BC3F3 derived from rice varieties Vandana/Moroberekan. Their work confirmed association of some of the same gene candidates, such as *oxalate oxidase* and *P14-3-3*, with rice blast disease resistance QTL.

In the Liu et al. (2004) study, the five candidate genes were shown to co-localize with QTLs against rice blast, and the accumulation of these five genes into advanced RILs reduced rice blast disease. The performance of RI lines carrying donor alleles from SHZ-2 with those carrying the alternative alleles from LTH was tested under field conditions in several locations. The accumulation of SHZ-2 regions containing the alleles corresponding to oxalate oxidase-like proteins, dehydrin, and PR-1 reduced DLA by 14.6 to 30.8%, with oxalate oxidase-like protein gene showing the strongest effects. *Chitinase 2a* and *P14-3-3* genes were the lowest contributors for blast resistance. In general, the more regions containing candidate genes that they accumulated into the lines, the more resistant those plants were against blast in five different locations in China and the Philippines, which demonstrated the broad spectrum nature of the resistance.

The contribution of the five candidate DR-genes identified in the Liu et al (2004) study was also validated in a different rice genetic background by backcrossing SHZ-2 with a susceptible recurrent parent Texianzhan 13 (TXZ-13) to produce a BC3F3 lines accumulating the candidate regions. BC lines shown by molecular marker analysis to contain the candidate gene regions showed high levels of resistance against rice blast in five locations. The BC3F3 line called BC116 was evaluated in field trials against rice blast for four years (8 crops) and is still showing high levels of broad spectrum durable resistance (Bin Liu, Rice Research Institute, Guandong, China; *personal communication*). The BC116 lines also have been tested for sheath blight resistance in Colombia and the USA. In both places the lines show good levels of resistance against sheath blight under greenhouse and field conditions (Fernando Correa, CIAT, Colombia, SA; *personal communication*; Jim Oard, Louisiana State Univ; *personal communication*).

Candidate genes such *PAL*, *oxalate oxidase-like* genes, and *P14-3-3* have been co-localized with QTLs against rice blast, bacterial blight and sheath blight in several rice mapping populations (Figure 1.1). Taken together, all of these studies implicate these genes as likely candidates explaining these QTLs.

Roles of DR gene candidates:

Phenylalanine ammonium-lyase

PAL plays a key role in linking primary metabolism to phenylpropanoid metabolism. PAL enzyme catalyzes the conversion of L-phenylalanine into E-cinnamic acid, which is the precursor of a great variety of phenylpropanoids. The reaction catalyzed by PAL is considered as a key step and potential site for pathway regulation in the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). Phenylpropanoid products such as lignins and isoflavonoid phytoalexins have important functions in plant defense against pest and predators (Bruce and West, 1989). Phenylpropanoids-derived compounds also act as UV protectants (Hahlbrock and Scheel, 1989), and as a signal molecule both internally and for communication with other organisms (Lynn and Chang, 1990).

Other phenylpropanoid products, the phytoalexins, are low-molecular weight antimicrobial compounds that are synthesized and accumulated in plant tissues upon pathogen attack. Two different types of phytoalexins, diterpenes and phenolics, have been identified and isolated from rice (Cartwright et al., 1981). The phenolic phytoalexin, flavanone sakuranetin, is produced in rice in response to UV irradiation and blast infection (Kodama et al., 1992). The rice phytoalexins are effective in inhibiting *in vitro* growth of the blast fungus *Magnaporthe grisea* and accumulate more rapidly in the incompatible interactions with the pathogen than in the compatible interactions (Song and Goodman, 2001).

Enhanced expression of *PAL* genes has been shown for many plant-pathogen interactions, including both compatible and incompatible interactions. Fritzemeier et al. (1987), found a rapid increase of mRNAs corresponding to *PAL* and *4-coumarate CoA ligase (4CL)*, another key enzyme in the phenylpropanoid pathway, in potato after the infection with virulent and avirulent races of *Phytophthora infestans (P.i.)*. The initial, transient increase in the amount of *PAL* mRNA

was almost identical in compatible and incompatible interactions. This increase in *PAL* mRNA coincided with the penetration of the *P.i.* hyphae through the inoculated epidermal cell layer. Cuypers et al. (1988) also demonstrated that there are spatial and quantitative differences in the induction of *PAL* mRNAs between compatible and incompatible interactions of potato plants infected with *P.i.* During compatible interactions (virulent pathogen) the induction of *PAL* mRNA was smaller and lasted longer compared with the incompatible interaction (avirulent pathogen).

In *Phaseolus vulgaris* infected with *Colletotrichum lindemuthianum*, the enzymatic activity of several enzymes of the phenylpropanoid pathway, including *PAL*, increased rapidly after elicitor treatments, and there were time and spatial differences between compatible and incompatible interactions (Cramer et al., 1985). Correlation between lignin accumulation and induction of genes such *PAL*, *cinnamyl alcohol hydroxylase (CAD)*, and *peroxidases (POX)* has been also found in potato infected with *Fusarium sambucinum* (Zeng and Hammerschmidt, 1992).

The role of the *PAL* gene in the regulation of the phenylpropanoid pathway and in several biotic stresses was studied in transgenic tobacco plants epigenetically suppressed for *PAL* activity (Elkind et al., 1990; Bate et al., 1994; Maher et al., 1994; Pallas et al., 1996). Infection of *PAL*-suppressed tobacco plants with the virulent fungal pathogen *Cercospora nicotinae* lead to more rapid lesion development than observed with wild type plants (Maher et al., 1994). *Cercospora* did not induce accumulation of phenylpropanoid compounds in sense-suppressed or in wild type plants, so the authors concluded that the pre-existing phenylpropanoid compounds play an important role in lesion limitation in response to the virulent pathogen. The authors suggested that the role of *PAL* during the compatible interaction is related to the preformed type of resistance rather than an induced type of resistance.

In tobacco plants infected with the avirulent *tobacco mosaic virus (TMV)*, the *PAL* activity and transcripts were induced and this primary infection was sufficient to induce systemic acquired resistance (SAR) against other pathogens (Pellegrini et al., 1994; Ryals et al., 1994). *PAL*-suppressed tobacco plants inoculated with the TMV were deficient in SA and after initial

infection with TMV, and they failed to induce SAR in systemic tissue (no reduction of lesion size) (Pallas et al., 1996). These mutants were also impaired in the induction of PR-genes in systemic tissue (Pallas et al., 1996).

In *Arabidopsis* using T-DNA insertional mutants, Rohde et al. (2004) demonstrated that a mutation in a single *PAL* gene significantly affected not only the phenylpropanoid pathway genes, but also genes in carbohydrate metabolism. This single mutation affects the transcriptome and metabolome levels. Using transcript-profiling approaches in three mutants, *Arabidopsis pal1*, *pal2*, and *pal1 pal2* double mutants, they showed that disruption of PAL function significantly affected the transcription of genes that encode enzymes of the phenylpropanoid, carbohydrate, and amino acid metabolisms. This work established a clear link between primary and secondary metabolism. Components of most of the pathways altered in these *Arabidopsis pal* mutants were previously described for their responsiveness to pathogen attack or elicitation.

The PAL enzyme is a tetrameric protein. In potato and maize, this enzyme is made up of four identical subunits (Havir and Hanson, 1973). Studies of PAL enzyme purified from wheat seedlings indicated that this enzyme is made up of two pairs of unidentical subunits, two subunits of molecular wt of 75 KDa and two subunits of 80 KDa, suggesting that the PAL proteins derive from two structural *PAL* genes (Havir and Hanson, 1973; Nari et al., 1972). Because there is a coordinated regulation of key genes from the phenylpropanoid pathway, such as *PAL*, *4-coumarate CoA ligase (4CL)*, and *cinnamate 4-hydroxylase (C4H)* in parsley and bean (Logeman et al., 1995; Hahlbrock and Scheel, 1989; Yamada et al., 1996), Logeman et al. (1995) suggested the possibility of a putative multienzyme complex formed by products of these three genes from the phenylpropanoid pathway. Havir and Hanson (1973) described the formation of high molecular weight aggregates of PAL enzyme in mustard seedlings in vitro with other proteins.

There are several lines of evidence that *PAL* genes are involved in disease resistance in rice. First, enhanced expression of *PAL* genes has been shown to be associated with disease and resistance in rice based in SAGE analysis (G.L. Wang, *personal communication*). Second, increased and more rapid accumulation of PAL enzymatic products, the phenolic phytoalexin

flavanone sakuranetin, in incompatible interactions with *Magnaporthe grisea*, the rice blast pathogen (Kodama et al., 1992) and these phytoalexins are effective in inhibiting *in vitro* growth of the blast fungus (Song and Goodman, 2001). Third, *PAL* genes have been shown to co-localize with disease resistance QTL (Wang et al., 2001). In spite of these lines of evidence, still little is known about the *PAL* genes, or the biochemistry and enzymatic activity of the *PAL* enzyme in rice.

Oxalate oxidase and oxalate oxidase-like genes

Oxalate oxidase (*HvOxOa*) and *oxalate oxidase-like* (*HvOxOLP*) genes are members of the germin-like subfamily that belongs to the functionally diverse cupin superfamily (Woo et al., 2000). The germin and germin-like proteins (GLP) are multimeric, and glycosylated enzymes, that have extreme resistance to heat and to chemical degradation by proteases or hydrogen peroxide (Woo et al., 2000). Germins and GLPs are known to play a wide variety of roles as enzymes, structural proteins, or receptors (Bernier and Berna, 2001). To date, the exact mechanism by which these proteins are involved in plant defense responses remains unclear. As enzymes, germins have oxalate oxidase activity (Lane et al., 1993; Lane, 2000) and some GLPs have superoxide dismutase (SOD) activity (Bernier and Berna, 2001), both of which produce hydrogen peroxide (H_2O_2) in plants (Lou and Baldwin, 2006). For example, the barley germin protein subfamily HvGER1a exhibits oxalate oxidase activity, whereas the HvGER4 (*HvOxOLP*) and HvGER5 GLP subfamilies exhibit superoxide dismutase (SOD) activity (Zimmerman et al., 2006).

H_2O_2 , the product of germin and GLP activity, is a central component of plant defense responses. Rapid generation of H_2O_2 and superoxide (O_2^-) occurs during the oxidative burst, and both molecules mediate the signal networks underlying systemic and local resistance responses to pathogen challenges (Alvarez et al., 1998; Lamb and Dixon, 1997). H_2O_2 plays a role in the oxidative cross-linking of cell wall proteins and lignification, which imply a role in the cell wall reinforcement and papillae formation against pathogen penetration (Bradley et al., 1992; Olson & Varner, 1993). H_2O_2 also is involved in the orchestration of hypersensitive response (HR), a localized defense response (Levine et al., 1994). In addition H_2O_2 is a diffusible signal during systemic acquired resistance (SAR) (Lamb and Dixon, 1997). Elevated H_2O_2 levels may trigger downstream components of the defense response pathway and induce expression of defense

response genes associated with SAR (Chen et al., 1993). Constitutive expression of a *glucose oxidase* gene (*GOX*) in potato and rice plants increases the endogenous levels of H₂O₂ and induces the expression of defense response genes. These transgenic plants overexpressing *GOX* gene show enhanced broad-spectrum resistance to both bacterial and fungal pathogens (Wu et al., 1995; Kachroo et al., 2003).

Various studies have demonstrated that germins and GLPs are involved with plant defense response against several pathogens (Lane, 2002). First, the genes encoding the germins or GLPs are induced during plant interactions with pathogens. A barley germin-like protein (HvOXOa) that has oxalate oxidase activity is strongly induced after infection with the powdery mildew fungus, *Erysiphe graminis* f.sp. *hordei* (Zhang et al., 1995; Dumas et al., 1995; Zhou et al., 1998; Zimmerman et al 2006). A pepper germin-like protein is induced after virus and bacterial infection (Park et al., 2004). Members of the barley *HvGER4* (formerly referred to *HvGLP4* and *HvOxOLP*) gene family are expressed predominantly in *B. graminis*-attacked leaf epidermal tissue (Schweizer et al., 1999; Wei et al., 1998). This expression pattern is supported by the fact that in barley there are 44 *HvGLP4* out of approximately 5,000 EST from pathogen-attacked epidermis compared with one of a total of 106,000 EST from 21 other cDNA libraries (Christensen et al., 2004).

Second, overexpression of germin genes in plants enhances their resistance to pathogens. The wheat germin (oxalate oxidase) enzyme expressed in soybeans (Donalson et al., 2001), and hybrid poplar (Liang et al., 2001) is able to degrade oxalic acid and confer resistance to pathogens that secrete oxalic acid as a pathogenicity factor. Expressing a barley oxalate oxidase gene in peanut enhances resistance against *Sclerotinia minor* (Livingstone et al., 2005). Transgenic sunflowers expressing the wheat oxalate oxidase gene show enhanced resistance against *Sclerotinia sclerotiorum* and this enhancement is correlated with elevated levels of hydrogen peroxide, salicylic acid, and defense gene expression (Hu et al., 2003). Overexpression of the barley GLP gene *HvGER4* (*HvOxOLP*) gene enhances resistance to powdery mildew (Zimmerman et al., 2006).

Third, reduced expression of germin or germin-like genes enhances susceptibility to pathogens. Consistent with the findings that overexpression of the barley *HvGER4* (*HvOxOLP*) gene enhanced resistance to powdery mildew, silencing of the gene induces hypersusceptibility to this disease. Interestingly, the subfamily *HvGER4* is the only one that was induced by the non-host pathogen soybean rust fungus in barley, suggesting that the SOD activity exhibited by this family may be involved in broad-spectrum resistance to several pathogens. Although silencing of *germin* and *germin-like* genes enhances susceptibility to pathogens, it may result in enhanced resistance to pests; silencing of a germin-like protein gene in *Nicotiana attenuata* increases resistance against herbivores (Lou & Baldwin, 2006).

Fourth, GLP genes appear to be associated with quantitative resistance. The interesting barley *HvGER4* gene subfamily is represented by a cluster of at least nine highly conserved duplicated members (Druka et al., 2002; Wei et al., 1998). The same may be true for wheat *TaGLP4* subfamily (Christensen et al., 2004). Wei et al., 1998 demonstrated that one member of this subfamily *HvGER4* (*HvOxOLP*) is highly associated with an epidermis/papillae formation in the defense response of barley attacked by the powdery mildew fungus. Christensen et al. (2004) determined that the *HvGER4* gene subfamily previously named *HvGLP4* in barley and the wheat *TaGLP4* were important components of quantitative resistance in barley and wheat, respectively, and have SOD activity. Overexpression of *HvGLP4* and *TaGLP4* in their corresponding susceptible barley and wheat enhanced the resistance against their corresponding fungal pathogens, *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici*, respectively, by reducing the frequency of fungal haustoria formation. Silencing of those genes by RNAi revealed enhanced susceptibility (haustoria formation) compared with the corresponding barley and wheat non silenced plants.

In rice, Carrillo et al. (2005) identified more than 40 GLPs by scanning the rice genome. Four of the GLPs are putative *oxalate oxidases* on chromosome 3 where a rice blast resistance QTL was identified (Ramalingan et al., 2003, Wu et al., 2004). Phylogenetic analyses clustered chr.3 *oxalate oxidase* with the barley *oxalate oxidase* (*HvOXOa*, accession no. Y14203). Several GLP sequences on chr. 8 were similar in conserved domain structure to the barley oxalate oxidase-like protein (*HvOxOLP*, accession no. X93171) and these GLPs were co-localized with

resistance rice blast QTL in several mapping populations and are the major contributors for the resistance phenotype (Ramalingan et al., 2003; Liu et al., 2004; Carrillo et al., 2005; Wu et al., 2004).

Using the barley oxalate oxidase-like gene sequence (*HvOxOLP*), we predicted 12 putative *oxalate oxidase-like* genes (*OsOXL*) clustered in a 2.8 Mb section on rice chr. 8 (Davidson et al., *submitted*). Davidson et al (*submitted*) demonstrated that several members of this GLP subfamily in rice were differentially expressed after wounding and *Mg* inoculation of the susceptible and resistant parents of the two mapping populations where those genes were co-localized with QTL. While the expression profiles did not clearly implicate a single GLP family member to confer resistance against *Mg*, the patterns did suggest a combination of members that are finely regulated during defense responses in rice. Only one member *OsOXL6* showed higher and more sustained induction of *OsOXL6* in the resistant parents (+ chr.8 QTL) compared with the susceptible parents (- Chr.8 QTL). Five of the genes *OsOXL5* and *OsOXL7-10*, had similar expression patterns among all lines and all were induced by both treatments. *OsOXL11* was also induced by both treatments. Only *OsOXL1*, 2, 4, and 12 appeared to have little influence in the defense response after rice blast infection.

Although associated with disease resistance governed by QTL (Faris et al., 1999; Ramalingan et al., 2003, Liu et al., 2004; Wu et al., 2005), and implicated by expression profiling in the defense response (Davidson et al., *submitted*), the direct contribution of the *OsOXL* genes in disease resistance governed by specific QTL has not been demonstrated.

P14-3-3

All plant biological processes are controlled by signal transduction and metabolism regulation that occur via phosphorylation-mediated transition of protein states. Kinases, phosphatases, transcription factors, and enzymes all can be influenced by phosphorylation. In many cases, to complete their regulatory actions, these phosphorylated proteins must physically associate with the specialized adapter proteins known as 14-3-3 phosphoserine/threonine binding proteins (Sehnke et al., 2002). One hallmark of signal transduction and metabolism events is the identification of regulatory pathways in which 14-3-3 proteins interact physically with

phosphorylated target (client) proteins as adapters, chaperones, activators, or repressors to execute an important step in signal transduction and metabolism (Chen et al., 2006).

The 14-3-3 proteins were characterized initially during a survey-and-catalog project of proteins that appeared to be specific to mammalian brain tissue (Moore and Perez, 1967). Proteins were given operational designations based on chromatography elution and starch-gel electrophoresis profiles. Several three-numbered proteins appeared to be brain specific, including 15-4-1, 4-4-2, and 14-3-3. The three numbered designation has persisted for the 14-3-3 proteins (Sehnke et al., 2002). One function of the 14-3-3 proteins is to support cell survival which is achieved, in part, by antagonizing the activity of associated proapoptotic proteins. Disruption of 14-3-3-ligand interactions leads to apoptotic cell death in mammalian COS-7 cells (Masters et al., 2002).

Initially it was assumed that 14-3-3 proteins from *Arabidopsis* have the same function as in mammalian systems based on the model of these proteins and the evolutionary conservation in the protein structure. Support for this hypothesis comes from complementation studies in yeast, which indicate that several *Arabidopsis* 14-3-3 proteins can substitute for essential 14-3-3 functions in yeast (van Heusden et al., 1996). However, two lines of evidence support the hypothesis that there are important functional differences among the various 14-3-3 proteins in multicellular organisms. First, higher organisms have fairly large and diverse *14-3-3* gene families. The central conserved areas of the sequence remains intact, but the gene sequences diverge in several areas at the termini. The second evidence is that biological phenotypes associated with altered 14-3-3 isoforms demonstrate properties that are difficult to explain in the absence of specificity, and various 14-3-3 isoforms have differential affinities for at least certain client proteins (Sehnke et al., 2002). With the release of the *Arabidopsis* and rice genomes, it was feasible to study the diversity of these proteins in these plants (Wu et al., 1997; DeLille et al., 2001).

There are several lines of evidence to show that 14-3-3s are involved in many metabolic and signaling pathways for plant growth regulation and responses to environmental stress (Roberts et al., 2002). In plants, phenotypes associated with *14-3-3* genes have been examined.

Overexpression and antisense constructions of *14-3-3* in potato suggested a role for these proteins in plant development and senescence (Markiewicz et al., 1996). *Arabidopsis* plants expressing antisense *14-3-3* accumulate unusual amounts of starch in the leaf (Sehnke et al., 2001). 14-3-3 proteins are involved in the regulation of the plasma membrane H⁺-ATPase, nitrate reductase (NR) and sucrose phosphate synthase (SPS) (Sehnke et al., 2002). 14-3-3 proteins also bind a range of transcription factors for their activation and translocation (Roberts, 2003). For example, the tobacco transcription factor RSG (repression of shoot growth) is bound to 14-3-3s and involved in GA biosynthesis (Ishida et al., 2004). One hallmark of signal transduction and metabolism events is to identify 14-3-3 participation in cellular regulatory pathways in which those proteins generally acting as adapters, chaperones, activators, or repressors, interact physically with target (client) proteins phosphorylated to execute an important step in signal transduction and metabolism (Chen et al., 2006).

14-3-3 proteins are involved in plant defense responses (Brandt et al., 1992; Finni et al., 2002; Zhang et al., 1997; Seehaus and Tenhaken, 1998; Hill et al., 1999; Roberts and Bowles, 1999; Zhou et al., 2000; Yan et al., 2002; Cooper et al., 2003; Chen et al., 2006). In rice, however, little is known about the 14-3-3- proteins and their role in resistance. Chen et al. (2006) made an important start by bioinformatically describing the *14-3-3* gene family in rice, and analysing their expression patterns after several abiotic and biotic stresses including rice blast and bacterial blight challenges. However, it is not known how the various members of the 14-3-3- are involved in disease resistance, and if their co-localization with disease resistance QTL (Liu et al., 2004) is meaningful. In this study by gene suppression, we determined the contribution of one of the eight *14-3-3* gene members (*GF14-e*), co-localized with a resistance QTL on rice chromosome 2 (Liu et al., 2004), to disease resistance.

FORWARD AND REVERSE GENETICS FOR CANDIDATE GENE VALIDATION

In this thesis project, the validation of candidate gene function in disease resistance governed by QTL required the means to assess the effect of altered gene expression for individual gene family members. The feasibility of doing these studies was enhanced in the past 10 years by the release of the rice genome sequence (Goff et al., 2002; Yu et al., 2002) and the development of many tools to meet the challenge for the post-sequencing era. This challenge is

to assign and understand the biological functions to each of the estimated 50,000 genes in order to use them for genetic improvement of rice or other cereals. Several approaches to assign gene functions have been based on genetics such as mutant identification and map-based gene cloning (Martin, 1998). However, one of the major disadvantages to the application of these techniques is that they are time-consuming strategies.

Mutants are essential tools in assigning function to unknown genes and in dissecting biochemical, metabolic, and regulatory pathways. Forward genetics begins with a mutant phenotype and is applied to identify the mutant gene causing this altered phenotype. This approach has been used for more than a century. However, considering the increasing availability of rice genomic sequences, reverse genetic approaches to discover gene functions is becoming more important. Reverse genetics begins with a mutant gene sequence and tries to determine if this altered gene sequence results in an altered phenotype.

Rice researchers around the world have been active in the production of rice mutants (Hirochika et al., 2004). One of the more popular approaches is the generation of mutants by insertional mutagenesis. This approach is based on the insertion of foreign DNA into the gene of interest to disrupt its gene function. The most important advantage of this approach is that the inserted element acts as a tag for gene identification. This system allows the production of many mutant lines at one time and the induced mutation can be detected by polymerase chain reaction (PCR). Insertional mutagenesis has been performed using transposon, T-DNA and retrotransposons in several plant species (Hirochika et al., 2004). *Arabidopsis*, whose entire genome sequence was available before the rice genome sequence, was the first plant model used to generate a big collection of insertional mutants. Several research teams around the world produced a large number of insertional lines using a variety of insertion elements, including T-DNA (Azpiroz-Leehan and Feldmann, 1997; Koncz et al., 1992; Krysan et al., 1999) or transposable elements such as Ac/Dc (Parinov et al., 1999), and the *Enhancer/Suppressor mutator* element *En/Spm* (Tissier et al., 1999; Wisman et al., 1998). All of these insertional lines have been successfully used for reverse genetic screening to identify insertions in targeted genes (Krysan et al., 1996; McKinney et al., 1995; Parinov et al., 1999).

Currently, many rice researchers are generating a large collection of insertional mutants using the same insertion elements that were used in *Arabidopsis*. Several groups around the world successfully produced T-DNA rice insertional lines (Hirochika et al., 2004). Moreover, T-DNA vectors have been modified to monitor the expression of tagged genes and screen for enhancers/promoters using different reporter genes (Jeon et al., 2000; Jeon et al., 2002; Wu et al., 2003). A T-DNA insertion mutant population of Hwayeong rice cultivar was used to isolate mutant rice plants with enhanced susceptibility to rice blast (Kim et al., 2005). Izawa et al. (1991) demonstrated the transposition of the maize transposable element *Ac* in rice. One of the problems in the use of this system is the unpredictable silencing of the *Ds* elements observed in later generations in the presence of *Ac transposase* (Izawa et al., 1997). Kolesnik et al. (2004) reported an efficient *Ac/Dc* tagging system in rice and several generations of parental and *Ds* insertion lines have been analyzed for transposon activity and none of these lines undergoes gene silencing. They also had shown that *Ac* itself was stable in successive generations. A large collection of *Ac* insertion mutants was produced by taking into account this advantage (Enoki et al., 1999; Greco et al., 2001).

In addition, an endogenous retrotransposon *Tos17* was used to generate a large collection of insertional mutants (Hirochika, 2001; Miyao et al., 2003). This system generates stable mutations because the integrated copies of the retrotransposon are never excised, thus inducing stable mutations. Miyao et al. (2003) demonstrated that the integration of *Tos17* in the genome is not random. *Tos17* insertions were distributed through the rice genome, and preferred genic regions for integration. Moreover, there are insertion hot spots for *Tos17* in the rice genome.

While insertional mutants offer several advantages in gene cloning and reverse genetics, they are several economical and practical limitations associated with transformation-mediated mutagenesis. Because insertional mutagenesis requires plant transformation, most of the rice insertional mutants have been produced in *japonica* rice because of its amenability to tissue culture and transformation. The function of genes often depends on the genetic background so we can only detect gene function if the mutations are created in a specific genotype. Deletion mutants produced by chemical or irradiation mutagenesis offers the advantage of producing a large number of functional variants in any genotype and with a low initial investment in

producing the starting lines. One of the major disadvantages in the use of deletion mutants is the considerable effort to isolate the gene deleted after a phenotype is identified because the deletion is not physically tagged. With advances in high throughput genotyping, the efficiency in detecting point mutations or deletions in genes has been significantly improved making feasible the use of deletion and point mutation stocks for forward and reverse genetics.

Several techniques have been developed to detect point mutations. PCR-RF-SSCP (PRS), which combined cleaved amplified polymorphic sequences (CAPS) and single-strand conformation polymorphism (SSCP) was successfully used to detect point mutations in rice waxy mutants (Sato and Nishio, 2003). The new TILLING (Targeting Induced Local Lesions in Genomes) was developed to screen point mutation in desired genes using ethyl methanesulfonate (EMS)-induced mutants in *Arabidopsis* (McCallum et al., 2000; Colbert et al., 2001; Till et al., 2003). Recently new approaches based in microarray technology were developed to screen deletion mutants (Salamon et al., 2000). Borevitz et al. (2003) described the use of *Arabidopsis* oligonucleotide (oligo) chip to detect single feature polymorphism (SFP) in ecotypes of *Arabidopsis*. Chang et al. (2003) applied this concept to rice and use a Rice SyngentaGeneChip genome array to detect deletions in rice mutants.

The concept of detecting deletions by high-throughout PCR based approach was first developed in nematodes (Liu et al., 1999). A similar approach was use in *Arabidopsis* were nested PCR was used to selectively amplify the DNA of the deletion mutant in DNA pools of mutants induced by fast neutron. An example of detecting a deletion in a rice gene was included to illustrate the generality of the PCR screening approach (Li et al., 2001). However, the technique developed was not efficient for detection of small deletions, such as those introduced by diepoxybutane mutagenesis in rice.

GENE SILENCING FOR CANDIDATE GENE VALIDATION

RNA silencing is a generic term describing related RNA-guided gene regulatory mechanisms that include post-transcriptional gene silencing (PTGS) in plants, quelling in fungi and RNA interference in animals (Ding et al., 2004). The first examples of RNA silencing were observed in several transgenic plants each containing the same ectopic transgene. Those plants

exhibited inhibition of expression of the silencing construct and also of the endogenous gene (Napoli et al., 1990). RNAi is a highly conserved mechanism found in almost all eukaryotes and believed to serve as an antiviral defense mechanism. The molecular details are becoming clear from combined genetic and biochemical approaches (Dillin, 2003). Two types of RNA play major roles in RNA silencing: dsRNA, which acts as a trigger for RNA breakdown, and short interfering (siRNA), which is involved in the actual degradation of target mRNA in the final step of the RNAi pathway (Hannon, 2002). On entry into the cell, the dsRNAs is cleaved by an RNase III like enzyme, DICER, into small interfering (21- to 23) RNAs (siRNAs) (Bernstein et al., 2001). Biochemical evidence indicates that the siRNAs are incorporated into a multisubunit protein complex, the RNAi-induced silencing complex (RISC), which directs the siRNA to the appropriate endogenous mRNA. The RISC complex may unwind the siRNA to help interactions with the target mRNA (Martinez et al., 2002). Alternatively, the siRNA can serve as a primer for an RNA-dependent RNA polymerase (RdRP), creating many more siRNAs to amplify the signal.

One aspect of RNA silencing that is not well understood is the transitive RNA silencing observed in *C. elegans* and plants. In *C. elegans*, a dsRNA trigger can produce new siRNAs 5' to the region that is initially targeted (Nishikura, 2001). These newly synthesized siRNAs can target other RNAs on the basis of sequence similarity. In plants, siRNA spread not only in the 3' to 5' direction but also in the 5' to 3' direction when the *green fluorescent protein (gfp)* or *B-glucuronidase (gus)* transgenes were used as targets for RNAi silencing (Braunstein et al., 2002).

PTGS is an important molecular biology tool for investigating protein function by modulating gene expression, using either antisense (Ecker and Davis, 1986) or sense constructs (van der Krol et al., 1990). Unfortunately, these approaches to the induction of PTGS generally produce effective gene silencing in no more than 10-20% of a transgenic population (Hamilton et al., 1998). Gene-silencing methodology was greatly improved after the discovery that ds-RNA is a more efficient inducer of PTGS than either sense or antisense RNA in both plants and animals (Fire et al., 1998). The frequency of PTGS in a population was increased greatly after transformation of plants with a construct composed of an inverted repeat of the gene of interest, with or without an intron between the inverted repeat elements (Smith et al., 2000). However, the

technical manipulations necessary to generate these inverted repeat constructs has limited their routine use as a high-throughput tool for functional genomics.

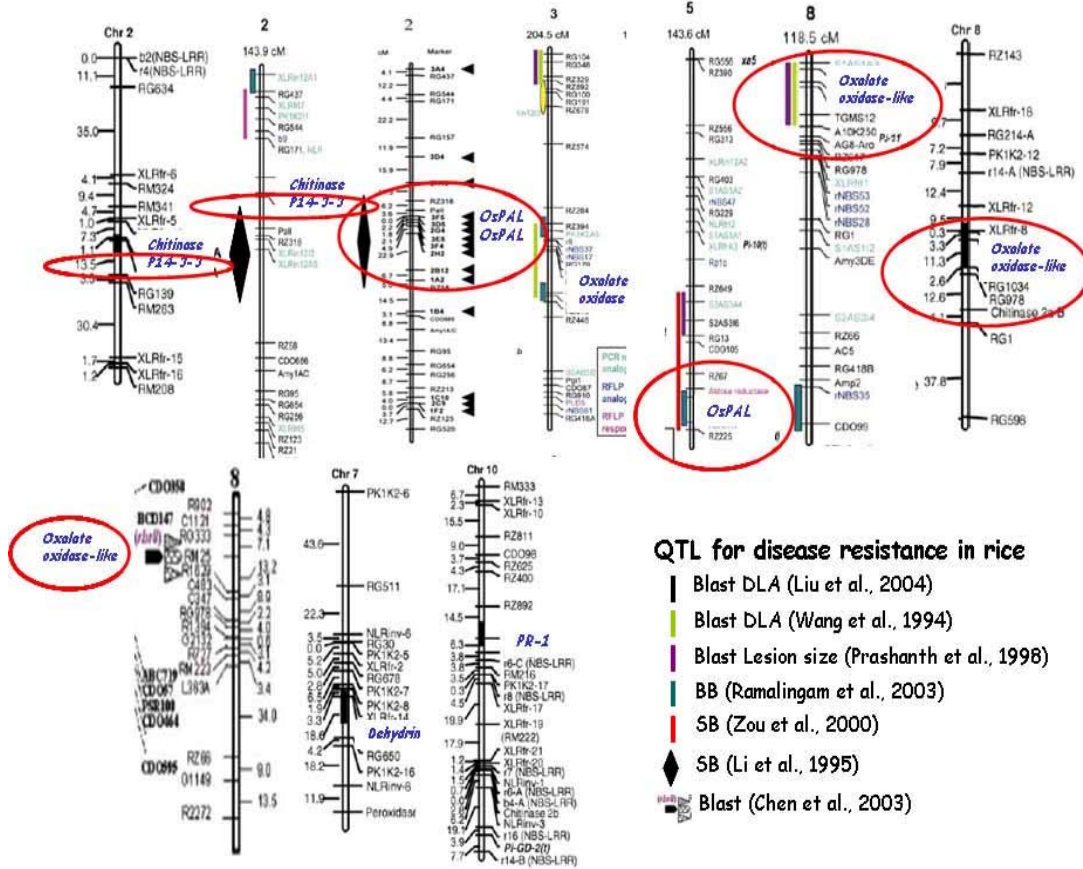
Brummell et al. (2003) described a method for the easy generation of an inverted repeat construct for the silencing of genes that is applicable to high-throughput studies. This improved procedure for high-efficiency gene silencing is specific for a target gene, but does not require inverted repeat DNA of the target gene in the construct. This method employs an inverted repeat of the 3'-UTR of a heterologous gene from *Agrobacterium tumefaciens* (*nopaline synthase, nos*). A target single region is cloned 5' to these nos inverted repeats. This method was developed based on the observations that siRNA molecules function as primers for an RNA-dependent RNA polymerase activity, and the RNA 5' to a dsRNA region is degraded. With this information as a basis, it was predicted that a molecule consisting of an inverted repeat of a 3' region corresponding to the nos gene located 3' of a single-stranded targeting sequence could induce highly efficient degradation of endogenous mRNA homologous to the transgene. Using this double nos terminator vector, Brummell et al. (2003) demonstrated that this vector efficiently suppressed gene expression of the targets in tomato and *Arabidopsis*.

In rice, (Miki and Shimamoto, 2004) developed a Gateway vector, pPANDA, for RNA interference of rice genes. This vector can be used for *Agrobacterium* transformation of rice and allows easy and fast construction of efficient RNAi vectors. (Miki et al., 2005) used this pPANDA vector to silence the *OsRAC* gene family members in rice. They tested this vector for gene-specific suppression of *OsRAC* by silencing multiple members of a gene family by putting gene-specific DNA regions together in a single construct, and, finally, gene suppression of most of the family gene members using a highly conserved region of the gene. The most important remarks in this paper were that they were able to suppress the expression of the entire *OsRAC* gene family in rice (7 members) with different efficiencies, by using a single region that was highly conserved among the family members. They also found that suppression levels generally correlated with the overall sequence similarity between the trigger region and the targets. However, they also suggested that other unknown factors must play a role in gene silencing processes because suppression efficiency did not exclusively depend on sequence homology between the trigger and target sequences.

Previous work has shown that several candidate defense response genes co-localize with disease resistance QTL in rice in several mapping populations. However, whether or not the function of these genes contributes to the disease resistance directed by the QTL has not been demonstrated. Therefore, the goal of my research was to determine if three candidate defense response genes contributed to QTL-based resistance in rice. My approach was to use the reverse genetics approach by using knock out mutants (diepoxybutane induced deletion mutation) and gene suppression strategy (RNAi) to assess if loss or reduction of gene function resulted in a reduction in disease resistance. The candidate genes I studied were members of larger gene families, including the gene families encoding oxalate oxidase-like proteins, phenylalanine ammonia lyase, and 14-3-3 proteins.

Figures and Tables Chapter 1

Figure 1.1 Candidate genes co-localized with QTLs against rice diseases



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**CHAPTER 2 - Mutation of a rice *phenylalanine ammonia lyase* gene
is associated with susceptibility to bacterial blight.**

Summary

Phenylalanine ammonia lyase (PAL) genes are putative plant defense response (DR) genes that co-localize with disease resistance quantitative trait loci (QTL) in rice. To study the roles of *PAL* and other target genes in rice defense responses, we optimized a DNA-pooling, polymerase chain reaction (PCR)-based screening strategy to enable identification of mutants in a collection of chemical-(diepoxybutane) induced rice mutants. A mutant line was identified (*ospal4*) that contains a 750 bp deletion in *OsPAL4*, which is one of the four rice *PAL* genes clustered on chromosome 2. Segregation of the *ospal4* mutation deviated from expected Mendelian ratios, probably due to poor transmission of the mutant allele. Inoculation of advanced generations of the *osPAL4* mutant line with a compatible isolate of the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae*, revealed that progeny lines carrying the *OsPAL4* deletion are more susceptible than those lines containing the wild type allele. These results are consistent with a role of the *OsPAL4* gene in the disease resistance governed by the QTL on chromosome 2.

Introduction

In general, plants contain several types of genes involved in defenses against pathogens (Hammond-Kosack and Jones, 1996). These include disease resistance (R) genes, which are involved in the detection of specific pathogen species or races, defense response (DR) genes, which include the enzymes that function in some way to inhibit pathogen spread, and other genes that relay the pathogen recognition signal and regulate the coordinated expression of the DR genes. DR gene expression may or may not be regulated by the disease R gene interactions. There is increasing evidence that, in some cases, DR genes may be responsible for resistance that is independent of R gene regulation. This may be the case for disease resistance that is governed by multiple genes with small effects, commonly referred to as quantitative trait loci (QTL). If true, this would be valuable information for crop plant improvement programs for several reasons. First, knowledge of the genes responsible for QTL-governed phenotypes would allow for the development of molecular markers that could be used in breeding strategies to facilitate incorporation of the QTL into important cultivars. Second, knowing that DR genes contribute to disease resistance QTL would shed light on whether the effects of individual QTL will retain their resistance effects over time and whether they should be expected to provide broad-spectrum (effective against a wide group of pathogens of the same or different genera) resistance.

Over the past several years, a number of lines of evidence implicate DR genes as the molecular basis for the broad-spectrum, durable disease resistance controlled by QTL in rice. Using several different rice mapping populations, a variety of DR genes have been shown to co-localize with QTL conferring resistance against rice blast, sheath blight, bacterial blight, and brown plant hopper (Wu, 2000; Wang et al., 2001; Ramalingam et al., 2003; Liu et al., 2004; Wu et al., 2004; Liu et al., 2005). Based on this information, Liu and coworkers (Liu et al., 2004) used marker assisted selection to accumulate regions associated with five DR genes into a rice cultivar, and demonstrated that the lines with the five candidates exhibited more resistance to rice blast in multilocation trials than lines with fewer genes. Although these studies are suggestive that DR genes function in QTL, evidence that a given gene or gene family member can contribute to resistance and the extent of that contribution are still lacking.

Mutants are valuable tools for assessing function of genes in processes such as defense responses. Insertional mutant collections of rice are now becoming available (Hirochika et al., 2004), and are ideal for reverse genetic studies because the inserted element acts as a tag for gene identification. However, the utility of these collections for the studies we describe here was limited because, although increasing in number, the insertion collections are not yet comprehensive enough to saturate the genome with mutations, limiting the chances of detecting a target gene mutation. Furthermore, most of rice insertional mutants have been produced in *japonica* rice because of its amenability to tissue culture and transformation. Most of the QTL based resistances that we have identified are in *indica* rice, which are more recalcitrant to transformation. Thus, to obtain mutants in defense response genes, we have relied on the large collection of chemical- and irradiation-induced mutants produced by the International Rice Research Institute in the *indica* rice cultivar IR64 (Wu et al., 2005), which is a source of several disease resistances QTL (Prashanth et al., 1998; Ramalingam et al., 2003).

A major disadvantage for the use of deletion mutants is that the deletion is not physically tagged with foreign DNA, which makes identification of the mutated gene, with or without a phenotype, very difficult. The concept of detecting deletion mutants by a high-throughput PCR based approach was first developed for nematodes (Liu et al., 2000). A similar approach was used to detect deletion mutants in DNA pools of *Arabidopsis* mutants that were induced by fast neutrons (Li et al., 2001). An example of detecting a deletion in a rice gene was included in that study to illustrate the generality of the PCR screening approach (Li et al., 2001). The rice FN induced deletion detected in that study was very large (2.5 kb). To enable detection of smaller sized deletions, such as those induced by diepoxybutane (DEB), in this study we modified the technique to optimize screening rice DEB-induced mutants for mutations in single genes.

Using this technique, we identified a mutant rice line that has a deletion in a putative DR gene, i.e., a member of the *phenylalanine ammonia lyase (PAL)* gene family. The enzyme PAL catalyzes the conversion of L-phenylalanine into cinnamic acid, which is the precursor of a great variety of phenylpropanoids. This reaction is a key step and potential site for regulation of the phenylpropanoid pathway (Bowles, 1990; Bate et al., 1994; Dixon et al., 1996; Hahlbrock and

Scheel, 1989). Phenylpropanoid products including lignins and isoflavonoid phytoalexins have important functions in plant defense against pathogens and insect pests (Bruce and West, 1989; Nicholson and Hammerschmidt, 1992; Hahlbrock and Scheel, 1989). Rice produces two different types of phytoalexins, diterpenes and phenolics (Cartwright et al., 1981) that are effective in inhibiting in vitro growth of the blast fungus (Song and Goodman, 2001). The phenolic phytoalexin, flavanone sakuranetin, has been shown to accumulate more rapidly in incompatible interactions with *Magnaporthe grisea*, the rice blast pathogen (Kodama et al., 1992). Other phenylpropanoid-derived compounds act as UV protectants (Hahlbrock and Scheel, 1989), as signal molecules both internally and for communication with other organisms (Lynn and Chang, 1990), or as structural barriers through cell wall reinforcement (Hahlbrock and Scheel, 1989). *PAL* is also a key enzyme for salicylic acid (SA) biosynthesis, which is a required secondary signal for the systemic acquired resistance (SAR) in plants (Lee et al., 1995, Malamy et al., 1990). SAR is a physiological state of enhanced defensive capacity, whereby the plant's innate defenses are potentiated against biotic challenges (Vallad and Goodman, 2004). Induced accumulation of *PAL* mRNA and increases of *PAL* enzymatic activity upon pathogen challenge has been demonstrated in several pathosystems (Cramer et al., 1985; Fritze et al., 1987; Cuypers et al., 1988; Becker et al., 1991; Zeng and Hammerschmidt, 1992; Joos and Hahlbrock, 1992; Logemann et al., 1995; Shiraishi et al., 1995; Cui et al., 1996; Kumar Jain, et al., 2004). *PAL* genes also have been co-localized with disease resistance QTL in several pathosystems (Faris et al., 1999; Geffroy et al., 2000; Trognitz et al., 2002), including interactions between rice and various pathogens (Wang et al., 2001).

Defense response genes in plants are commonly members of gene families. For example, in rice there are several *peroxidase* genes, but only a few have been shown to be associated with disease resistance (Chittoor et al., 1997; Chittoor et al., 1999; Hilaire et al., 2001). While some plants have only a single copy of *PAL*, such as loblolly pine (Whetten and Sederoff, 1992), most plants have several *PAL* family members, ranging from four genes in *Arabidopsis* (Rohde et al., 2004) to more than forty genes in potato (Joos and Hahlbrock, 1992). Understanding which family members are functioning as DR genes is important if we are to target these alleles for breeding programs.

Given the putative role of *PAL* genes in defense response against pathogens and the co-localization of particular *PAL* genes with QTL in rice, they are interesting candidates for the genes responsible for resistance conferred by QTL (Cui et al., 1996; Joos and Hahlbrock, 1992; Logemann et al., 1995; Blilou et al., 2000; Kumar Jain, et al., 2004; Fritzemeier et al., 1987; Shiraishi et al., 1995; Faris et al., 1999; Geffroy et al., 2000; Wang et al., 2001; Trognitz et al., 2002).

To determine if a specific rice *PAL* (*OsPAL*) gene family member or members might contribute to disease resistance in rice, we identified *OsPAL* gene family members in the rice genome sequence, developed *OsPAL* gene family specific primers, and used these primers to screen the IR64 mutant collection for mutations in members of the *OsPAL* gene family. We identified a rice mutant line that harbors a deletion in a specific family member of the *PAL* gene family clustered on chromosome 2, where a QTL against sheath blight has been mapped (Wang et al., 2001). This mutant, called *ospal4*, exhibits increased susceptibility against a virulent strain of *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight disease in rice.

Results

Optimization of high throughput mutant screening using PCR amplification of DNA pools and screening with DR gene primers.

To determine the optimal pool size to allow detection of a mutant amplicon among wild-type amplicons, we performed a reconstruction experiment using a natural deletion in the *Xa21* gene in rice line IR24 (Guo-Liang Wang, personal communication). To first characterize the mutation, gene specific primers, which were designed using the complete coding sequence (cds) of the *Xa21* gene (accession number AB212798), were used to amplify genomic DNA from IR24 and the isogenic line carrying the wild type *Xa21* gene, IRBB21. The *Xa21* specific primers amplified a band of the expected size (1377 bp) from IRBB21 line, but the amplicon from IR24 DNA was approximately 100 bp smaller. Sequence analysis of the PCR products from IRBB21 and IR24 lines revealed that the mutation spans 135 bp at the 3' untranslated region of the *Xa21* gene in the IR24 rice line (Figure 2.1a). For the reconstruction experiments using this mutation, we mixed IR24 genomic DNA in different ratios with IRBB21 DNA (1:1, 1:10, 1:100, 1:200, 1:1000), and used PCR to amplify the *Xa21* locus. Figure 2.1b shows that the *Xa21* mutant amplicon was detected at up to a dilution of 1:200.

Based on this information, we organized the DNA from a total of 3,000 DEB-mutagenized IR64 lines so that DNA of each line was included in a pool of 10 lines and a pool of 100 lines (superpools) (30 DEB super pools total). Using available sequence information of several putative DR genes, 33 primers corresponding to 26 DR genes were designed to amplify a single gene or gene family member (gene-specific) or to amplify several members of a gene family (gene family-primers) (Table 2.1). These primers were used to amplify DNA in each super pool (100 lines), and the amplicons were separated in 4% polyacrylamide gels and visualized with silver staining. If a putative mutant band, i.e., a band that migrates further than the wild type amplicon, was found in the products of a super pool, PCR analysis was carried out on the component ten-line pools. Finally, once the deletion was identified in the ten-line pool, PCR was performed in the 10 corresponding individual lines to identify the specific mutant line (Figure 2.2). Of the 26 DR gene primer pairs tested, only one set of primers identified a mutation, the primers that amplified gene family members of the rice *phenylalanine ammonia lyase* gene family located on chromosome 2 (see below).

Development of primers to detect deletions in the rice phenylalanine ammonia lyase gene (OsPAL) gene family.

Nine putative *phenylalanine ammonia lyase* genes were predicted in the rice genome using the public rice sequence databases <http://www.ncbi.nlm.nih.gov/> and <http://www.tigr.org> (Figure 2.3a). Multiple sequence alignment of the nine predicted *OsPAL* genes revealed genomic DNA sequence identities between members ranging from 36% to 92% and amino acid sequence identity from 68% to 95%. Predicted gene sizes ranged from 2,400-4,005 nucleotides, and predicted proteins ranged from 690-718 amino acids. Most of the genes are predicted to have two exons and one intron with the exception of genes *OsPAL3* and *OsPAL7*, which are predicted to contain one exon (Figure 2.3b).

Phylogenetic reconstruction using the unrooted neighbor-joining method revealed the sequence identity and evolutionary relationships among all the *OsPAL* gene members (Figure 2.3c). *OsPAL* gene members group into three major gene clusters. The first cluster contains *OsPAL1* and *OsPAL5*, which share 87% identity in predicted amino acid sequences. The second and largest cluster contains five gene family members, *OsPAL2*, 4, 6, 7, and 3. Three closely related members of this group *OsPAL3*, 4, and 7, share from 89% to 94% amino acid sequence similarity. The third cluster contains *OsPAL8* and *OsPAL9*, which share 95% amino acid similarity, and are the most closely related genes among the nine members.

OsPAL gene family primers were designed in the most highly conserved region among all the *OsPAL* gene members (Figure 2.3b). The distance between the primers for most of the genes was 923 bp, with an exception being *OsPAL8*, in which the expected amplicon was 938 bp. These primers were used to amplify the DEB-mutant DNA super pools. After amplification, two super pools (17 and 18) revealed polymorphisms. The mutant amplicons were approximately 200 bp (Figure 2.4a). After amplification of the ten-line pools that composed superpools 17 and 18, pools 169 and 178, respectively, showed the same polymorphisms (Figure 2.4b). After PCR amplification of the 10 individual lines corresponding to pools 169 and 178, a single individual mutant line 1982, corresponding to pool 169, was found to contain the variant fragment (Figure 2.4c). Individual lines containing the variant fragment could not be detected in lines composing pool 178 probably due to mistakes during the pooling process. Both wild type and mutant fragment were amplified from the single mutant line 1982, suggesting that the line is

heterozygous for the deleted allele or that the wild type fragment correspond to other gene members that can be amplified with this gene-family primers. The IR64 background of the *ospal* mutant line (1982) was confirmed by genotyping this line with several simple sequence repeat (SSR) markers (data not shown).

Characterization of the mutation at the OsPAL locus.

To determine if the *OsPAL* genes predicted using the *japonica* cv. Nipponbare database were different from their orthologs in the *indica* rice database; *OsPAL* genes were predicted using the BGI *indica* variety 93-11 and the *O. sativa ssp. indica* WGS contigs from The Institute for Genomic Research (TIGR) and NCBI databases, respectively. Most of the *OsPAL* genes predicted in Nipponbare were predicted in 93-11 with only two exceptions (*OsPAL8* and *OsPAL9*). The genomic sequences from 93-11 shared from 98-100% homology with their corresponding orthologs from Nipponbare.

To obtain the sequence of the mutant allele, we excised the variant fragment from the polyacrylamide gel, reamplified that fragment using gene-family primers, and cloned the amplicon prior to sequencing. The mutant fragment was identical to the *OsPAL4* gene family member predicted from cv. 93-11 (AAAA02006954-7) (Figure 2.5). We also amplified the wild type allele using gene-specific primers for the mutant allele and this wild type fragment also showed a 100% homology with the mutant fragment and the *OsPAL4* gene predicted from cv. 93-11. In the mutant amplicons, we detected a deletion between nucleotides 889 and 1,639 in *OsPAL4* (Figure 2.5). The *OsPAL4* gene predicted from Nipponbare has 99% identity with the corresponding 93-11 (Accession number: AAAA02006954-7).

Two bands were consistently detected in the acrylamide gels, gel purification, reamplification. Sequencing of the two fragments indicated they were identical in sequence. Since additional copies of *OsPAL4* were not predicted in the rice genome, we assumed that the presence of the double band is an artifact of the polyacrylamide electrophoresis. However, attempts to clarify this by the use of several additives in the PCR reaction, such as dimethylsulfoxide (DMSO) and formamide, as well as gradient PCR reactions, which should eliminate secondary structure that might cause the fragment to resolve into two bands, did not resolve the amplicon in the *OsPAL* mutant segregant lines into one band (data not shown).

Internal primers designed within the deleted region were used to amplify DNAs from IR64 wild type and the segregating mutant population (the heterozygous *ospal4* mutant and mutants with the *OsPAL4* wild type allele) to confirm the region that was deleted (Figure 2.6, 2.5). As expected, the primers located in the deleted region did not amplify the mutant band in any of the lines including the heterozygous segregant *ospal* mutant lines. The wild type *OsPAL* band was amplified in all lines (Figure 2.6).

To confirm which *OsPAL* gene family member was altered, gene specific forward primers corresponding to each predicted *OsPAL* gene family member were designed and used with a common reverse primer to amplify genes from the IR64 parent, M4 *ospal4* mutants, and M4 segregants not deleted in the *OsPAL4* gene (Figure 2.7a). Only the specific gene primers corresponding to *OsPAL4* on chromosome 2 amplified the smaller, deleted fragment in the two heterozygous *ospal4* mutant lines; the mutant-sized fragment was not amplified in segregant plants that contained the wild type *OsPAL4* allele. Furthermore, the gene specific primers corresponding to other *OsPAL* gene members did not amplify the mutant fragment in any line (Figure 2.7b). Several primers were designed to amplify the *OsPAL9* gene but none amplified this gene family member from any of the rice mutant lines or from the IR64 control (data not shown). Using gene-specific primers for the mutant allele, both wild type and mutant bands were amplified in the *ospal4* mutant lines (5, 6), indicating that those lines were heterozygous for the deleted allele (Figure 2.7b).

Phenotypic characterization of the ospal4 mutant line

Considering that *PAL* genes are involved in plant defense responses and disease resistance in several pathosystems, we tested if the *ospal4* rice mutant lines were more susceptible to a virulent strain of *Xanthomonas oryzae* pv. *oryzae* (PXO339), the causal agent of bacterial blight. In two screens, individual plants in progeny lines (M3 and M4) were genotyped for the presence of the mutation in *OsPAL4*, and grouped into two groups, those with (*ospal4* mutants) and without the altered gene fragment (*OsPAL4* segregant). In the first screen (M3 plants), the group with the altered *ospal4* showed a higher level of susceptibility to *Xanthomonas oryzae* pv. *oryzae* PXO339 (lesion length Least Square Mean 11.1 cm) than those with only wild type fragments (lesion length Least Square Mean 8.9 cm; $P=0.068$; Figure 2.8). In the second screen, the *ospal4* mutant group also exhibited increased susceptibility (lesion length Least

Square Mean 17.1 cm) when compared with the *OsPAL4* segregant group (lesion length Least Square Mean 10.2 cm; $P < 0.0001$; Figure 2.8). The results were consistent across generations in two different locations, and were not significantly different between the two experiments ($P = 0.31$) allowing us to analyze the data from two generations together to increase the statistical power of the analysis. An F-test confirmed that the Least Squares Mean (LS Mean) of the lesion length corresponding to the *ospal4* mutant group from both generations (14.1 cm) is significantly different ($P < 0.0001$) from the LS mean corresponding to the wild type *OsPAL4* segregant group (10 cm) (Figure 2.8). Thus, the M3 and M4 segregating lines with the altered *OsPAL4* gene are more susceptible to *X. o. pv. oryzae* isolate PXO339 than the segregating lines with only the wild type *OsPAL4* allele.

Genetic segregation of the ospal4 mutation

To understand the inheritance of the *ospal4* mutation, we determined the segregation of the *OsPAL4* mutation in 91, 122 and 52 individual progeny of the M3, M4, and M5 generations of the 1982 line, respectively. The segregation of the variant band in the progeny did not fit the 3:1 Mendelian ratios in the three generations indicated by the Chi-square test ($P < 0.000001$). To determine if lines could be established that were homozygous for the variant allele, 10 progeny lines were selected corresponding to each of the 20 M3 plants that were presumed to be heterozygous for the variant allele since they showed both variant and parental sized fragments. We also selected 10 lines corresponding to 20 M3 plants that did not have the variant allele. DNA was extracted from these progenies and amplified fragment sizes examined. Each of the 20 M3 lines presumed to be heterozygous segregated the variant allele among their progeny. None of these M3 lines tested showed the variant band in all individuals of their progeny, indicating that these 20 lines were heterozygous for the variant allele. On the other hand the 20 M3 lines that did not have the variant alleles did not segregate for the mutation in their corresponding progeny lines, indicating that those lines containing only the wild type allele *OsPAL4*. The same experiment was repeated using the progeny of 10 M4 lines presumed to be heterozygous, and again we were not able to detect a family that segregated the variant allele in the progeny. The inability to identify individuals homozygous for the variant allele and the deviation from 3:1 indicate that the variant allele has poor transmission through the male, female or both types of gametes. To test for gametophytic lethality, we attempted reciprocal crosses using a M4

heterozygous *ospal4* mutant line and the variety Kitaake. When the plant with the variant allele was used as a male and Kitaake as a female; the *ospal4* deletion was found in all five of the progeny plants that were verified to be F1 progeny using 5 SSR markers (RM266, RM334, RM278, RM333, and RM224) (Figure 2.9, 2.10). This result indicated that gametophytic lethality is not associated with the pollen because we obtained heterozygous hybrid lines for the *OsPAL4* deletion. In contrast, no seed was obtained in the reciprocal crosses where the plant with the variant allele was used as the female. The fact that the rice inflorescence of the rice *ospal4* mutant and the F1 hybrids between mutant line and Kitaake exhibit seed abortion (less grains filled in the inflorescence) supports the hypothesis of some kind of lethality associated with the *OsPAL4* mutation (Figure 2.11).

Discussion

Several lines of evidence from multiple pathosystems suggest that *phenylalanine ammonia lyase* plays a protective role in induced plant defense responses (Hahlbrock and Scheel, 1989). Higher levels of induction of *PAL* genes and accumulation of PAL enzymes during defense responses relative to disease responses have been shown for many pathosystems (Fritzemeier et al., 1987; Moerschbacher et al., 1988; Cuyper et al., 1988; Geetha et al., 2005), including rice (Zhu et al., 1995; Tanaka et al., 2003; Wang et al., 2004). In tobacco it was shown that overexpression of a bean PAL gene made plants less susceptible to pathogens (Shadle et al., 2003) whereas comprehensive silencing of all tobacco PAL gene family members rendered plants more susceptible (Maher et al., 1994; Felton et al., 1999). Although there is significant correlative evidence for *PAL* gene involvement in plant defense, still lacking for any crop or cereal plant system was evidence that the absence of function of particular members of this gene family would render the plants incrementally more susceptible to disease agents. The identification of a rice mutant harboring a deletion in one of the four *PAL* genes on chromosome 2 (*OsPAL4*) in this study provided a tool to assess the impact of inactivation of a single gene family member on the plant defense response. We demonstrated that plants harboring the *ospal4* mutation exhibit enhanced susceptibility to the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*. This evidence is consistent with the *OsPAL4* gene involvement in disease resistance in plants.

The enhanced susceptibility measured in our study was indicative of a reduction in non-race specific resistance (also referred to as general resistance), as opposed to cultivar-specific resistance, because we challenged the *ospal4* mutant plants with a virulent isolate of *X. o.* pv. *oryzae*. General resistance is considered to result from the activity of multiple genes, and is thought to be governed by quantitative trait loci (QTL). Consistent with our findings, *PAL* genes are co-localized with QTL for pathogen resistance in several pathosystems (Faris et al., 1999; Geffroy et al., 2000; Trognitz et al., 2002), including interactions between rice and various pathogens (Wang et al., 2001). In fact, the cluster of four *OsPAL* genes on chromosome 2 that contains *OsPAL4* maps to a region with rice QTL for sheath blight resistance (Wang et al., 2001). Other *OsPAL* genes in the same cluster appear to contribute to disease resistance.

OsPAL1 was detected by SAGE analyses as upregulated in moderately resistant rice plants inoculated with sheath blight (Guo Liang Wang, *personal communication*), and an activation tagging rice line that overexpresses *OsPAL1* exhibited enhanced resistance to *Xanthomonas oryzae* pv. *oryzae* (Pamela Ronald, *personal communication*). The evidence from these studies is consistent with contributions from *OsPALs*, and in particular *OsPAL1* and *OsPAL4*, in the resistance governed by the disease resistance QTL on chromosome 2.

In this study, we predicted and described a family of nine closely related genes (>69% of amino acid sequence similarity) encoding *phenylalanine ammonia lyase* (*OsPAL*) from *Oryza sativa*. Four genes (*OsPAL1*, 2, 3, and 4) are located clustered on rice chromosome 2. Two of these genes (*OsPAL1*, 2) have several alternatively-spliced forms as documented in the TIGR database. There are two *OsPAL* genes tandemly arranged on chromosome 4 (*OsPAL5*, 6), and a single gene on chromosomes 5 (*OsPAL7*), 11 (*OsPAL8*), and 12 (*OsPAL9*). In most plant species, several copies of *PAL* genes are present per genome, with the most frequently occurring number being 3 to 4 genes (Lois et al., 1989; Minami et al., 1989). The extremes are more than forty-fifty copies per haploid genome in potato (Joos and Hahlbrock, 1992), and a single gene in loblolly pine (Whetten and Sederoff, 1992). Nine *OsPAL* genes were predicted in the *japonica* Nipponbare but two genes (*OsPAL8* and *OsPAL9*) were not found in the *indica* cv. 93-11. The eight genes (*OsPAL1-OsPAL8*) could be amplified using gene specific primers in IR64 (*indica*) and the mutants lines (Figure 2.7), suggesting that the reason *OsPAL8* was not predicted from the 93-11 sequence was due to the poor quality of the sequence in this chromosomal region of 93-11. Attempts to amplify *OsPAL9* using several different gene specific primers were unsuccessful. This *OsPAL9* gene member was amplified in Nipponbare and other *japonica* varieties such Kitaake and LTH using the same gene-specific primers (data not shown), suggesting two possibilities: 1) *OsPAL9* gene member is indeed missing in the *indica* genome, and that this is a true difference between *indica* and *japonica* genomes; and 2) *OsPAL9* is highly divergent at the 5'UTR where the gene specific primers were designed resulting in a lack of amplification in *indica* varieties.

Rice contains *OsPAL* gene clusters on chromosomes 2 and 4. This is consistent with the organization of highly similar *PAL* gene members in clusters on chromosomes of potato (Trognitz et al., 2002) and wheat (Liao et al., 1996). The number of *PAL* genes in *japonica* rice

(nine) and the clustered arrangement of the genes on the rice chromosomes suggest a redundancy in functions of the *OsPAL* gene family members. Ober (2005) described gene duplication as the major driving force for the recruitment of genes for secondary metabolism. Gene copies are gradually modified to create genes with new specificities and expression patterns adapted to the needs of the pathway in which they are involved. The modifications among these highly conserved duplicated genes may reflect differences in regulation (promoter), subcellular localizations, differences related to enzyme properties, inhibition and even feedback regulations. Mutation of one of the nine predicted *OsPAL* genes in rice could sufficiently affect expression of the other *OsPAL* gene copies, which in turn could affect the phenylpropanoid and other related pathways. Using transcript-profiling approaches, Rohde and coworkers (Rohde et al., 2004) demonstrated that a single *PAL* mutation could profoundly affect the transcription of a plethora of genes outside of the phenylpropanoid pathway. They found that *Arabidopsis pal1*, *pal2*, and *pal1 pal2* double mutants exhibited significant changes in the transcription of genes that encode other enzymes of the phenylpropanoid pathway as well as enzymes in carbohydrate, and amino acid metabolism, establishing a clear link between primary and secondary metabolism. Components of most of the pathways altered in these *Arabidopsis pal* mutants were previously described for their responsiveness to pathogen attack or elicitation. However, in this study these mutants were not tested for their response after pathogen challenge. Epigenetically *PAL*-suppressed tobacco lines with reduced levels of *PAL* activity were more susceptible to the virulent fungal pathogen *Cercospora nicotinae* as well as to avirulent *Tobacco Mosaic Virus* (TMV) and those plants were also impaired in SAR induction due to SA deficiency (Maher et al., 1994; Pallas et al., 1996). We demonstrated that mutation in a single *OsPAL* member increases susceptibility to the bacterial blight pathogen.

The *PAL* enzyme is a tetrameric protein. In potato and maize this enzyme is made up of four identical subunits (Havir and Hanson, 1973). In wheat, the enzyme is made up of two pairs of different subunits, two subunits with a MW of 75 kDa and two subunits of 80 kDa. This suggests that the wheat *PAL* proteins were derived from at least two structural *PAL* genes (Havir and Hanson, 1973). Coordinated regulation of key genes from the phenylpropanoid pathway such as *PAL*, *4-coumarate CoA ligase (4CL)*, and *cinnamate 4-hydroxylase (C4H)* has been reported for potato, parsley and bean (Fritzemeier et al., 1987; Logemann et al., 1995; Hahlbrock

and Scheel, 1989; Yamada et al., 1996). Logeman et al. (1995) suggested the possibility that PAL functions as part of a multienzyme complex formed by these three gene products from the phenylpropanoid pathway. Supporting this hypothesis, Harvis and Hanson (1973) described the formation of high molecular weight aggregates of PAL enzyme with other proteins in mustard seedlings in vitro. Little is known about the biochemistry and enzymatic activity of the PAL enzyme in rice. However, if rice enzyme is made of two different subunits that are encoded by two different genes, and if the rice PAL enzymes form aggregates with other enzymes from the same or unrelated pathways, mutation of a single *PAL* gene family member could result in severe changes in the plant metabolism and responses to plant stresses including pathogen challenge. This could explain why the variant *OsPAL4* gene has the phenotype of a heterozygote, ie. if a variant *OsPAL4* protein aggregates with other components to form an inactive multienzyme complex.

The *OsPAL4* gene deletion spans ~750 bp, which results in deletion of most of the second exon of the gene, and likely abolishes the function of the encoded *OsPAL4* enzyme. No gross morphological differences were observed between the *ospal4* mutant lines and the wild type *OsPAL4* segregants lines. However, the *ospal4* mutant lines showed not only low seed germination but also low seed production as indicated by the poor grain filling and seed abortion in the inflorescences of the rice mutant lines, compared with the wild type lines or mutants segregants containing the wild type *OsPAL4* allele (Figure 2.11).

Segregation ratios of the *OsPAL4* mutation were determined in three different generations derived from a single seed *ospal4* mutant line. In the M3, M4 and M5 progenies derived from self pollination, the deletion segregates at a 1:1 ratio in the progeny, which is not the expected 1:2:1 Mendelian segregation ratio. Moreover, an *ospal4* homozygous mutant was never detected in the progeny and the heterozygous *ospal4* mutant lines exhibited poor germination and some pollen infertility (data not shown). The inflorescence of the mutant lines showed less grain filling possibly due to seed abortion (Figure 2.11). Based on these observations, we inferred that transmission of the *OsPAL4* mutation is impaired through some kind of gametophytic lethality (Howden et al., 1998). Gametophytic mutations affect the haploid cycle and thus it is typical for gametophytic mutations to show segregation distortion, that is,

altered transmission ratios through the male, female, or both. This is consistent with the fact that we did not obtain a homozygote mutant, but the mutation was able to be maintained as a heterozygote (Johnson-Brousseau and McCormick, 2004).

Specific flavonols derived from the phenylpropanoid pathway are known to play an essential role in pollen fertility (Taylor and Helper, 1997), and mutants in enzymes of the phenylpropanoid pathway, including PAL, can lead to defective or sterile pollen. PAL activity in immature anthers has been positively correlated with pollen fertility in male fertile and cytoplasmic male-sterile strains of *Brassica oleracea* (Kishitani et al., 1993). Tapetum-specific sense and antisense expression of a sweet potato PAL cDNA in tobacco resulted in partial male sterility and abnormal pollen grains devoid of flavonols (Matsuda et al., 1996). *Arabidopsis pal1 pal2* double mutants have abnormal inflorescence and are sterile (Rohde et al., 2004). Reduced pollen viability was also observed in tobacco transgenic down-regulated for PAL (Elkind et al., 1990). Maize and petunia mutants deficient in chalcone synthase (CHS), an enzyme in the phenylpropanoid pathway, produce flavonol-deficient pollen that is defective in germination or pollen tube growth (Mo et al., 1992).

The inability to detect a homozygous *PAL* mutant is not sufficient to infer that the mutation is homozygous lethal. This can be concluded only if transmission of the mutation through both the male and the female is normal, yet the progeny of a selfed heterozygote yields no homozygotes (Johnson-Brousseau and McCormick, 2004). We carried out reciprocal crosses with *ospal4* to resolve if one or both parents were responsible for the observed segregation distortion. Seeds were only obtained in crosses where the *ospal4* mutant line was used as a male and no problems with transmission were detected. Thus, it is likely that the transmission of the mutation is affected through the female. Further studies are required to explain the segregation distortion found in this study, and to determine if the mutant transmission is through the male or female.

The *ospal4* mutant line was identified by using a PCR-based DNA pooling strategy. Since, relative to insertion mutants, deletion mutants are easy to produce in large quantities, regardless of genotype and with a low initial investment cost, having a reliable strategy to identify these untagged mutations is particularly valuable for genetic studies. The PCR-based

screening strategy used for deletion mutants involves amplification of both the wild type and mutant gene, because both primers are specific to the targeted locus. We used a 100 bp deletion in reconstruction experiments to optimize detection because the mutagen used to generate the collection we studied was DEB which is predicted to induce small deletions (<1 kb, Recio et al., 2001; Lee et al., 2002; Wang et al., 2004) relative to FN (~0.8-12 Kb (Shirley et al., 1992; Okubara et al., 1994; Bruggemann et al 1996). We reliably detected the 100 bp deletion in pools of 1:200 (mutant to wild type DNA), however, to be conservative, we used 1:100 pools for the full screening experiments, and we resolved the PCR products in polyacrylamide gels to ensure detection of small deletions. Li et al. (2001), using the known 5 kb deletion mutant *gal-3* in *Arabidopsis* (Sun et al., 1992), demonstrated detection of the deletion in pools of 1:1000 mixture of mutant:wild type *Arabidopsis* DNA. In that same study, they demonstrated the detection of a 2.5 kb FN-induced mutation in a megapool of 2,592 lines.

Our modified PCR-pooling strategy involves one PCR reaction using primers specific to the targeted locus. Li et al. (2001) used two pairs of primers per gene target to screen the FN-induced libraries from *Arabidopsis* and rice, i.e., they first amplified with a pair of primers that flanked the targeted gene, followed by amplification with a pair of nested primers (located inside the original primers). Using this strategy they identified one deletion mutant in 24660 lines. In a population of 3,000 DEB-induced rice deletion lines, we identified one mutant for 26 DR gene primers tested. Using the formula $N = \ln[1-P] / \ln[1-F]$ (Li et al., 2001), where N is the population size, P is the probability of isolating a deletion, and F is the frequency of deletions that can be isolated using the deletion-based reverse genetics system, the probability of isolating a deletion in a DR gene using this strategy was 4%. Thus, 224,703 and 345,000 DEB rice mutant lines would need to be screened to detect deletions in target genes with a probability of success of 95% and 99%, respectively. Using the same equation, Li et al. (2001) estimated that they would need a population size of 84,825 and 130,397 FN *Arabidopsis* lines to have 95% and 99% probability of detecting deletions in targeted genes, respectively. In *Arabidopsis* for a median gene length of 2.1 kb, ~180,000 T-DNA insertions are required to have a 95% chance of mutating a particular gene (Krysan et al., 1999). In rice where the mean size of genes is ~2.6 Kb, ~460,000 insertions are required for a 95% chance of mutating any gene (Hirochika et al., 2004), which is approximately 2.6 times more than the number of *Arabidopsis* insertions required to

detect a mutated gene with the same probability. The estimation of the population size of rice DEB mutants in this study to detect deletions with a 95% or 99% probability is 2.6 times more than the population size of the *Arabidopsis* FN mutants estimated for the same probabilities (Li et al., 2001). We screened for 26 gene targets, and detected only one deletion mutant in 3,000 DEB rice mutants while Li et al., (2001) screened for 5 gene targets in 24,660 rice FN mutants and identified only one deletion mutant. Considering that FN likely causes larger deletions than DEB, our data are consistent with those of Li et al. (2001).

We used a PCR-pooling strategy to detect a deletion in the gene encoding *phenylalanine ammonia lyase* (*PAL*). Prior to this report, no *PAL* mutants were reported in rice. The *OsPAL4* mutant identified in this study will provide important insights into rice primary and secondary metabolism and stress responses. Studies with *PAL* mutants from other plants have provided important insights into the function of *PAL* genes under stress conditions and also the relationships among *PAL* gene family members and other genes involved in the phenylpropanoid and other pathways (Elkind et al., 1990; Bate et al., 1994; Maher et al., 1994; Rohde et al., 2004; Pallas et al., 1996). Further characterization of the rice mutation, including expression analysis, inheritance analysis, histochemistry studies, and responses against other important rice pathogens like *Magnaporthe grisea* (rice blast), and *Rhizoctonia solani* (sheath blight) are in progress.

Experimental Procedures

Plant material and genomic DNA extraction

Rice deletion mutants used in this study were generated by the International Rice Research Institute using IR64 as a breeder seed (Wu, 2005). About 3,000 M2 diepoxybutane (DEB) mutants were screened with several primers corresponding to different defense response genes. IR64 *indica* rice line was used as a wild type control in all experiments. The production of M2 and M3 mutant seeds are as described (Wu, 2005); we produced M4 and M5 seed using progeny derived from a single M3 and M4 line, respectively. Near-isogenic rice lines IRBB21 and IR24 were used for the reconstruction experiment of the *Xa21* gene deletion.

The japonica variety Kitaake was used to make the reciprocal genetic crosses with the *PAL* mutant rice line. Seeds were germinated in petri dishes at 28 °C and rice seedlings were planted in a greenhouse. Rice leaves were harvested and ground in liquid nitrogen. Rice genomic DNA was extracted using a modified hexadecyltrimethylammonium bromide (CTAB) procedure (Saghai-Marooof et al., 1984). Genomic DNA was quantified by UV absorbance using the NanoDrop ND-1000 spectrophotometer (Rockland, DE, USA).

Characterization of the Xa21 natural deletion in rice cultivar IR24 and reconstruction experiments

To optimize the PCR-based DNA pooling strategy, we used a naturally occurring deletion in the *Xa21* gene observed in rice cultivar IR24. The *Xa21* gene polymorphism between IR24 rice line and IRBB21 was detected by PCR using gene specific primers Xa21-f (5'ATAGCAACTGATTGCTTGG 3') located in the kinase domain and Xa21-r (5'CGATCGGTATAACAGCAAAC 3') located in the 3'UTR (accession number AB212798.1). PCR reactions were performed using HotStar Taq DNA Polymerase (Qiagen Inc., Valencia, CA, USA) and 20 ng of genomic DNA as a template in a final volume of 25 ul. PCR reactions were subjected to 35 cycles at 95°C (30 sec), 55°C (30sec), 72°C (1 min), with a final extension at 72°C (8 min) using PTC-200 DNA Engine Thermal Cycler (MJ Research, Waltham, Massachusetts, USA). PCR products were resolved in 1% agarose gel and visualized with ethidium bromide. PCR fragments corresponding to IRBB21 and IR24 lines were excised from

the gel and purified using QIAquick Gel Extraction Kit (Qiagen). The products were sent for sequencing at the DNA Sequencing Facility (Kansas State University, Manhattan, KS, USA). Sequences were aligned using the ClustalX algorithm (Thompson et al., 1997) to determine the deletion junctions. PCR was performed using the Xa21-f and Xa21-r primers as described above. DNA templates for the PCR reactions were 20 ng of IRBB21 DNA, mixed independently with IR24 DNA (20, 2, 0.2, 0.4, and 0.02 ng) to obtain the following wild type: mutant ratios: 1:1, 1:10, 1:100, 1:200, and 1:1000 respectively. The PCR products were analyzed in 1% agarose gel stained with ethidium bromide.

Bioinformatics and primer design

Based on literature searches and database mining, several plant defense response (DR) genes shown to be associated with disease resistance (Ramalingam et al., 2003) were selected for screening in the rice mutant collection. Rice gene annotated sequences corresponding to DR-genes were obtained from the National Center for Biotechnology Information (NCBI) and The Institute for Genomic Research (TIGR) databases. All primers in this study were designed manually. Primer properties were analyzed using IDT's Oligo Analyzer 3.0 (<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/>), and primer specificity was verified by performing blastn analyses of the primer sequences against the rice genome database. Gene specific primers were designed manually in the 5' or 3' untranslated regions (UTR) or in the gene introns. For multigene families, several sequences corresponding to different members were aligned using CLUSTAL W. 1.8 in the BCM Search Launcher Interface (Baylor College of Medicine HGSC, <http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>). Gene family primers were designed in the highly conserved regions among gene members. Two *Hordeum vulgare* mRNA sequences for *oxalate oxidase-like (HvOXOLP)* and *oxalate oxidase (HvOXOa)* genes (accession numbers: X93171, Y142203 respectively) and the *Oryza sativa* sequences for three *phenylalanine ammonia-lyase (PAL)* genes (accession numbers: X16099.1, X87946, Z15085) were used as queries for the tblastx search (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) using The High Throughput Genomic Sequences (HTGS) database. The FGENESH program (<http://www.softberry.com/berry.phtml>) was used to predict the putative *OsOXL* and *OsPAL* genes from the significant rice BAC hits. Phylogenetic reconstruction was prepared using PAUP 4.0 B10 neighbor-joining algorithm

(Sinauer Associates, Inc. Sunderland, MA). Unrooted neighbor-joining analysis was performed, and tree branch scaling was based on number of amino acid changes.

PCR-pooling screening

Genomic DNAs corresponding to 3,000 M2 DEB mutants were extracted, aliquoted, and organized into pools. Each pool contains DNA samples representing 10 lines; and each super-pool contains DNA from 100 lines. The smaller pools allow rapid deconvolution once deletion mutants are found in a particular superpool. PCR using DR-gene specific or gene family primers were done using all superpools. PCRs were performed using HotStar Taq DNA Polymerase (Qiagen). PCR products were separated in 4% polyacrylamide-7.5M urea gels and the bands were visualized by silver staining. PCR amplifications of the mutant line using several simple sequences repeat (SSR) markers, that differentiate the variety IR64 from others, were done to confirm the purity of the mutant line.

Characterization of the PAL deletion

Wild type and mutant bands detected with the PAL primers were excised from the polyacrylamide gel using a scalpel. The gel slices were transfer to a 1.5 microfuge tube containing distilled water. The DNA was allowed to diffuse out of the gel into solution at 4°C overnight. 10 ul of this solution was used as a template to reamplify the bands with the gene family primers PAL-F2 (5' AAGCTGCTCAACGCGAACG 3'), PAL-R2 (5' TTGACGTCCTGGTTGTGCTGC 3') using HotStart DNA Polymerase (Qiagen). The PCR products were separated in agarose and the fragments were excised from the gel and purified using QIAquick Gel Extraction Kit (Qiagen). These fragments were inserted in pCR 2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) and introduced in the TOP10 One Shot Chemically Competent Cells (Invitrogen). DNA plasmid extractions of the positive clones were performed using QIAprep Spin Miniprep Kit (Qiagen) and were sequenced. Sequences corresponding to the wild type and mutant fragment were aligned using MultAlin program to determine the deletion junctions. Several internal primers were designed in the deletion region. Gene specific forward primers for each of the 9 OsPAL gene members predicted from the rice database were designed manually in the most divergent regions and used with a common reverse primer PAL-F2 to amplify genomic DNA corresponding to four M4 non-mutant

lines, two M4 mutant lines, and the IR64 control. The PCR products were resolved in 4% polyacrylamide and visualized with silver staining.

Bacterial blight inoculation

The presence of the *OsPAL* deletion was assessed in 182 M3 progeny of the mutant 1982 rice line and 85 M3 derived M4 progeny which allowed distinction of the lines into two groups: *OsPAL4* segregants and *ospal4* mutants. A virulent strain of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) PXO339 was used to inoculate the M3 and M4 genotyped plants in the greenhouse. The second youngest fully expanded leaf from M3 and M4 plants were inoculated with a bacterial suspension (5×10^9 CFU/ml) using the leaf clipping method (Kauffman et al., 1973). Disease was assessed at 2 weeks after inoculation by measuring the lesion length. Differences between Least Squares Means were tested individually in M3 and M4 mutant generations using the GLM Procedure and the analysis of both generations together were done by the F- test using the Mixed Model Procedure in SAS Version (SAS Institute 2004).

Segregation analysis and genetic crosses

A total of 91 M3 progeny, 122 M4 progeny derived from a single M3 plant, and 52 M5 progeny derived from a single M4 plant were genotyped by PCR amplification to determine the presence or absence of the *ospal4* mutation. M3 progeny was genotyped using *OsPAL* gene-family primers used to screen the rice mutant collection. The M4 and M5 progeny was genotyped using gene specific primers corresponding to *OsPAL4* gene member. PCR products were resolved in 4% polyacrylamide gel and visualized by silver staining. Segregation ratios (3:1, 2:1, and 1:1) were tested by Chi Square (X^2) test. Reciprocal genetic crosses were performed under greenhouse conditions between an *OsPAL* mutant line and the variety Kitaake. When 50% of the panicle had emerged from the boot, emasculation was carried out by suction using a vacuum emasculator. Pollination was done when maximum anther extrusion from the florets occurred. The pollinated panicles were bagged. The collected F₁ seeds were germinated in petri dishes and seedlings were transferred to soil and grown under greenhouse conditions. F₁ plants were screened for the *PAL* mutation and several SSRs markers were used to confirm that the F₁ plants were hybrids. The products of the PCR using SSR markers were resolved in 3% agarose gels and stained with ethidium bromide.

Figures and Tables Chapter 2

Figure 2.1 Reconstruction experiments using a natural 135 bp deletion in the *Xa21* gene of rice line IR24.

(a) Wild type copy of the *Xa21* gene present in IRBB21 rice line. The gene is 4623 bp long and has two exons indicated by the boxes. The arrows show the positions of the primers, the gray triangle indicates the position of the natural deletion in the IR24. The distance between the two primers in the wild type is 1377 bp and in the mutant is 1242 bp. (b) PCR was performed to determine the optimal pool size for the rice mutants. DNA was diluted with variant amount of wild type DNA solutions to generate pools with ratio of 1:10, 1:100, 1:200, and 1:1000. These DNA pools were used as a template for the PCR reaction (lanes 3-7) together with the IRBB21 (lane 1) and IR24 (lane 2) DNA. The PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. 1Kb plus DNA ladder (M) was loaded in lane 8. The IR24 mutant band was detected in all the pools through 1:200.

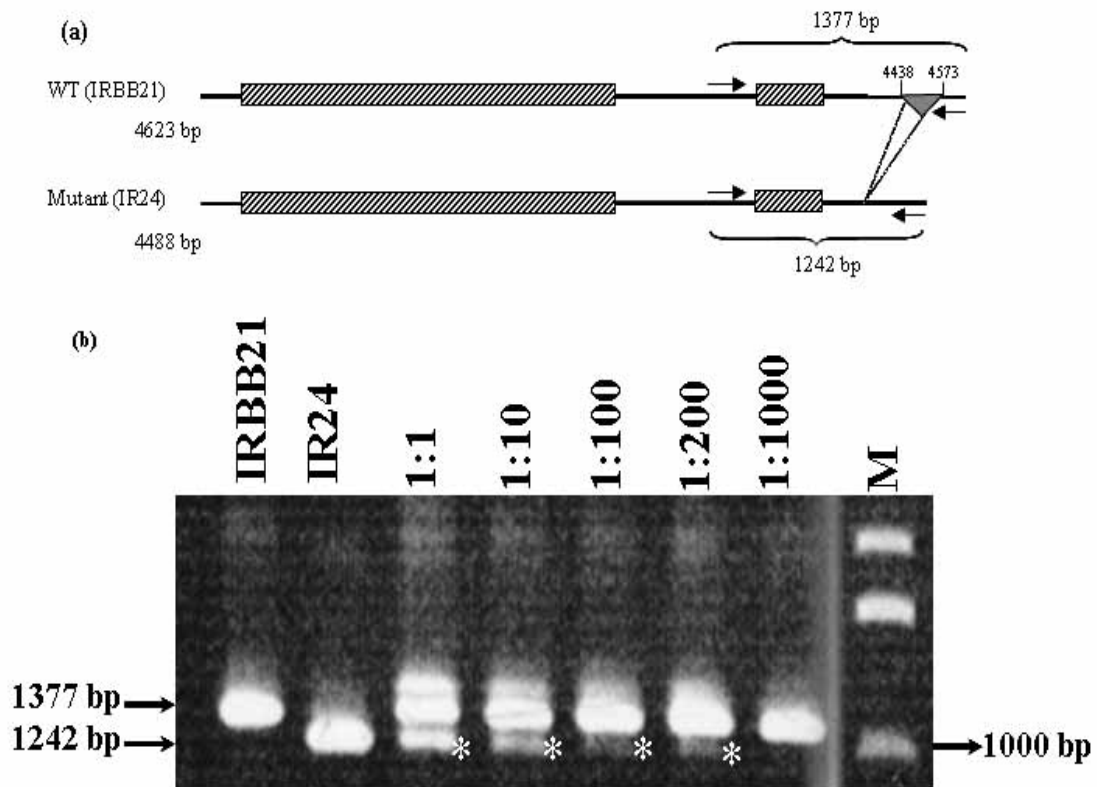


Figure 2.2 Pooling and screening strategy

Flow chart of the pooling and PCR screening processes showing the steps from the mutagenesis to the characterization of the gene deletion in the individual mutant line

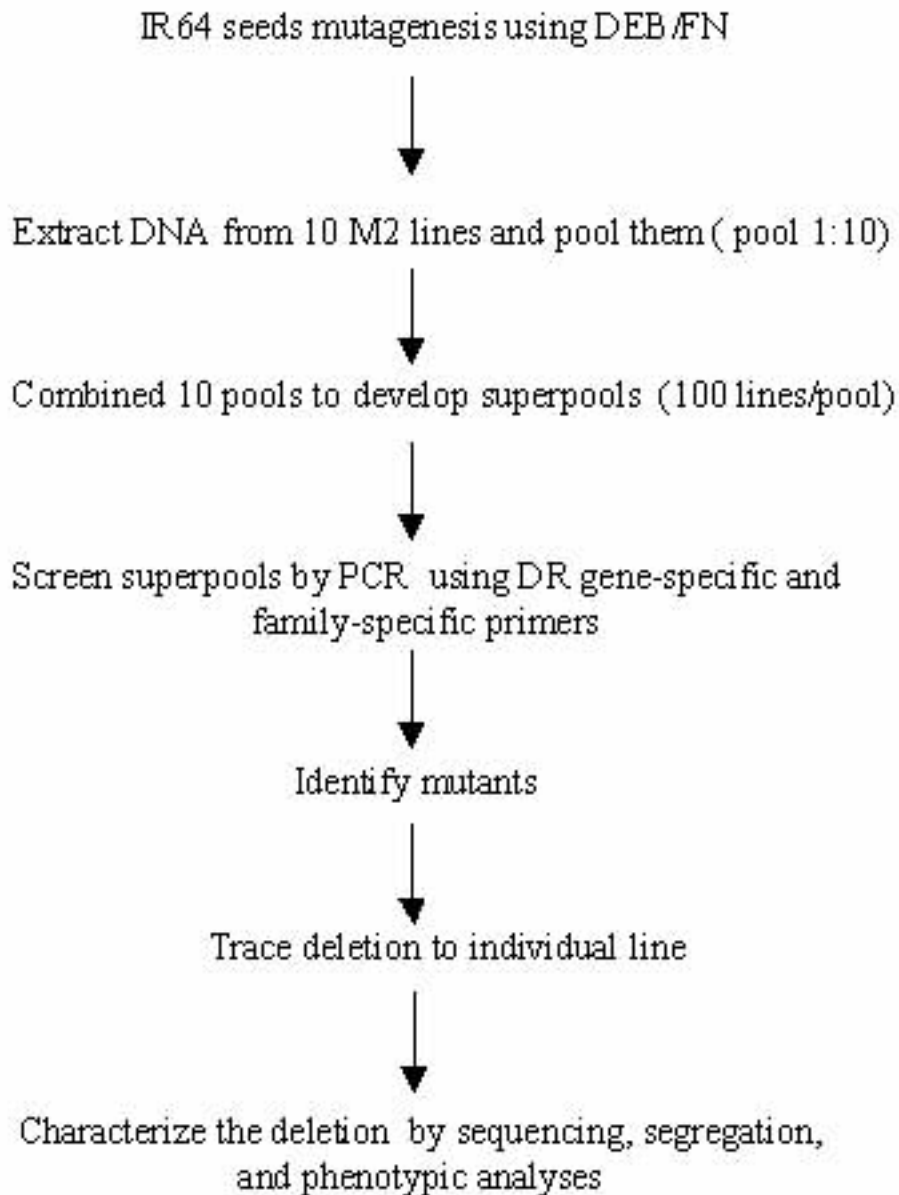


Figure 2.3 Gene prediction and structures of rice *phenylalanine ammonia-lyase (PAL)* gene family

(a) *OsPAL* genes predicted in the rice genome and their corresponding TIGR ID locus, BAC name and the positions in the BACS which they were predicted.

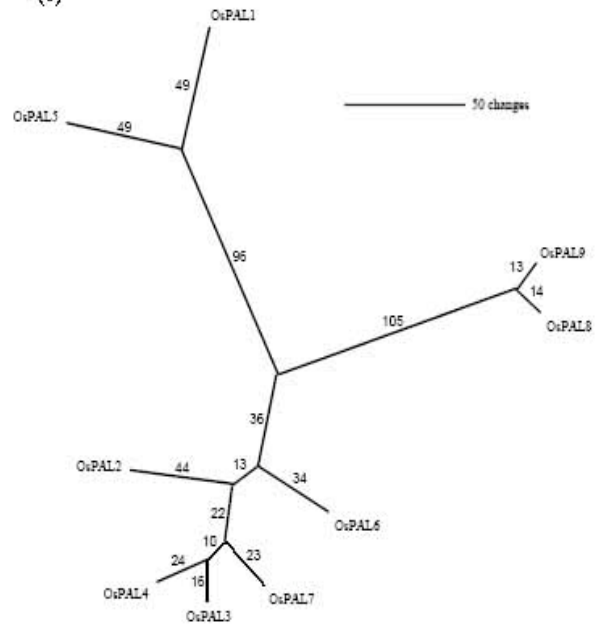
(b) Gene structures of *OsPAL* genes. The majority of coding regions corresponding to the exons indicated by boxes are highly conserved among the members; the 5' and 3' UTR are highly divergent. The arrows show the position of the primers that were used to screen the rice mutant collection and those primers are located at the most highly conserved region among all *OsPAL* genes. The expected amplicon size for most of the genes is 923 bp; the exception is *OsPAL8* which amplicon is 938. Lengths in bp of the predicted genes are showing at the right.

(c) Phylogenetic tree of *OsPAL* gene family. The dendrogram was generated using the complete amino acid sequences of all the *OsPAL* genes using PAUP 4.0 B10 neighbor-joining algorithm.

(a)

GENE	TIGR Locus ID	BAC clone	Position (bp)
<i>OsPAL1</i>	OS2g41630.1	P0042D01	5172-8768
	OS2g41630.2		
	OS2g41630.3		
<i>OsPAL2</i>	Os02g41650.1	P0042D01	17041-20869
	Os02g41650.2		
	Os02g41650.3		
<i>OsPAL3</i>	Os2g41670	P0042D01	24302-26895
<i>OsPAL4</i>	Os2g41680	P0042D01	36929-40602
<i>OsPAL5</i>	Os4g43760	OSJNBa0073E02	97029-101032
<i>OsPAL6</i>	Os4g43800	OSJNBa0073E02	117292-120586
<i>OsPAL7</i>	Os5g35290	OSJNBa0044P19	122339-124901
<i>OsPAL8</i>	Os11g48110	OSJNBb0026K20	107056-110793
<i>OsPAL9</i>	Os12g33610	OSJNBa0050D07	175626-178025

(c)



(b)

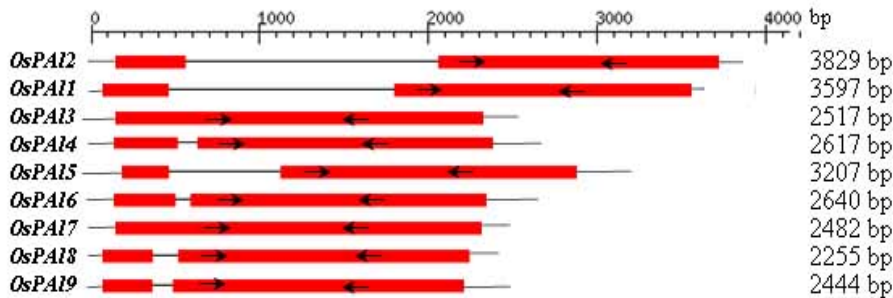


Figure 2.4 Identification of a deletion in an *OsPAL* gene family member detected in rice DEB mutants

- (a) Amplification of 30 DEB 1:100 pools and IR64 (wild type) using the *OsPAL* gene family specific primers showed polymorphisms bands, in pools 17 and 18. Wild type band (Wt) is 931 bp and the mutant bands (m) are ~200 bp.
- (b) Amplification of the 1:10 pools 169 and 178 that correspond to pools 17 and 18 respectively, showed the same polymorphisms.
- (c) Ten single individual plants corresponding to pools 169 and 178 were analyzed by PCR. Individual mutant 1982 contains the variant bands.

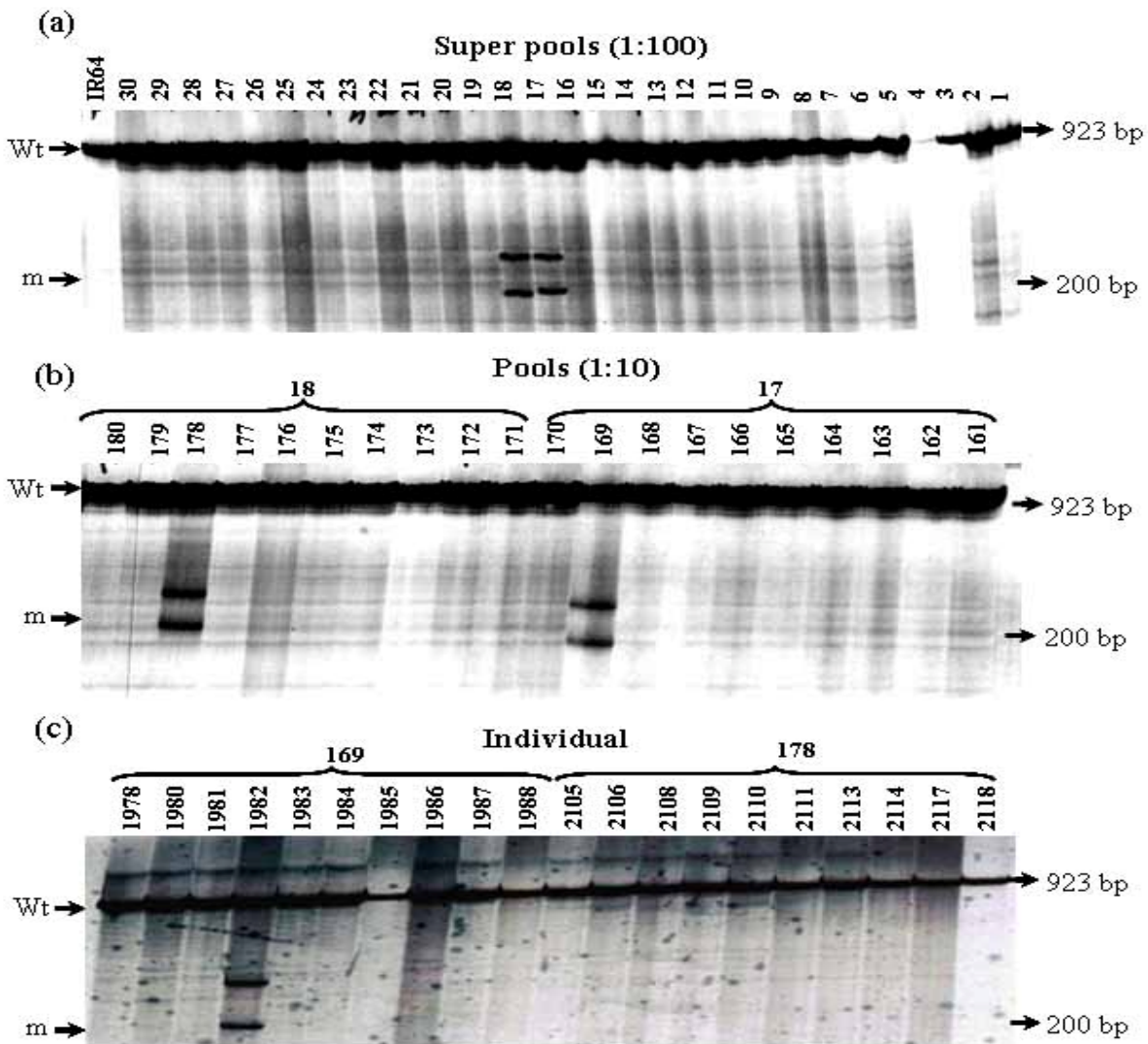


Figure 2.5 Characterization of the *OsPAL* deletion

Sequence analysis indicated that the deletion occurs between 889-1639 nucleotides of the *PAL* Japonica *OsPAL4* and the *PAL Indica* AAAAA02006954.7 genes predicted. Black bars indicate the *OsPAL* primers used for reamplification. Multiple sequence alignment of wild type band (1982WT) and mutant band (1982Mt) with the predicted *OsPAL* members from Japonica and Indica genomes showing that the candidate gene to be mutated is *OsPAL4* member.

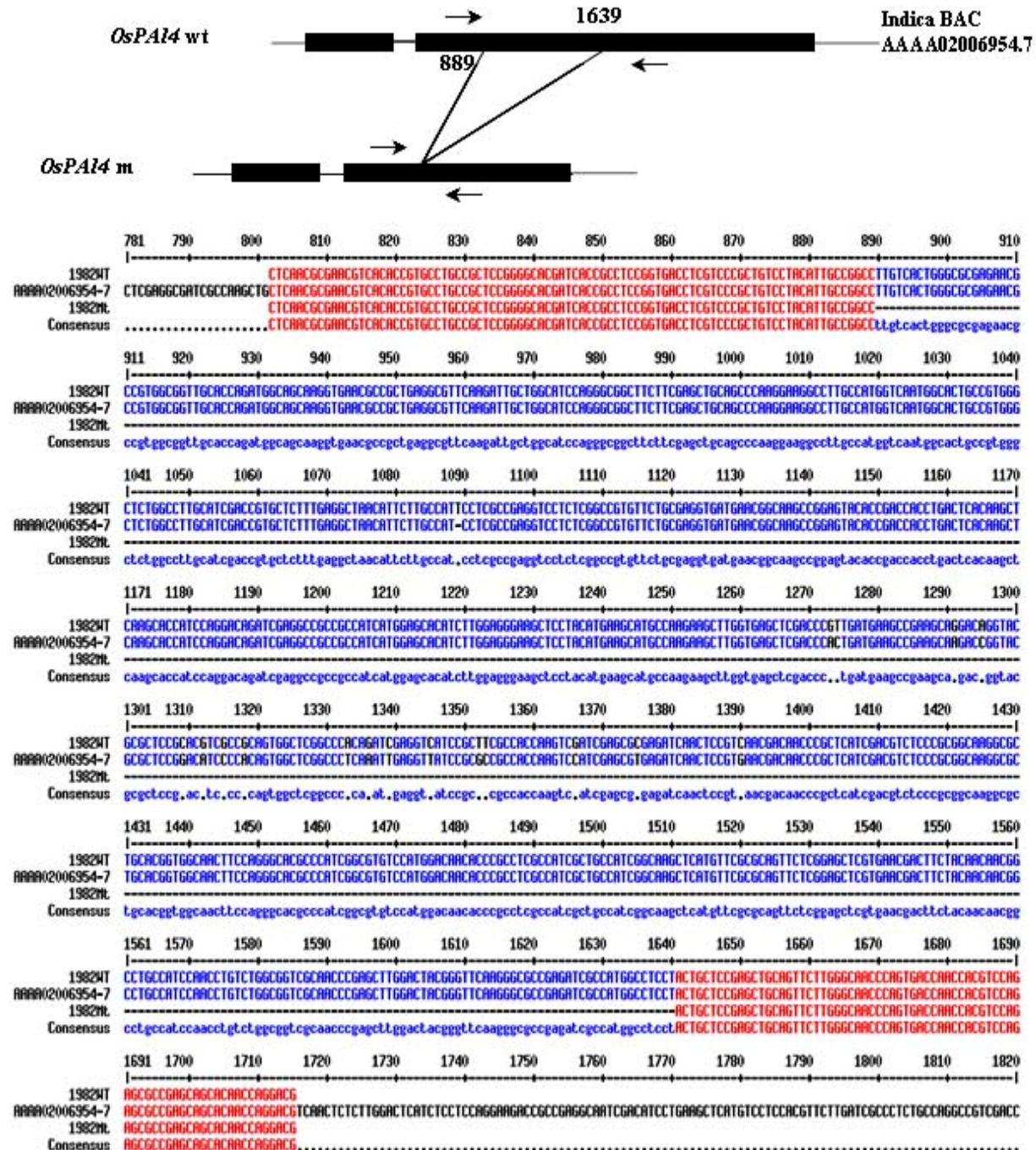


Figure 2.6 Confirmation of the mutation using nested PCR

Amplification of IR64 (lane1), two M3 segregating *OsPAL4* non mutant lines (lanes 2,3) and two M3 *ospal4* mutant segregating lines (lanes 4,5) with the primers *PALF2/R2* showing the wild type *OsPAL* gene fragment (wt) and the mutant fragment (m). The same individuals were used to confirm the deletion by PCR, using an internal reverse primer (*PALR6*) designed in the deleted region with a common forward primer (*PALF2*). The *PALF2/R6* combination as expected failed to amplify the deleted fragment in the *ospal4* segregant lines. Several internal primers designed in the deleted region were tested and all of them failed to amplify the deletion in the *ospal4* mutants (data not shown).

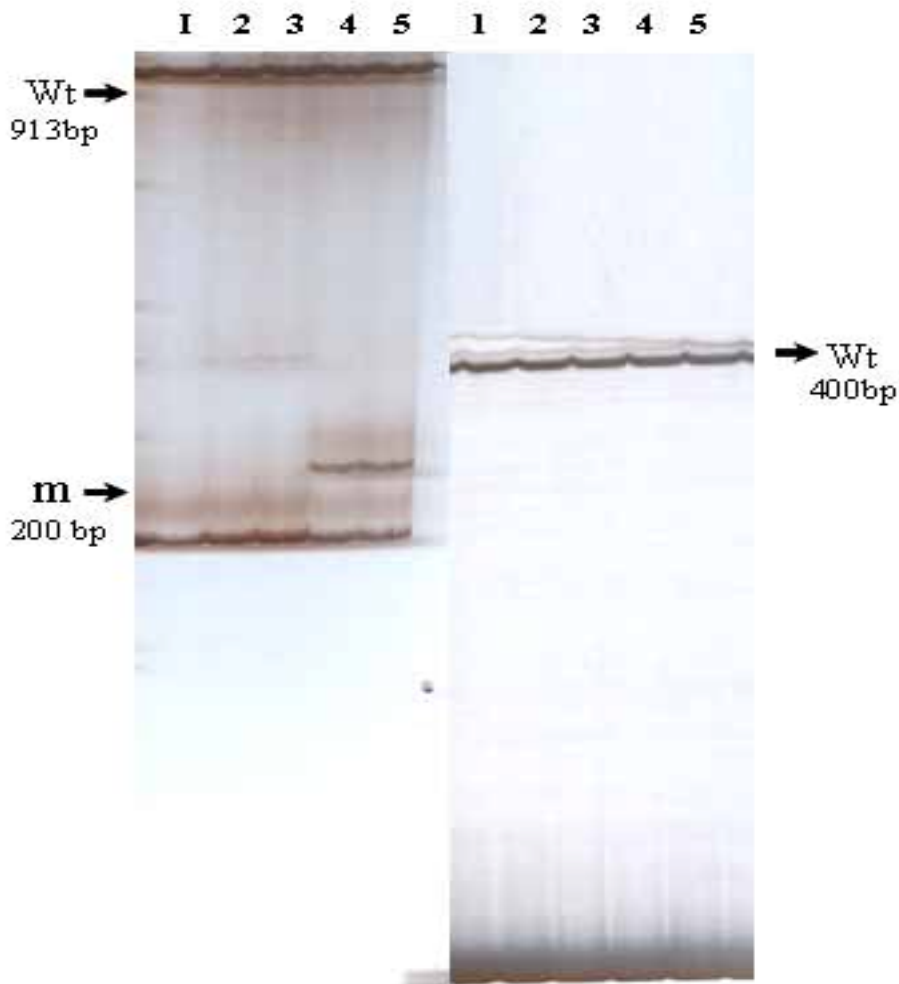


Figure 2.7 PCR confirmation of the mutated *OsPAL4* on chromosome 2

(a) Gene specific forward primers for all the predicted *OsPAL* genes were designed in the divergent regions and were used with a common reverse primer designed in a highly conserved region. Forward primer sequences, nucleotide position and gene location as well as expected PCR product size obtained are shown. (b) 4% polyacrylamide electrophoresis of the PCR products using the *OsPAL* gene specific primers. Primers were used to amplify seven plants (1, 2, 3 not deleted *OsPAL4* segregants; 5, 6 *ospal4* mutants; I (IR64)). Only the primers specific for the *OsPAL4* amplified the smaller fragment in the two mutant plants but not in the plants with wild type allele *OsPAL4* (lanes 1, 2, 3, and IR64). Furthermore, the primers designed to amplify the other *OsPAL* members do not show the polymorphism from any plant. The PCR product size of the wild type and the mutant bands are indicated in bp at the left.

(a)

Primer name	Gene	Primer sequence 5' → 3'	Position	Location	Size product (bp)
PALF10	<i>OsPAL6</i>	TGCTGTTTCGTCTGGTGAGAGC	556	Intron	1148
PALF12	<i>OsPAL1</i>	GTTTGGGCTGCAACTTGGCAG	1664	Intron	1199
PALF13	<i>OsPAL5</i>	CAACGGCTCCGATGGCAACTC	1285	5' 2nd exon	1052
PALF19	<i>OsPAL3</i>	GAACGGTCAGGTTGCTGCCGAT	154	5' 2nd exon	1460
PALF22	<i>OsPAL7</i>	CCGACGGCCACGTCC	566	Exon	1044
PALF24	<i>OsPAL8</i>	CGTACGTACTCGGCGACCTGTACGTGCCGC	1286	5' UTR	1658
PALF25	<i>OsPAL2</i>	AGTCGGCACGGCGGCAGTGTGTATGTAC	1905	Intron	1231
PALF8	<i>OsPAL4</i>	TAACGTTTACCTGGTCACTGC	558	Intron	1168
PALF15	<i>OsPAL4</i>	TCACACCGTGCCTGCCGCTCC	814	5' 2nd exon	912
PALF21	<i>OsPAL4</i>	TTCTCTCGACGCTTTCTGTGCTAGG	84	5' UTR	1500

(b)

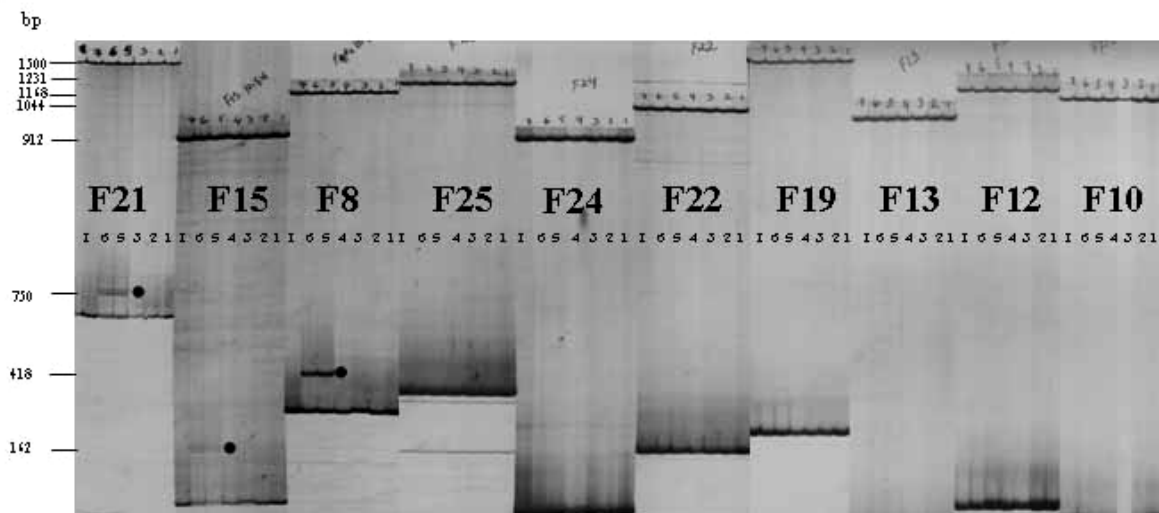


Figure 2.8 The *OsPAL4* deletion is correlated with disease susceptibility to the virulent strain of *Xanthomonas oryzae* pv *oryzae* (*Xoo*).

182 M3 rice progeny of the heterozygous *ospal4* mutant 1982 rice line and 85 M3 derived M4 progeny were genotyped for the presence of the *OsPAL4* deletion and grouped into two groups (*OsPAL4* segregants and *ospal4* mutants). Those plants were inoculated in the greenhouse with a virulent *Xoo* isolate PXO339. M3 was inoculated at KSU 2004 and M4 at CSU 2005. Plants inoculated with *Xoo* were scored for disease (lesion length in cm) in the second youngest leaf (L2) and simple correlations were computed using SAS. Probability values are shown for each data.

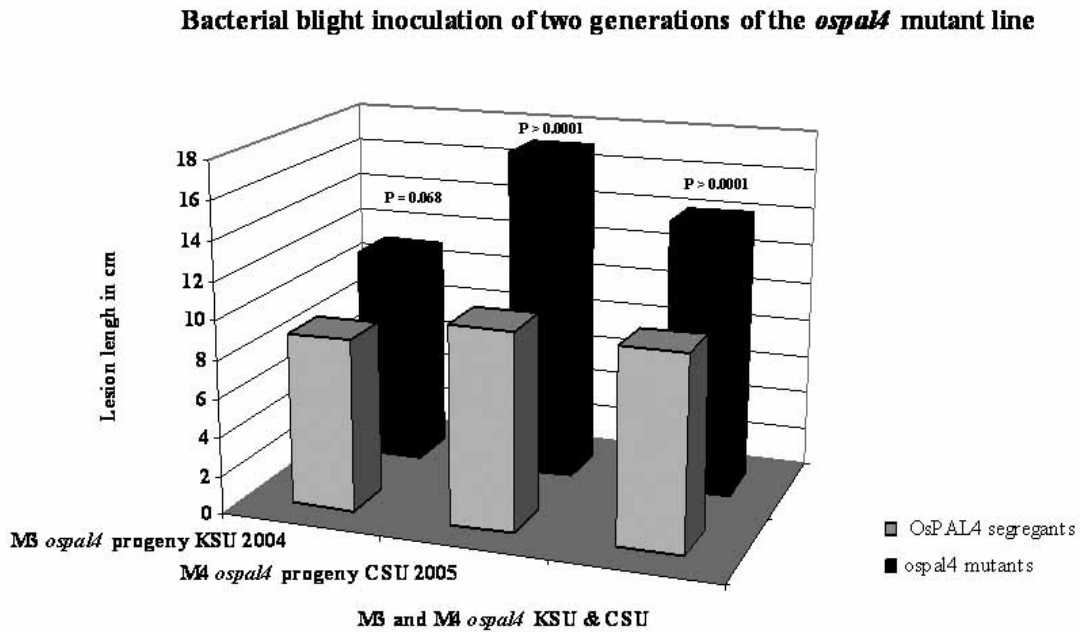


Figure 2.9 *ospal4* mutant line cross with Kitaake transmit the *ospal4* deletion in the progeny

DNA amplification of progeny (1, 2, 3, 4, 5, 6, 7) derived from the pollinization of the Kitaake variety with pollen from *ospal4* IR64 mutant (m) using gene specific primers for the *OsPAL4* gene. PCR products runned in 4% polyacrylamide show that the deletion in *OsPAL4* gene is transmitted by the male gamete to the progeny. DNA of IR64 also was amplified as a control.

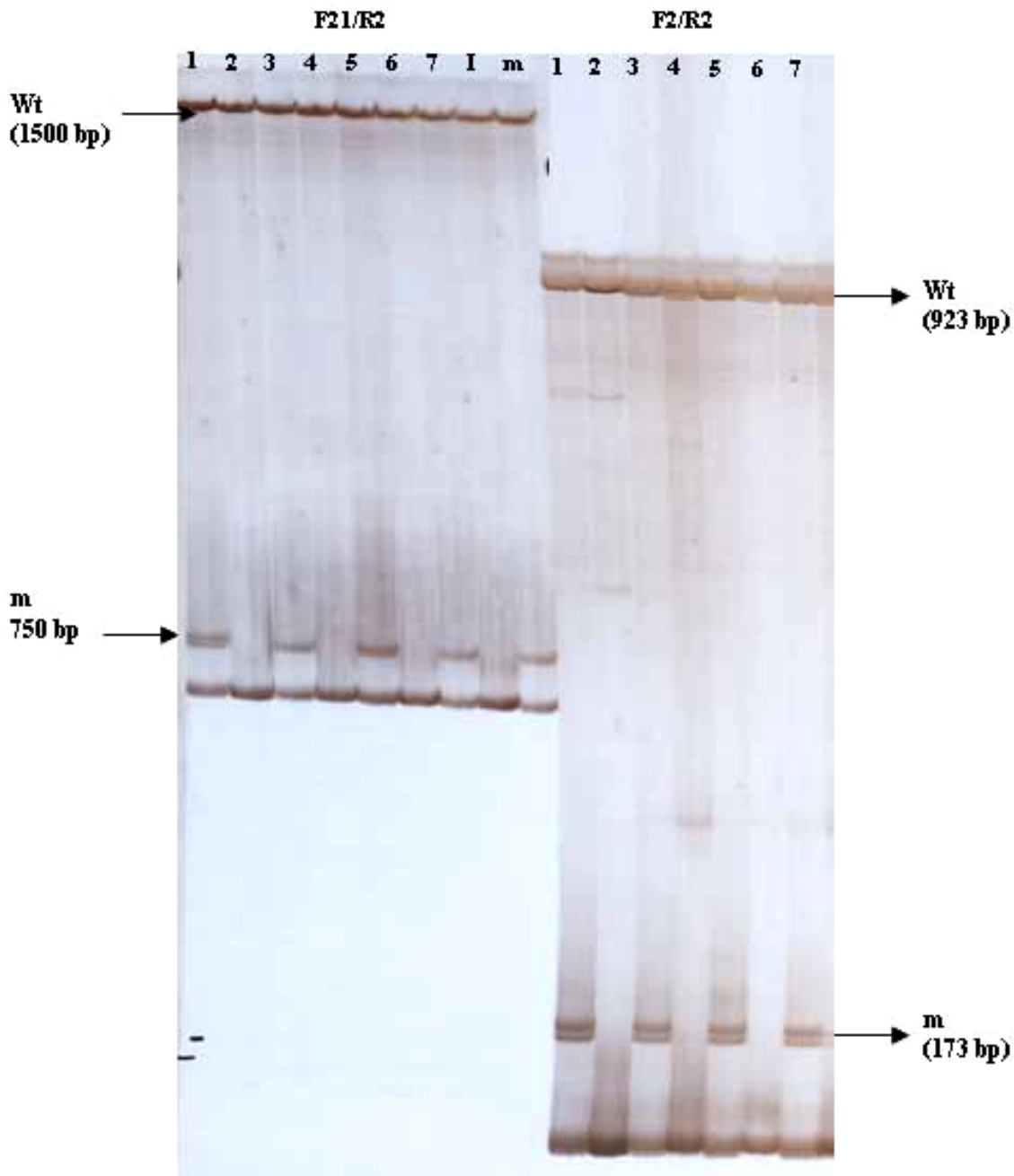


Figure 2.10 SSR amplification to identify F₁ hybrids between Kitaake and *ospal4* mutant line.

Five SSR markers were used to confirm the hybrid nature of the progeny obtained from the cross. Out of the seven progeny plants analyzed five of them are truly hybrids as shown by the SSR markers, those hybrids are the same individual containing the *OsPAL4* deletion. I (IR64), K (Kitaake).



Figure 2.11 Rice inflorescence phenotype between wild type and *PAL* mutants

Rice panicles of the wild type IR64, the wild type Kitaake, the rice segregant mutant containing the wild type *OsPAL4* allele, the *ospal4* mutant, and two F₁ hybrids from the cross using the pollen of the *ospal4* mutant line to pollinize a Kitaake line.

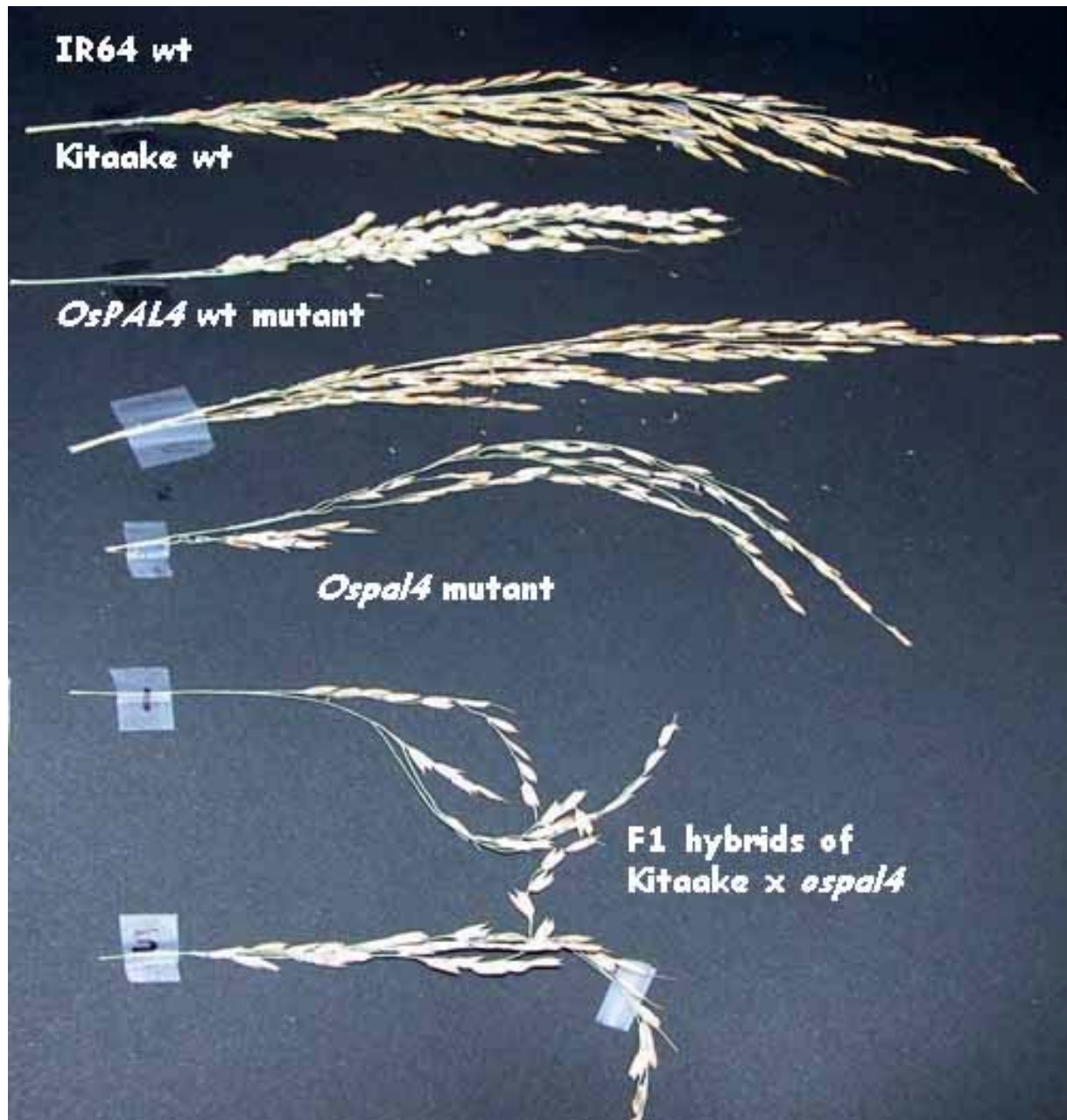


Table 2-1 Primers for candidate genes used to screen the rice mutant collection.

Gene Target	Primer Name	Sequence 5->3
Lipoxygenase	LOX1	CAACCCAAGCCAAGCTCAC
Lipoxygenase	LOX2	TGTCTCATCAGATGGATGTGC
Peroxidases	POX8.1 AND PXOgX9	ACG TTC TAC GAC ACG TCG
Peroxidases	POXgX9	ATT CGA AAC TAG CAC TTC ACT TG
Peroxidases	POXf	ATC AAG AGC GCC GTG AC
Peroxidases	POXr	TGT TGG AGG CGA AGT TCC
Bax inhibitor-1 (BI-1) gene	OsBI-1	AAA ATT CGA AGC CAT CCA CCA
Bax inhibitor-1 (BI-1) gene	OsBI-1	TTT TTT TTT TTT TGG GGA AAG GAT CAT C
zinc finger protein	LSD1f	TCT GTT GCT AAC TTG TAC AGA AGT AG
zinc finger protein	LSD1r	TTA CTC CAA TTT CAG TGA CAA AGA G
PR-10a	PR 10a-f	CTAGCTACAGGCATCAGTGGTC
PR-10a	PR 10a-r	CACGAAATTACTCTCACGGACTC
3-Hydroxy 3 methylglutaryl CoA Reductase	HMGRCoA-f	AGCCGACAGCTGTAAACTGG
3-Hydroxy 3 methylglutaryl CoA Reductase	HMGRCoA-r	AAGGTGACCAGAGGCAAGAG
Phenylalanine ammonia-lyase	PAL-f	CAC TCC TCC AGG GAT ACTC
Phenylalanine ammonia-lyase	PAL-r	CAA GAG AGT TGA CGT CCTG
Oxalate	AP005505-1-F	GGCACTACACAAAATATGGCTCT

oxidase-like		
Oxalate oxidase-like	AP005505-1-R	ACGCACAAAGGAACAATCAAG
Oxalate oxidase-like	AP005505-2- F	GTT CTC ATC TGA AAT TGT GCA TGC
Oxalate oxidase-like	AP005505-2- R	GCATCAATTGATTAGGGAGAATCAG
Oxalate oxidase-like	AP005505-3-F	AAGCCTTGAGCTCACAACCTTAG
Oxalate oxidase-like	AP005505-3-R1	GTA ACA CAA AAG CTA ATA CAT TG
Oxalate oxidase-like	AP005505-4-F	CTGAAATGCTATGGAACATATGCG
Oxalate oxidase-like	AP005505-4-R	GATTCACGGTATGCCAACAAC
Oxalate oxidase-like	AP005505-5-F1	CATGTACATATGGGTGAGAATATTG
Oxalate oxidase-like	AP005505-5-R1	GCAGAATAGAACTTATACATAGTATAAC
Oxalate oxidase-like	AP005505-6 F1	CATGCATTCAGCCATTTTCA
Oxalate oxidase-like	AP005505-6 R1	CATAGAGTCCGTAAGCGGAC
Oxalate oxidase-like	AP005505-7F	TTACA TTT ATG ATG AGAAA CACTC
Oxalate oxidase-like	AP005505-7R	GTATGCATAACAAGTACAAACTCC
Oxalate oxidase-like	AP005505-8-F1	GGGGGCTTTATATATATAATCTAAAATAATTGG AC
Oxalate	AP005505-8-R1	AACAAACACCTTGAAGAACGG

oxidase-like		
Oxalate oxidase-like	AP005531-1-F1	TTCTTGCATATACGATAAGCAGC
Oxalate oxidase-like	AP005531-1-R1	ATGTAGTATACTGTCCTTGATGGTG
Oxalate oxidase-like	AP004559-1-F	CAGCTGTCATATCTTAATTGTTGTCC
Oxalate oxidase-like	AP004559-1-R	CCTCCCATACACAAAAGCAC
Oxalate oxidase	Oxox1F	AGCTTGTCACTGCGCTTCTT
Oxalate oxidase	Oxox1R2	CAGCTTGTCACTGCGCTTCT
Oxalate oxidase	Oxox2F2	AGCTCGTCACTGCTACTGTC
Oxalate oxidase	Oxox2R3	GTACGCTGCTCGTAGCGAAG
Oxalate oxidase	Oxox3F2	CCATAGCACCAAAGCTAGGC
Oxalate oxidase	Oxox3R2	TCATTCCCACAACATGGGAGG
Oxalate oxidase	Oxox4F2	ACTACTGCCTCTGCCAGGTG
Oxalate oxidase	Oxox4R2	TCATTTCGTGGCCTGCGATGG
P14-3-3	AP004003-F1	AGTTGATCTGACTGCCATGTA
P14-3-3	AP004003-R1	AGGTAGAAGACCTTGGACTCTG
Chitinase	Chit-F	TTC TAC ACC TAC GAG GCC TTC
Chitinase	Chit-R	GGC CTC TGG TTG TAG CAG T
Chitinase I	Chit1-F	CAA CAG GCT CTG CTG CAG
Chitinase I	Chit1-R	CGC AGT AGC GCT TGT AGA AC
Chitinase II	Chit2-F	AGM TCG CYG CCT TCT TCG
Chitinase II	Chit2-R	ATC CAG AAC CAG AKS GCC
Chitinase III	Chit3-F	CAR CTA CGG CCT GTC STC
Chitinase III	Chit3-R	CTC ARA CGC TGS WCT TCA C
Chitinase 1 and endochitinase	Chit1a-F	GGC TTA ATT TGC ATT GCA TC
Chitinase 1 and	Chit1a-R	CAT TGT ATT TTA TTC GTC TGC

endochitinase		
Glucanase	X58877F	TAG AGA GGT TGC AAC GAC AC
Glucanase	X58877R	CAG CAC AAT TCA TTC ATT TAG
Glucanase	GluF	GGC AAC ATC TAC CCS TAC
Glucanase	GluR	GTT GAA CAT GGM GAA SAC
Thaumatococcus	X68197F	GTA GAG TAA ATT GTT AAT GGC G
Thaumatococcus	X68197R	CGA TCA AGG TCT CGT TTT ATT C
Thaumatococcus	PR5F	ACC TTC MSC ATC ACC AAC
Thaumatococcus	PR5R	CAT GGC GAS GTT GTA GCC
Dehydrin-like protein	AP005055-f1	ACTCGTCACAGCTCAAACAAGT
Dehydrin-like protein	AP005055-r1	AAAGGCACATAATCGTATCCACC

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**CHAPTER 3 - *Oxalate Oxidase-Like* Genes Contribute to Rice Blast
Disease Resistance Governed By QTL**

Summary

Valuable agronomic traits such as nutritional quality and disease and drought resistance are frequently governed by quantitative trait loci (QTL). However, use of these QTL to improve crop species is hindered because the genes contributing to the trait, which could be used in marker aided selection, are not known. Mapping studies have shown that defense response (DR) genes such as the *oxalate oxidase like*-genes (*OsOXL*) co-localize with QTL conferring disease resistance to rice blast disease in several rice mapping populations, and are the major contributors for the phenotypic variance. We used RNA interference to determine if any of the twelve *OsOXL* genes predicted within the rice blast QTL region on chromosome 8 contributed to blast disease resistance. A single portion of the coding region that is highly conserved among the *OsOXL* genes was sufficient to silence from a few to all expressed *OsOXL* family members in T₀ plants and T₁ transgenic rice lines. Challenge with *Magnaporthe grisea*, the rice blast pathogen, in a spot inoculation on rice detached leaf assay revealed that the more chromosome 8 *OsOXL* genes suppressed in the transgenic lines, the more susceptible the plants were to disease. Of the 12 genes, *OsOXL 6, 7, 8, and 9* are the major contributors to resistance against *M. grisea*, with *OsOXL6* providing the largest contribution. Thus, multiple *OsOXL* genes contribute to the resistance governed by the rice blast QTL on chromosome 8, and the contributions of certain gene family members are more critical than others.

Introduction

Protection of agronomic crops from losses caused by plant pathogens has largely been possible through the use of host genetic resistances in plant breeding programs. In major food crops such as rice, qualitative single gene-based resistance has been highly effective for some diseases. However, some pathogens such as *Magnaporthe grisea* (*Mg*) are highly variable and can adapt rapidly to overcome the single gene resistances (1). A viable solution to the unsustainability of qualitative resistance is to build a base of quantitative resistance, which, because of its multigenic nature, is predicted to confound the evolution of pathogens to virulence (2). In addition, quantitative resistance is predicted to be broad-spectrum because it apparently is not strain specific (3). Incorporating quantitative trait loci (QTL) into germplasm, however, has been hindered by the lack of knowledge of which genes are contributing to the QTL. No QTL for plant disease resistance have been cloned to date; the QTL that have been cloned are related to other traits, such as *Arabidopsis* flowering time, tomato fruit characteristics, rice heading time, and maize plant architecture (4-5). Without knowledge of the genes that contribute to disease resistance QTL, plant breeders cannot develop the molecular markers needed to track and select for the genes in their crop improvement programs.

Our goal is to understand the molecular basis for QTL-governed disease resistance in plants. Using various mapping populations derived from rice cultivars with demonstrated variation in multigenic resistance, regions conferring quantitative resistance to several important rice diseases such as bacterial blight, sheath blight, and rice blast were identified (6-11). The small effects of many QTL make precise mapping difficult, and therefore, the use of map-based cloning to identify the genes contributing to these QTL would be difficult (5-12). An alternative approach is a candidate gene approach wherein a systematic analysis of candidate plant defense genes in linkage disequilibrium with resistance, associated with QTL, or both, would allow the more precise identification and localization of genes conditioning quantitative resistance and may identify suitable targets for marker-assisted selection, cloning, or both (12). Towards this approach, we demonstrated the association of candidate defense response (DR) genes with disease resistance QTL in several mapping populations (7-9). DR genes are those plant genes whose mRNAs and/or enzymatic activity are induced or increase after pathogen challenge (13).

One of the several DR genes associated with QTL conferring resistance to rice blast was an *oxalate oxidase like*-gene marker (*HvOxOLP*) that co-localized with a disease resistance QTL with LOD scores from 7.1 and 10 on rice chromosome 8 in several rice mapping populations. It was the major contributor for the phenotypic variance of 30% DLA (7-10). *Oxalate oxidase* (*HvOxOa*) and *oxalate oxidase-like* (*HvOxOLP*) genes are members of the germin-like subfamily that belong to the functionally diverse cupin superfamily (14). The germin and germin-like proteins (GLP) are multimeric, and glycosylated enzymes, that have extreme resistance to heat and to degradation by proteases or hydrogen peroxide (14). Germins and GLPs play a wide variety of roles as enzymes, structural proteins or receptors (15). Various studies have demonstrated directly or indirectly that germins and GLPs are involved in plant defense responses against several pathogens (16). Expression of particular germin and GLPs was induced to higher level after infection with pathogens, feeding of insects, or application of various chemicals such as salicylic acid (SA), H₂O₂, and methyl jasmonate (17-21). Plants engineered to express or overexpress heterologous oxalate oxidase enzymes exhibit enhanced resistance to pathogens that secrete oxalic acid as a pathogenicity factor (22-25). Silencing of a *Nicotiana* *GLP* increased resistance against an herbivore pathogen (18). In a comprehensive study, Zimmermann et al. (26) demonstrated that overexpression of representatives of the six barley GLP subfamilies resulted in enhanced resistance to the powdery mildew fungus *Blumeria graminis* f.sp *hordei*, whereas silencing of the genes resulted in enhanced susceptibility to the pathogen.

To date, the mechanisms by which these proteins are involved in plant defense responses remains unclear. Germins have oxalate oxidase activity (27-28), and some GLPs have superoxide dismutase (SOD) activity (15). Both of these activities produce hydrogen peroxide (H₂O₂) in plants, which may relate to their function in defense responses (18). Rapid generation of H₂O₂ and superoxide (O₂⁻) during the oxidative burst are recognized as a central component of plant defense responses to pathogen challenge (29). H₂O₂ also is involved in oxidative cross-linking of cell wall proteins and lignification, which implies a role in the cell wall reinforcement and papillae formation against pathogen penetration (30-31). H₂O₂ also is involved in the orchestration of the HR (32) and is a diffusible signal in systemic acquired resistance (SAR)

(29). Elevated H₂O₂ levels are suggested to trigger downstream components of the defense response pathway and induce expression of DR genes associated with SAR (33). Both O₂ and H₂O₂ mediate signal networks underlying systemic and local resistance responses (34). Over expression of a glucose oxidase gene (*GOX*) in potato and rice plants increases the endogenous levels of H₂O₂, induces the expression of DR genes, and confers enhanced broad-spectrum resistance to both bacterial and fungal pathogens (35-36).

As in other crop species, there are many GLPs in rice, and the co-localization of several of them with disease resistance QTL suggests a role in defense responses in rice. Carrillo et al. (2005) scanned the rice genome and identified more than 40 GLPs. Four GLPs with putative oxalate oxidase function co-localized with a rice blast resistance QTL on chromosome 3 (7- 9), and are related to the barley *oxalate oxidase* (*HvOXOa*, accession no. Y14203). Several GLP sequences co-localized with a rice blast resistance QTL on chromosome 8 in several mapping populations (7-10); these are most similar to the barley oxalate oxidase-like gene (*HvOxOLP*, accession no. X93171). Within the region of the chr. 8 QTL, we predicted twelve putative *oxalate oxidase-like genes* (*OsOXL*) clustered within 2.8 Mb (Davidson et al., *submitted*). Several members of the chr. 8 *OsOXL* were differentially expressed after wounding and *Mg* inoculation in the susceptible and resistant parents of the two QTL mapping populations (Davidson et al., *submitted*). No single gene family member was implicated as most important for resistance against *Mg* by expression profiling; rather the data suggested a combination of members contributed to defense responses in rice. *OsOXL3, 5, 6, 7, 8, 9, 10*, and *11* are the most directly related gene members to the defense response after wounding and rice blast infection. *OsOXL6* is the only gene member exhibiting highly induction after wounding and *Mg* inoculation in the resistance parents in comparison with the susceptible parents.

From our earlier studies, three lines of evidence suggested that *OsOXL* genes contribute to blast disease resistance governed by the chr. 8 QTL. First, the *OsOXL* co-localize with a disease resistance QTL on chr.8 (7-10). Second, accumulation by breeding of regions harboring the *OsOXL* genes into rice cultivars enhanced blast resistance (8). Third, the induced expression of some family members within the cluster correlated with disease resistance (Davidson et al., *submitted*). However, still lacking was evidence that the *OsOXL* genes indeed function to

contribute to the QTL-governed disease resistance. Here, we use RNAi interference to silence the twelve predicted *OsOXL* gene family members clustered within 2.8 Mb of rice chr. 8 to confirm their contribution to rice blast disease resistance by gene suppression analysis. Our data show that the more *OsOXL* gene members are suppressed, the more susceptible the transgenic lines are to *Mg*. Among the gene members clustered on chr. 8, we observed functional redundancy, with *OsOXL6*, 7, 8, and 9 contributing the most to resistance, and, of these, *OsOXL6* having the greatest effect. We conclude that members of the *OsOXL* indeed contribute to resistance controlled by the chr. 8 blast resistance QTL.

Results

RNAi Silencing of OsOXL Genes on Rice Chromosome 8 Using a Highly Conserved Coding Region Construct

The *HvOxOLP* cDNA and a related cDNA corresponding to the barley *oxalate oxidase* (*HvOxOa*) (accession no. Y14203) were used to scan the rice genome sequence for *oxalate oxidase-like* and *oxalate oxidase* genes, respectively. We predicted 41 gene members of these families scattered on rice chromosomes 1, 2, 3, 4, 5, 8, 9, 11, and 12 (Figure 3.1). All of these proteins contain exact or slight variations of the characteristic sequence, PHIHPRATEI, known as the germin box (data not shown) (37). Multiple sequence alignment of the encoded proteins by these 41 predicted rice GLP genes were compared with the gene members representative of the known GLP subfamilies in barley (*HvGER1*, *HvGER2*, *HvGER3*, *HvGER4*, *HvGER5*, and *HvGER6*) (21, 29) and with the two members used to scan the rice genome (Figure 3.1). The dendrogram resulting from the multiple sequence alignment of the rice and barley GLP members allowed us to subdivide the rice GLPs into several subfamilies that, as in barley, often contain two or more duplicated genes of high sequence similarity. The *OsGER4* subfamily in rice contains at least 7 gene members. The most closely related subfamily to *OsGER4* is the *OsGER3* subfamily, which contains at least 3 gene members. Twelve highly conserved *OsOXL* gene members on chr. 8, named *OsOXL1* through *OsOXL12*, are predicted within the blast QTL region on chr. 8 (Davidson et al., *submitted*) and are members of the rice *OsGER4* and *OsGER3* GLP subfamilies. *OsOXL* on chr. 8 are more closely related with *OsOXL* gene members on chr.12 (*OsOXLXII-1*, 2, 3, 4) than the others (Figure 3.1).

To determine whether a specific, highly conserved coding region among the *OsOXL* members on chr. 8 could effectively suppress the expression of only the 12 family members on chr. 8 but not the others, we used the highly conserved coding region of *OsOXL3* as the trigger for RNAi. The relative nucleotide identity exhibited between this 500 bp region and the other eleven members on chr. 8 and four members on chr.12 were from 69% to 85% (Figure 3.2, Table 3.1). The 500 bp region contained several contiguous stretches of nucleotides in which 22 to 26 bases are identical (allowing one or two mismatches) between *OsOXL3* and the other *OsOXL*

members on chr. 8 and chr. 12 (Figure 3.2, Table 3.1). The 500 bp region was cloned 5' of an inverted repeat of a 3'-UTR region of the *nopaline synthase* (*nos*) gene from *Agrobacterium tumefaciens* in the pTSi-1 RNAi vector (39-40) (Figure 3.3a, b) and used for *A. tumefaciens*-mediated transformation of the japonica rice variety Kitaake. We demonstrated the genome insertion of the transgene in the T₀ most susceptible plants, including the ones which we advanced to T₁ generation, by PCR using a vector primer and a primer corresponding to the transgene (data not shown).

Reverse transcription (RT)-PCR analysis of the *OsOXL* members in transgenic T₀ rice plants showed that most of the members on chr.8 were suppressed with different efficiencies among the T₀ plants (Figure 3.4). The constitutive levels of mRNA of *OsOXL3* were very low in untransformed plants and, in the T₀ plants that expressed the conserved RNAi trigger region, the expression levels were even lower (data not shown). The mRNA levels of the other *OsOXL* members on chr.8 were reduced to variable degrees compared with the untransformed control Kitaake (Figure 3.4). *OsOXL4* and *OsOXL10* genes were not expressed under these conditions in the transgenic plants or the untransformed control; *OsOXL3*, and *OsOXL8* were expressed at very low levels (data not shown).

Our results show that silencing can still occur even with one or two mismatches within the target region. Moreover, all of the sequences share a similar degree of homology with the RNAi target region and this region was able to silence most of them. For example, although *OsOXL12* shares 85% sequence similarity with the target region and has several stretches of 26 nucleotides with perfect matches, among the transgenic plants the silencing of this family member is less compared with family members having lower sequence similarities (Figure 3.2, Table 3-1, and Figure 3.4). Overall, these results demonstrated that the pTSi-1 RNAi vector was efficient in silencing a highly conserved gene family in rice. Moreover, a highly conserved region among gene family members was sufficient to suppress the targeted members and the levels of silencing depended not only on sequence similarity.

The *OsOXL*-suppressed transgenic plants are phenotypically indistinguishable from the Kitaake untransformed plants. However, some Kitaake suppressed T₀ plants failed to produce seeds (data not shown).

OsOXL Suppressed T₀ Kitaake Transgenic Plants are More Susceptible to M. grisea

We used a detached rice leaf-spot inoculation assay that had been developed to assay qualitative disease resistance responses (41) to evaluate the reliability of the assay for assessing quantitative resistance in rice-blast interactions (7-8). The detached leaf inoculation assay efficiently distinguished the control resistant and susceptible responses (Figure 3.5) and these results were in agreement with data from the commonly used spray inoculation assays (data not shown). Using the detached leaf assay, quantitative resistance to *Mg* Che86061 in Kitaake, the host for our silencing studies, was reliably measured. The *OsOXL* T₀ Kitaake suppressed plants with confirmed gene silencing were evaluated for their responses to *Mg* Che86061 using the detached leaf assay. The degree of susceptibility was scored primarily based on the amount of mycelia present in the lesion compared with untransformed plants. Accordingly, we classified T₀ plants as highly susceptible (SS), susceptible (S), moderately susceptible (MS), moderately resistant (MR), and resistant (R) (Figure 3.6a, b). In the Kitaake T₀ transgenic plants, increasing susceptibility to *Mg* was observed as more chr.8 *OsOXL* genes were silenced (Figure 3.6a). *OsOXL6*, 7, and 9 gene family members contribute the most to resistance because the plants scored as SS, S, and MS have at least one or a combination of these gene members highly suppressed (Figure 3.6a, b). The fact that the most susceptible lines have more gene members suppressed indicated functional redundancy among these gene members.

RNAi Silencing of Oxalate Oxidase-Like Genes in T₁ Kitaake Transgenic Lines

We selected T₀ plants 12A1 and 12A3, in which all expressed *OsOXL* genes were suppressed for advancement to the T₁ generation (Figure 3.4). T₁ lines were analyzed for gene silencing by RT-PCR and RNA levels were compared with the untransformed control Kitaake (W). Most of the genes silenced in the T₀ parental plants were differentially silenced among the T₁ progeny (Figure 3.7a). The overall effect of silencing was reduced in the T₁ generation compared with the T₀ parent plants, as shown by the number of genes suppressed among T₁ lines (Figure 3.7a, 3.4). *OsOXL4* and *OsOXL10* were not expressed and *OsOXL1* and *OsOXL3* were

expressed at very low levels under these conditions (data not shown). *OsOX12*, which has 85% similarity with the trigger region of the RNAi construct, showed strong suppression in the T₁ lines compared with the T₀ plants. *OsOXL5*, with 74% similarity and a 24 nucleotide perfect match with the RNAi trigger region, was not suppressed in T₁ lines although it was suppressed in the parental T₀ plants (Figure 3.7a, 3.4). These results show the levels of silencing depend not only on sequence similarity, but also on other unknown factors.

We also tested for silencing of the closest related members to the chr. 8 *OsOXL* family, which is a group of four *OsOXL* genes in tandem array on chr. 12 (Figure 3.1). Of the four *OsOXL* genes predicted on chr.12, one was not expressed under our conditions (*OsOXLXII-4*). The other three *OsOXL* genes on chr.12 were suppressed in the T₁ lines (Figure 3.7 b). The distantly related *oxalate oxidase* genes on chr.3 (Figure 3.1) were not silenced by the conserved region from the chr.8 *OsOXL3* member (data not shown).

Overall, our results demonstrated that a highly conserved sequence targeted RNAi gene silencing of most of the *OsOXL* gene family members. The suppression of each gene member among the transgenic lines is variable, probably depending in several factors such as sequence similarity and abundance of mRNA, among other unknown factors. The silencing effect is carried through the first generation, but gene suppression is reduced in T₁ generation.

OsOXL Suppressed T₁ Kitaake Transgenic Lines are More Susceptible to M. grisea

The *OsOXL* T₁ Kitaake lines tested for gene silencing were evaluated for their responses to *Mg* Che86061. The T₁ lines that have more *OsOXL* gene members silenced were more susceptible to infection with *Mg* (Figure 3.8a, b). However, in accordance with their silencing patterns that indicated a reduction in the gene suppression in this generation, only two lines were considered highly susceptible (SS), compared with six lines (SS) obtained in the T₀ plants (Figure 3.6a, b). In this experiment *OsOXL6*, 7, 8, and 9 gene members stand out as the most important contributors to resistance against *Mg* (Figure 3.8a, b). Note that A3-16 line, the most susceptible, is the only line that has total suppression of *OsOXL6*.

We hypothesized that the two most suppressed and most susceptible lines (A3-16 and A1-7) (Figure 3.8a, b) were homozygous for the presence of the transgene. The most susceptible line A3-16 did not produce T₂ seeds (seed abortion). The other highly susceptible line A1-7 is homozygous for the transgene based on the presence of the hygromycin gene in T₂ progeny (data not shown). The three gene members on chr.12 (*OsOXLXII-1, 2, 3*), which are closely related to the chr. 8 *OsOXL*, were silenced in this generation, but their silencing was not correlated with increasing susceptibility (Figure 3.8a, b).

Based on inoculation studies with the T₀ and T₁ generations, there is not a single member responsible for the blast resistance governed by the chr. 8 QTL, but rather a combination of members contribute, with the most important being *OsOXL6, 7, 8, and 9*. Among these members *OsOXL6* provided the strongest contribution to resistance. These results also indicated a redundancy in function among the closely related family members in plant defense responses because several members must be suppressed to render the line highly susceptible.

Discussion

We demonstrated that multiple rice *OsOXL* genes belonging to *OsGER4* subfamilies contributed to resistance governed by the chromosome 8 QTL against rice blast. Using the barley *HvOXOLP* cDNA sequence (accession no. X93171), we predicted a cluster of 12 *OsOXL* (*Oryzae sativa* pv. *oryzae oxalate oxidase-like*) genes in a 2.8 Mb region that co-localized with the rice blast QTL on chr.8 (Davidson et al., *submitted*). Based on mRNAi silencing studies, we conclude that certain gene family members are more critical than others, with the most important being *OsOXL6*, 7, 8, and 9 (Figure 3.6a, b; 3.8a, b). According to our T₁ data, *OsOXL6* is a major contributor because the A3-16 transgenic line that was most susceptible to rice blast is the only one where the expression of this gene member is totally suppressed (Figure 3.8a, b). This is in agreement with our previous studies of gene expression profiles of the 12 gene members after wounding and blast inoculation where *OsOXL6* was the only member that showed higher and more sustained induction in the resistant parents (+ chr.8 QTL) compared with the susceptible parents (- Chr.8 QTL) against rice blast (Davidson et al., *submitted*). *OsOXL6* gene is one of the most closely related gene member to the barley genes *HvOXOLP* and *HvGER4d* (Figure 3.1); these two barley genes which are associated with resistance to virulent and non-host pathogens in barley and one is associated with papillae formation (42-44).

All forty-one genes encoding putative germin-like proteins (GLP) were predicted to contain the germin box or a slight variation of this box (PHIHPRATEI). Germin and germin-like proteins (GLPs) constitute a large family of proteins ubiquitously distributed in the plant kingdom. They are part of the superfamily of proteins now called “cupins” that also includes seed storage globulins, sucrose-binding proteins as well as microbial and animal proteins (45). Various studies have demonstrated directly or indirectly, that germins and GLPs are involved in plant defense responses against several pathogens (16). Expression of particular germins and GLPs was induced to higher levels after infection with pathogens including bacteria, fungi, insects and virus (17-21-24-37-42-44-46-47). Some germins and GLPs can be induced after application of various chemicals such as salicylic acid (SA), H₂O₂, and methyl jasmonate (17-21).

We demonstrated that a highly conserved region among the chr.8 *OsOXL* gene members specifically suppressed the mRNA expression of all the expressed gene members on chr.8 and the closely related family members on chr.12 to variable degrees (Figures 3.4, 3.7a, b). The gene suppression was effective, but less efficient, through the T₁ generation. The suppression efficiency was not related only to the level of homology between the trigger and target sequences (Figure 3.2, Table 3-1) because some members (*OsOXL2*, 6, 7, 8, 9, and 11) with one or two mismatches with the target region were silenced in the T₀ and T₁ generation (Table 3-1, Figures 3.2, 3.4, 3.7a, b) whereas *OsOXL5*, which had stretches of 24 nucleotides in perfect match with the target region was not suppressed in the T₁ plants although it was in T₀ plants (Figure 3.4, 3.7a). Miki, et al. (48) reported similar observations in their studies of RNA silencing of the rice *OsRac* gene family members using a highly conserved region among these members as a trigger for gene silencing. They silenced all the *OsRac* gene members to variable degrees among transgenic lines by using a highly conserved region. Although they did not advance their materials to the T₁ generation, their work with T₀ plants confirmed that gene suppression efficiency was not exclusively dependent on sequence homology between the target and the RNAi trigger sequences. Other factors might play a role during the gene silencing processes. For example, if the initial concentration of the target mRNA is higher, it will result in easier access of these mRNA to the SiRNA and RISC complex. Alternatively, if the mRNA is initially in low quantities, siRNAs can target other members that have high sequence similarity. mRNA secondary structures might also play a role during gene silencing. For example, some secondary structures on the mRNA in the target genes might reduce the efficiency in which the siRNAs will find their endogenous targets to undergo the gene silencing processes.

In several suppressed T₀ plants and T₁ lines we observed that the level of expression of some *OsOXL* genes was higher than the expression of the Kitaake untransformed control plants (Figures 3.4, 3.7a). In the T₀ plants, these differences may be because of developmental variation between the transgenic T₀ plants and the untransformed Kitaake plants. This is because the transgenic plants were transferred to the soil as seedlings while the control Kitaake seed were germinated in petri dishes and then planted in soil when the T₀ plants were transferred to soil. Variations in the expression profile of some genes such *OsOXLI* and 8 between T₀ and T₁ RNAi

silencing patterns (Figures 3.4, 3.7a) also might be explained by developmental variations. In the T₁ experiments, all seeds corresponding to transgenic lines and Kitaake control plants were germinated and planted in soil at the same time, however higher expression levels of some *OsOXL* genes were observed in T₁ suppressed lines compared with Kitaake controls. It is possible that the suppression of some *OsOXL* gene members induces the expression of other gene members to compensate for gene suppression. More studies need to be done to determine the effects of altered expression (reduced) of specific *OsOXL* gene members on the other gene family members.

The rice *OsOXL* gene members suppressed in this study are closely related to the genes of the GLP subfamily 4 (*HvGER4*) from barley whose corresponding GLP proteins exhibit superoxide dismutase (SOD) activity (73 to 87% of amino acid similarity) (26) (Figure 3.1). The *OsOXL* gene members that are most clearly associated with resistance against rice blast (*OsOXL6*, 7, 8, 9) are the closest related members to the barley *HvGER4* members (85 to 86% amino acid similarity, Figure 3.1) and are tightly clustered on the QTL region on rice chromosome 8 (Davidson et al., *submitted*). The *HvGER4* subfamily of proteins from barley, including the *HvOxOLP*, is an important component of quantitative resistance in wheat and barley (42-44). Members of this subfamily of GLPs from barley and wheat were strongly induced by virulent pathogens and non-host pathogens (26-42). Wei et al. (44) isolated a cDNA clone corresponding to *HvOXLP* and determined that this *oxalate oxidase-like* from barley is expressed in compatible and incompatible interactions between barley and powdery mildew. Moreover, this gene is expressed mainly in the epidermis and its spatial and temporal expression patterns correlate with papillae formation (a general resistance barrier). Christensen et al. (2004) (42) determined that the *HvGER4* gene subfamily members previously named *HvGLP4* in barley and *TaGLP4* in wheat were important components of quantitative resistance in barley and wheat respectively, and that these gene products have superoxide dismutase (SOD) activity. When *HvGLP4* and *TaGLP4* were overexpressed in a panel of barley and wheat cultivars that were susceptible to the fungal pathogens *B. graminis* f. sp. *hordei* and *B. graminis* f. sp. *tritici*, respectively, resistance was enhanced by the reduction in the frequency of fungal haustoria formation. Silencing of those genes by RNAi revealed enhanced susceptibility (increased haustoria formation) compared with the corresponding control plants.

Zimmermann et al. (26) studied the function of six GLP subfamilies in barley using a single gene member to represent each subfamily and studying their gene expression profiles as well as overexpression and gene silencing studies in barley and *Arabidopsis*. Four members corresponding to four GLP gene subfamilies (*HvGER1*, *HvGER3*, *HvGER4*, and *HvGER5*) were induced in barley by the powdery mildew fungus *B. graminis* f.sp *hordei*. Only one protein, HvGER1a, exhibited oxalate oxidase activity; proteins from two other subfamily genes *HvGER4* (*HvOxOLP*) and *HvGER5* exhibited superoxide dismutase (SOD) activity. Overexpression and silencing experiments of the barley genes representing two subfamilies, *HvGER4* and *HvGER5*, demonstrated their role in resistance against powdery mildew, with overexpressing plants exhibiting enhanced resistance and silenced plants exhibiting hypersusceptibility. The *HvGER4* subfamily is the only one that was induced by the non-host pathogen soybean rust fungus in barley. Thus, this subfamily with SOD activity may be involved in broad-spectrum resistance to several pathogens. Members of the barley *HvGER4* (formerly referred to as *HvGLP4* and *HvOxOLP*) appear to be expressed predominantly in *B. graminis*-attacked leaf epidermal tissue (44-49). This expression pattern is supported by the fact that in barley there are 44 *HvGLP4* out of approximately 5,000 EST from pathogen-attacked epidermis compared with one of a total of 106,000 EST from 21 other cDNA libraries (42). Because of the high amino acid similarity with the *HvGER4* subfamily in barley, we speculate that the OsOXL proteins encoded by the gene members on rice chr. 8 might have SOD enzymatic activity (Figure 3-1). If so, the H₂O₂ produced by the putative SOD activity and their putative apoplasmic localization (21, 42) may be responsible for the reinforcement of the cell wall at the penetration site of pathogen, either through papillae formation and callose deposition or in the formation of lignin. Ultimately, this H₂O₂ could be used as secondary messenger signaling for the activation of downstream defense responses.

Our results in rice show that the more chr. 8 *OsOXL* genes suppressed in the T₀ and T₁ Kitaake transgenic rice lines, the more susceptible the plants were to rice blast in a detached leaf assay (Figures 3.6a, b; 3.8a, b). This indicates functional redundancy among these closely related gene members. This functional redundancy was also shown in barley (26-46). Independent overexpression of two members of the *HvGER4* subfamily protected barley from the powdery

mildew pathogen (26). In barley *HvGLP4* represents a cluster of at least nine duplicated genes (38-44), while in rice we predicted 12 clustered gene members on chr.8 and four on chr.12. Clustering of gene family members involved in plant defense responses has been described for *Arabidopsis thaliana* and *Solanum tuberosum* (50-51) as well as for *Oryza sativa* (52-54). Because of the frequent occurrence of gene paralogs with very high sequence similarity within the coding sequence (CDS) and because of the functional redundancy among these members, we suggest that the CDS might have diversified after duplication events. This duplication might have resulted in gene members that encode proteins with different enzymatic properties such as optimal pH for activity, cofactor requirements, and inhibition requirements. This variation could reflect differences in protein activation and location to different cell compartments. In addition to diversification within the CDS of these *OsOXL* gene members, their promoter regions also might have diversified to create spatial and temporal differences in their specific expression patterns under several stimuli. Ober et al. (55) described gene duplication as the major driving force for the recruitment of genes for secondary metabolism. Gene copies are gradually modified to create genes with specificities and expression patterns adapted to the needs of the pathway in which they are involved. In barley, H₂O₂, the product of most of *HvGER* proteins, and exogenous application of H₂O₂ was found to lead to transcript accumulation of several *HvGERs*. These results indicated a possible feedback loop of signaling mechanisms during defense responses involving these gene members (26).

We also showed a complex picture of the fine regulation and functional redundancy of the rice *OsOXL* members during defense responses against the rice blast fungi *M. grisea*. Davidson et al. (*submitted*) showed that the most closely related *OsOXL* paralogs on chr.8 have similar global expression patterns after wounding and *Mg* inoculations. Because of the regulation and overlapping functions among the gene members of this GLP subfamily in rice, and their contribution to resistance against rice blast, we proposed that the *OsOXL* gene members clustered on rice chr.8 explain at least part of the resistance against rice blast governed by the QTL on chr. 8. The variation in expression of the chr.8 *OsOXL* gene members and location of their encoded enzymes may complicate the ability of the pathogen to overcome that resistance, and therefore result in more durable resistance.

The predicted roles of the *OsOXL* gene members during general resistance against pathogens are consistent with these genes conferring broad-spectrum resistance, or resistance against multiple pathogens, in rice. Our preliminary results indicate that the *OsOXL* T₀ suppressed lines are also more susceptible to sheath blight caused by *Rhizoctonia solani* and to aphids (data not shown). Experiments are underway to test the hypothesis that these *OsOXL* gene members are associated with broad-spectrum resistance and to characterize the enzymatic activity as well as determine the defensome profile in plants silenced for these interesting enzymes.

Methods

Bioinformatics

Two *Hordeum vulgare* mRNA sequences corresponding to *oxalate oxidase* (*HvOXOa*) and *oxalate oxidase-like* genes (*HvOXOLP*) (accession no: Y142203 and X93171, respectively) were used as queries for tblastx search (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) using The High Throughput Genomic Sequences (HTGS) database. The FGENESH program (<http://www.softberry.com/berry.phtml>) was used to predict the putative *oxalate oxidase* (*OXO*) and *oxalate oxidase-like* genes (*OsOXL*) from the significant rice BAC hits. All sequences corresponding to different members predicted using these two barley sequences were aligned using CLUSTAL W. 1.8 in the BCM Search Launcher Interface (Baylor College of Medicine HGSC, <http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>). Sequence similarities among the predicted proteins were compared with known GLP proteins from barley and the phylogeny reconstruction was performed using CLUSTAL W. 1.8. The phylogenetic tree was generated using the TreeViewX program (R. D. M. 1996) and bootstrap values were calculated using the program PAUP 4.0b10 (Swofford D, 2002).

Construct for RNAi Silencing and Agrobacterium Mediated-Transformation

A 500 bp region in the second exon, that is highly conserved among *OsOXL* gene members on chr. 8 was selected as trigger for RNAi gene silencing. This fragment was amplified from IR64 genomic DNA using the following amplification primers: OXOF2R2, 5'-TGGGTTTCCTTGCAAGAACC-3' and 5'-TTCTTCTCCACTTGAAATGCC-3'. The PCR product was ligated in the antisense orientation into *XcmI*-digested pTSi-1 (40) (Figure 3.3b). pTSi-1 vector was digested with *NheI* and the fragment containing the RNAi vector with the antisense transgene was ligated into *XbaI*-digested pCambia 1305 binary vector and transformed into *Agrobacterium tumefaciens* EHA105. *Agrobacterium* transformed cells were cultured overnight at 28 °C in MGL medium containing kanamycin (50mg/ml). A 1 ml aliquot of this overnight culture was added to 15 ml of TY medium with 200 uM acetosyringone and the culture was incubated overnight at 28 °C. The overnight culture was diluted to 0.1 to 0.25 O.D at 600 nm and used to transform rice calli following the protocol for rice transformation described by (57) and modified by Patrick Canlas (UC Davis, *personal communication*). The presence of the

transgene was confirmed in 25 independent T₀ transgenic rice lines by PCR amplification with the reverse primer that is specific to the 2xCaMV35S promoter (5'-CGTGCTCCACCATGTTGGCAAGC-3') and the forward primer specific to the transgene (OXOF2R2, 5'-TGGGTTTCCTTGCAAGAACC-3').

DNA and RNA Analysis

Rice genomic DNA was isolated from leaf tissue using a modified CTAB procedure (58). Genomic DNA was quantified by UV absorbance using a NanoDrop ND-1000 spectrophotometer (Rockland, Denver). PCR reactions were performed using HotStar Taq DNA Polymerase (Qiagen). Trizol reagent (Invitrogen) was used to isolate total RNA from leaf tissues of T₀ transgenic plants (two weeks after transfer to soil), T₁ transgenic plants (two weeks after emergence), and wild type Kitaake plants (same developmental stage as the transgenic plants) using the manufacturer's protocol. RNA concentrations were determined by UV absorbance. Total RNA was treated with one unit of DNase (Promega) per ug total RNA. cDNA was synthesized from total RNA (500 ng) using Superscript III reverse transcriptase kit (Invitrogen). The cDNA was amplified with a pair of gene specific primers (10 pmol of each primer) developed for each *OsOXL* gene on chr. 8 using HotStar Taq DNA Polymerase (Qiagen) (Davidson et al., submitted). Gene specific primers were designed in the 5' and 3' untranslated regions (UTR) of the gene family members on chr. 12 and 3 (Table 3.2). *EF-1a* (*elongation factor 1 alpha*) and *ubiquitin* genes were amplified as internal controls for each cDNA samples. A PCR cycle gradient (15, 20, 25 and 30 cycles) performed with internal control primers (Table 3.2) determined that 25 and 30 cycles were the optimum conditions for unsaturated PCR reactions for *ubiquitin* and *EF-1alpha*, respectively (data not shown). *Hygromycin* primers were used to determine the transgene presence (Table 3.2).

Plant and Fungal Growth and Inoculation Methods

The reliability of a detached leaf assay (41) for assessing resistance governed by quantitative traits relative to the standard spray inoculation method (59), was determined by inoculating several rice cultivars with *Mg* isolates known to elicit disease or resistant responses. We compared a *japonica* (Azucena, susceptible), and two *indica* varieties (IR64, moderate resistance; SHZ-2, resistant) after inoculation with *Mg* PO6-6, and two *japonica* lines (Kitaake,

resistant; Nipponbare, susceptible) after inoculation with *Mg* Che86061. *Mg* was grown on oatmeal agar media under constant light at 26°C for two weeks (detached leaf assay) or 21 days (spray assay).

T₀ transgenic lines that had been transferred to soil and T₁ lines germinated from seed were grown with a photoperiod of 16 h light/8 h dark in a growth chamber with photon flux of 135 $\mu\text{molm}^{-2}\text{s}^{-1}$ and day/night temperatures of 28°/26°C. Two weeks after transfer to soil or after seed emergence, T₀ and T₁ lines, respectively, were inoculated with 5×10^4 spores/ml suspension of *Mg* strain Che86061 using the detached leaf method.

Figures and Tables Chapter 3

Figure 3.1 Dendrogram of the GLP proteins from rice and barley.

Multiple sequence alignment of rice GLP predicted proteins in this study and the barley GLPs (accession no. X93171, Y14203, TC140112, TC146914, TC131410, TC147149, TC147527, TC141367). Bootstrap values are indicated.

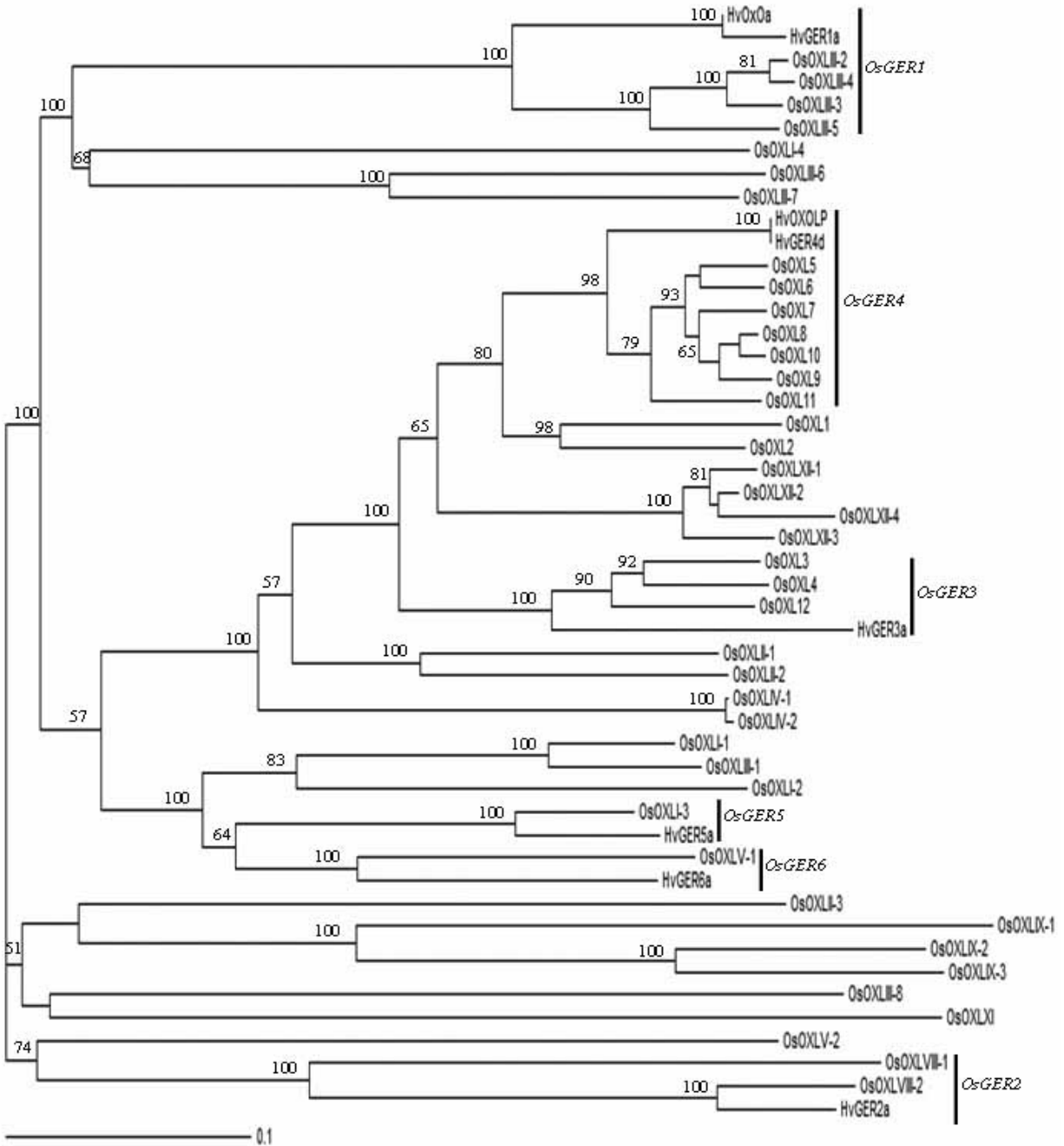


Figure 3.2 Multiple sequence alignment of the Chr. 8 *OsOXL* gene members and the closest related members on chr. 12.

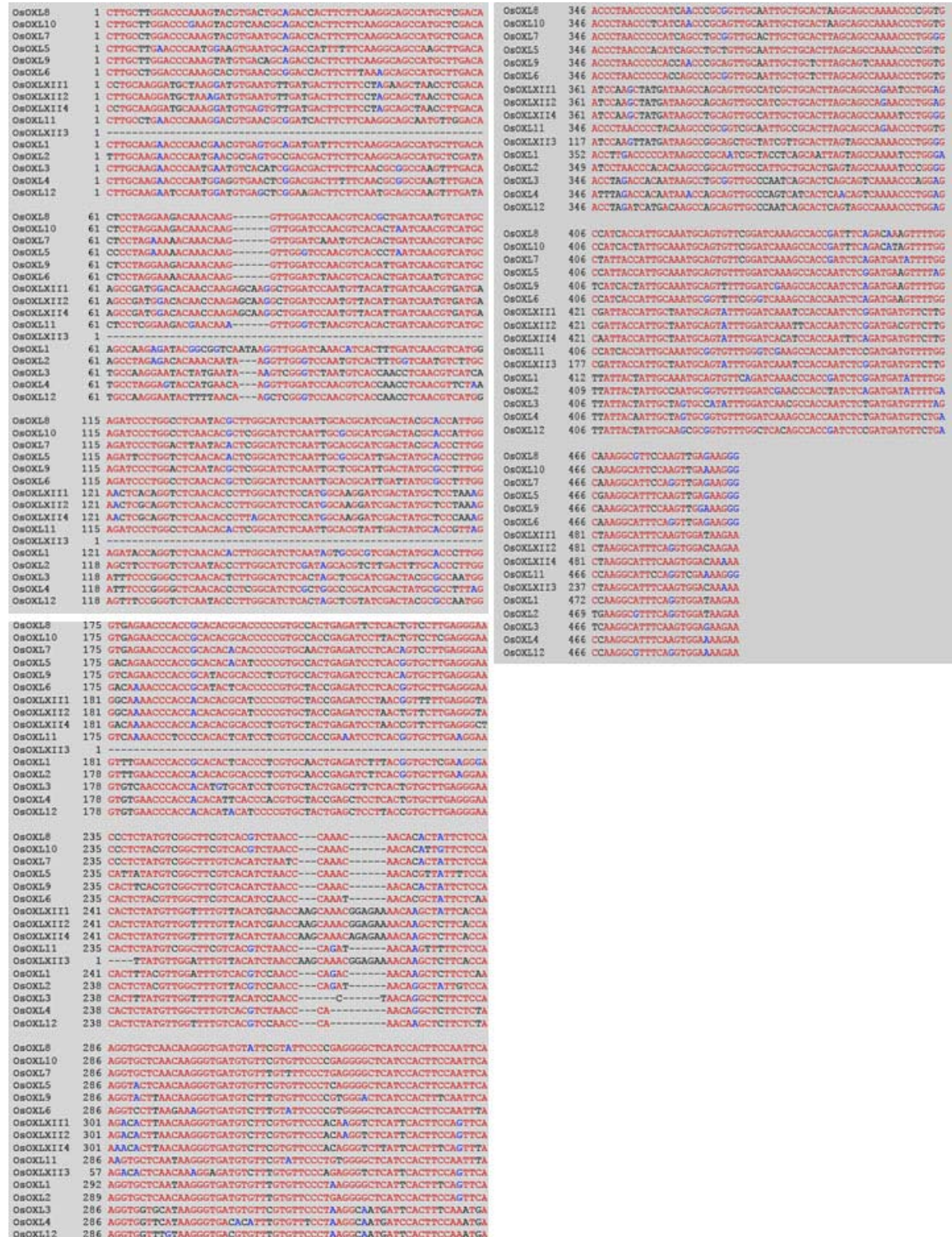


Figure 3.3 RNAi silencing vector pTSi-1 map.

(a) The HBT35S promoter includes a minimal CaM35S enhancer plus the 5' UTR of maize *CDDPK* gene. The inverted *NOS* carry the antisense sequence of the *Agrobacterium NOS* terminator plus an 81 bp fragment of the 3' end of the *GFP* gene. A pair of *XcmI* sites are used to generate "T" 3' extensions for cloning PCR products. Two *NheI* sites can be used to introduce the RNAi vector into *XbaI*-digested pCambia 1305. (b) Plasmid *pTSi-OsOXL* was constructed by inserting an antisense 500 bp corresponding to the second exon of *OsOXL3* at the *XcmI* sites. *NheI*-digested *pTSi-OsOXL* vector was inserted into *XbaI*-digested pCambia 1305 for *Agrobacterium* transformation.

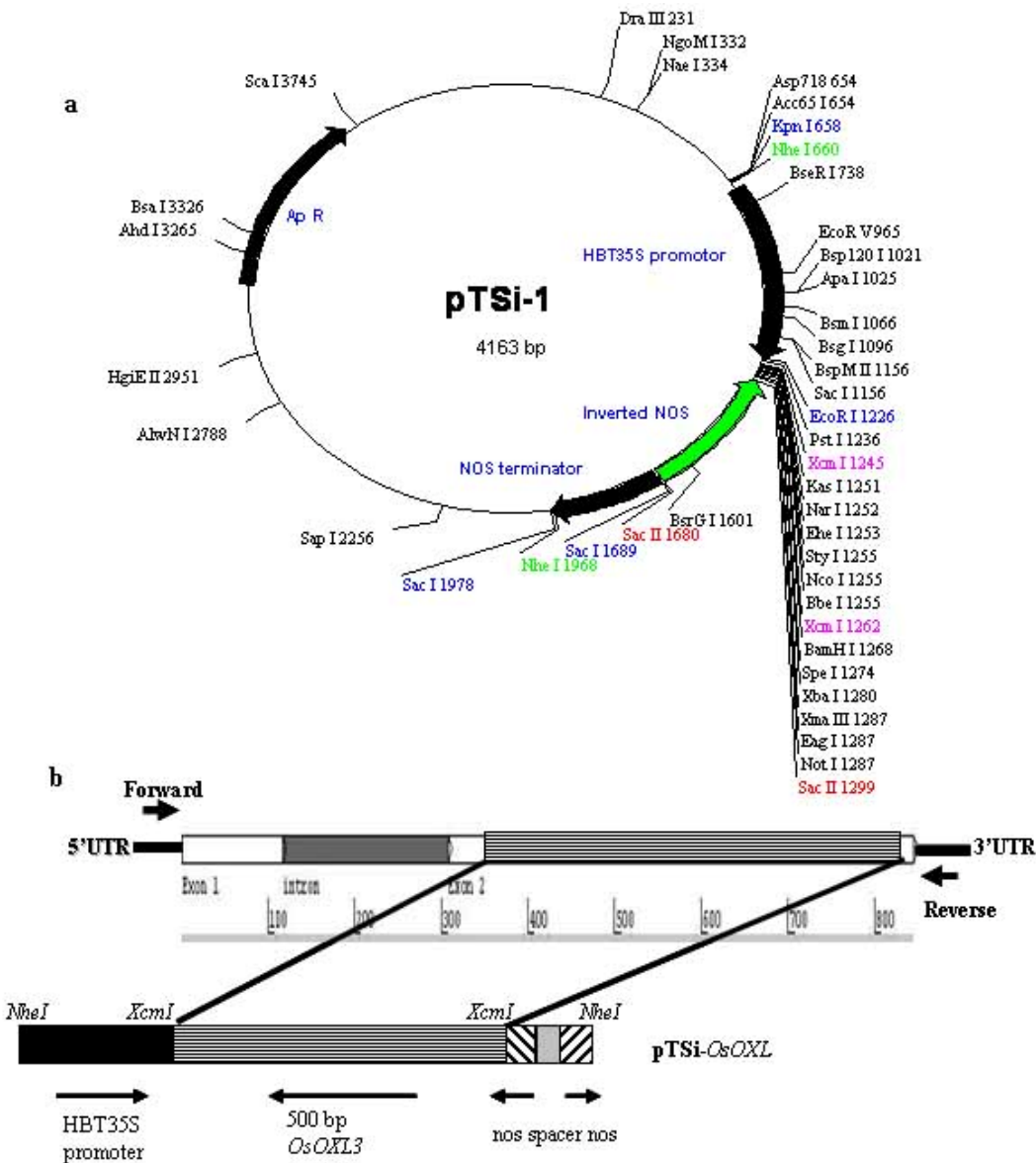


Figure 3.4 RNAi silencing of the 12 chr. 8 *OsOXL* genes in independant T₀ Kitaake transgenic plants varied among the different T₀ plants.

RT-PCR was performed using gene-specific primers for each *OsOXL* gene family member in uninoculated plants. Amplification results from uninoculated plants and an untransformed Kitaake line (W) were compared. Amplification of the *hygromycin* gene indicated the presence of the transgene. *EF-1 alpha* (*EF1-a*) and ubiquitin (*UBIQ*) genes were amplified as internal controls. Seed from lines 12A1 and 12A3 were used to generate T₁ plants.

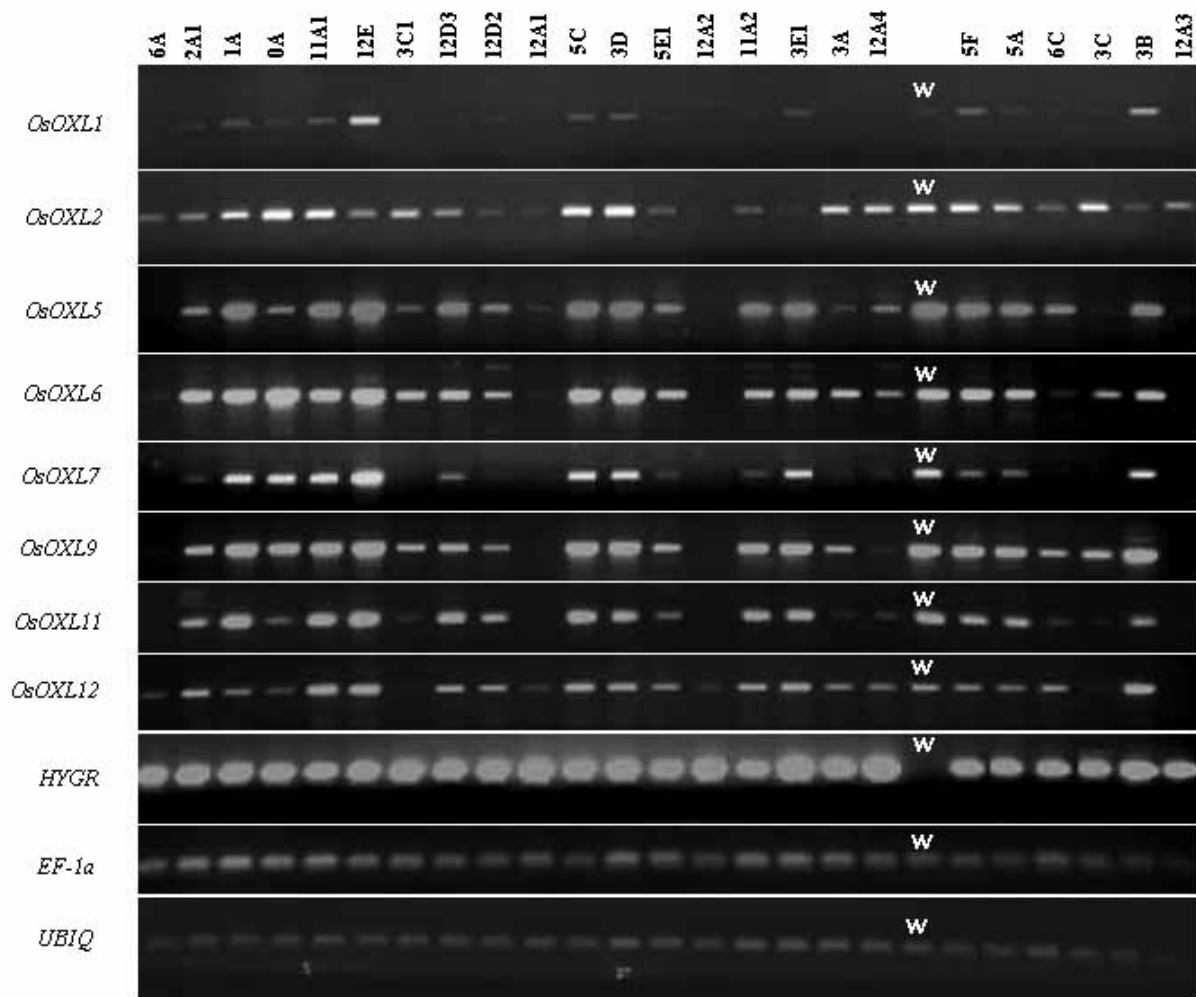


Figure 3.5 *In vitro* spot inoculation of rice detached leaves with *Magnaporthe grisea* (*Mg*). Cultivars that were known to exhibit QTL-governed resistance or susceptibility to *Mg* isolates PO6-6 and Che86061 in traditional spray inoculation assays were shown to exhibit comparable responses using the detached leaf assay. Azucena is highly susceptible to *Mg* PO6-6, whereas IR64 is moderately resistant and SHZ is highly resistant. Kitaake is resistant to *Mg* Che86061 while Nipponbare is susceptible.

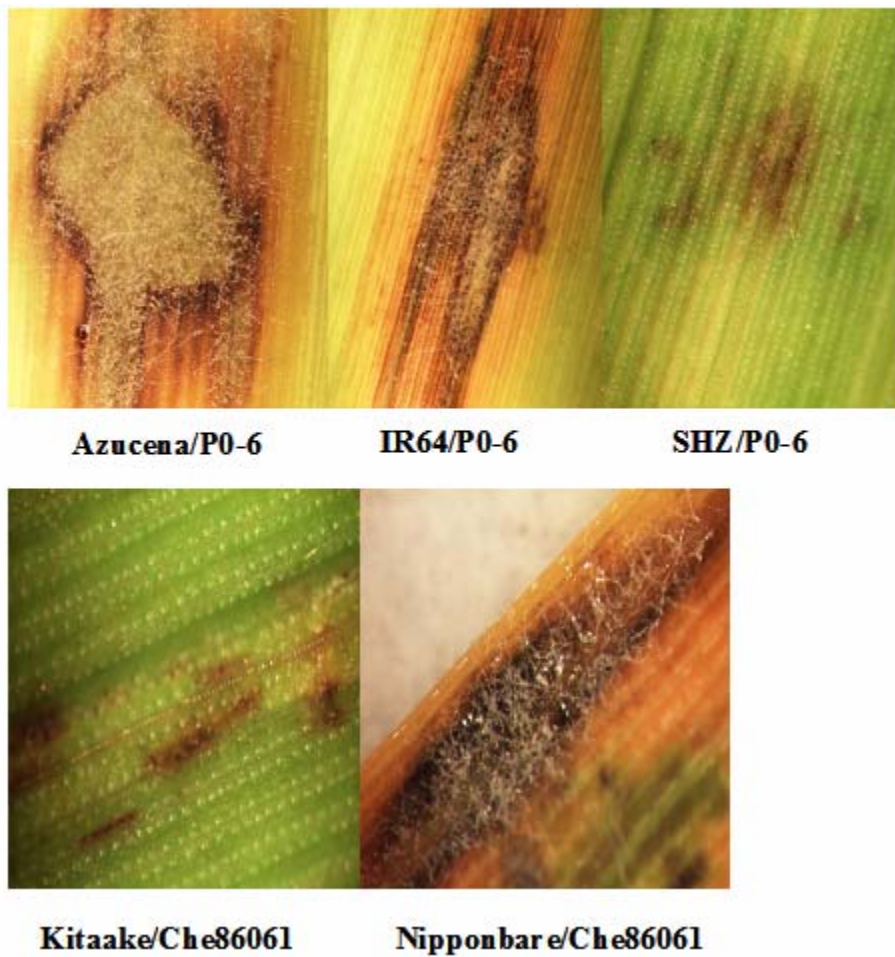
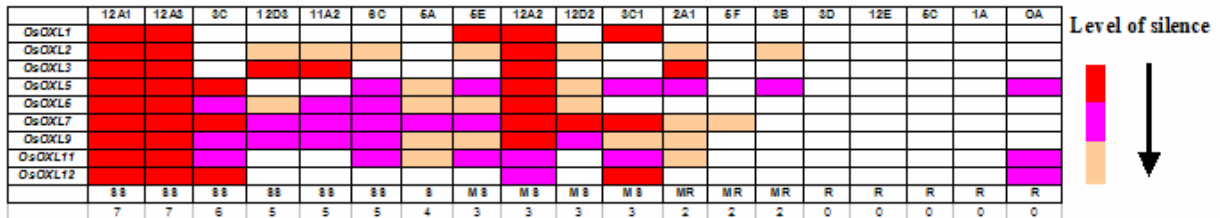


Figure 3.6 Suppression of multiple *OsOXL* gene in T₀ Kitaake lines correlates with increased susceptibility to *M. grisea*.

(A) The relative level of suppression for the *OsOXL* gene members expressed in uninoculated T₀ Kitaake lines is indicated as colored boxes and was determined from Fig. 3.4. Levels of suppression ranged from undetectable (red boxes) to strongly suppressed (dark pink), to slightly suppressed (light orange), to not suppressed (white). (B) Two weeks after transfer to soil, the second youngest leaves of the T₀ transgenic and wild type plants (same developmental stage) were detached and spot inoculated with *Mg* Che86061. Symptoms were assessed and photographed 7 days after inoculation and plants were classified as very susceptible (SS), susceptible (S), moderately susceptible (MS), moderately resistant (MR), and resistant (R) according to our established disease scale (7-0) which is based on the severity of the lesion and the presence of *Mg* mycelia. Wild type Kitaake (R) and Nipponbare (SS) plants of approximately the same stage were used as controls.

A



B

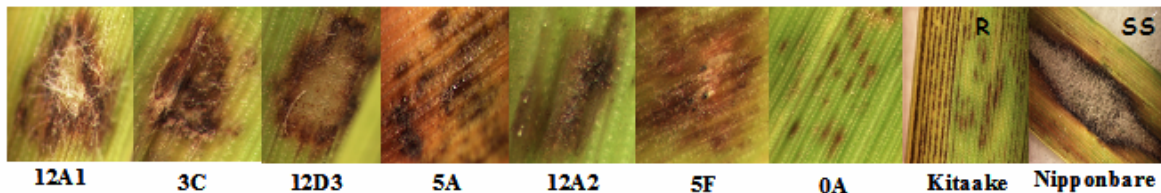


Figure 3.7 T₁ Kitaake transgenic lines exhibited various levels of silencing of the chr. 8 *OsOXL* genes.

(A) RT-PCR amplification using gene-specific primers for the chr. 8 *OsOXL* genes revealed different levels of silencing for different *OsOXL* gene family members. RNA was extracted from the third youngest leaf of two-week-old T₁ transgenic and Kitaake wild type plants (W). The levels of gene suppression are based on comparisons of amplification with the Kitaake untransformed line. *EF1-alpha* was used as internal control for the RT-PCR reactions. Left 1 Kb ladder (L) and right DNA amplification control (D). (B) RT-PCR for the closely related chr. 12 *OsOXL* family members using the same cDNA samples revealed differential silencing of those gene family members.

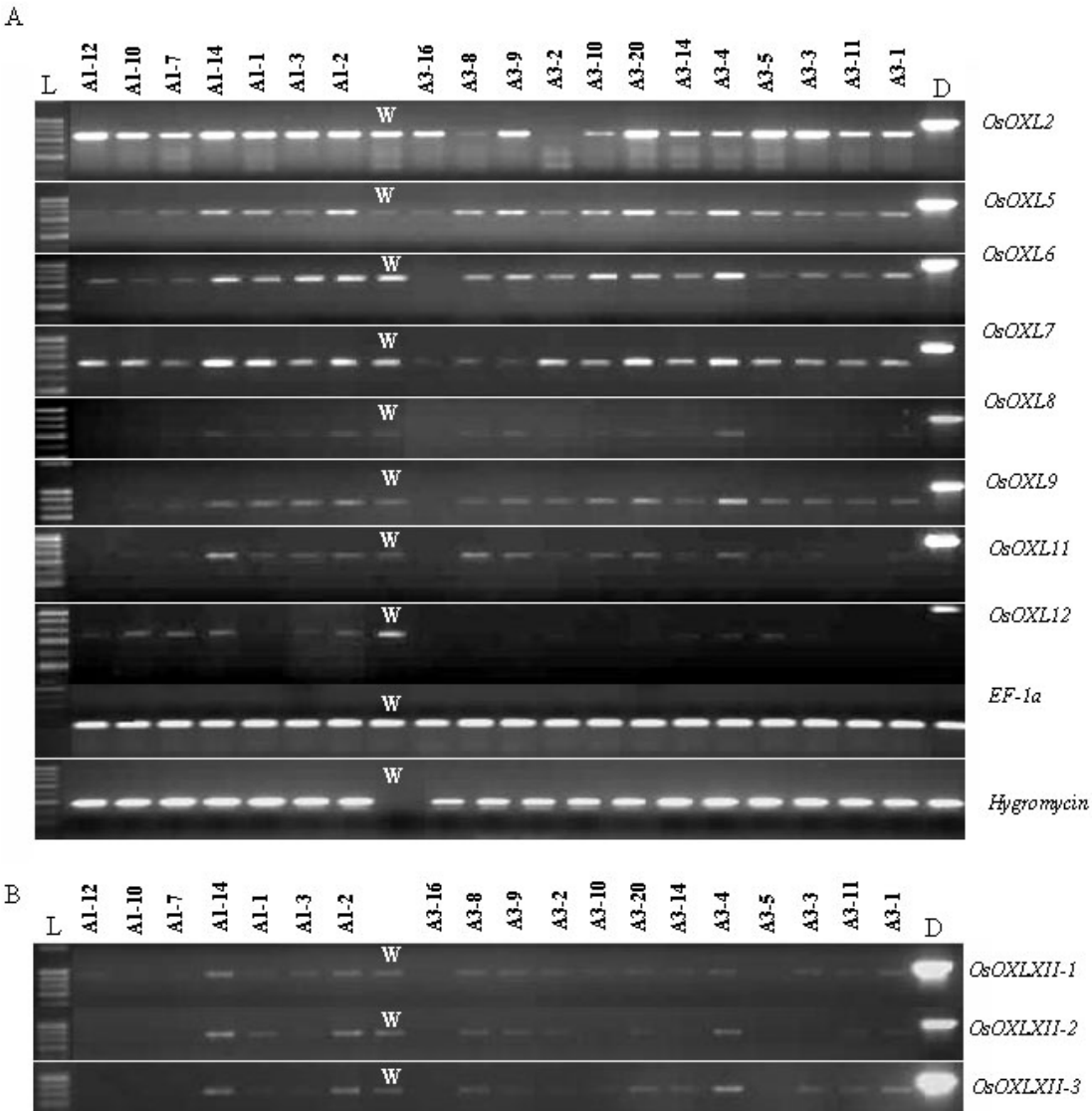


Figure 3.8 Suppression of multiple *OsOXL* gene in T₁ Kitaake lines correlates with increased susceptibility to *M. grisea* Che86061.

(a) The relative level of suppression of *OsOXL* gene members on chr.8 and chr.12 in uninoculated plants is indicated as colored boxes coded as in figure 3.6a. Suppression was determined from figure 3.7a, b. Suppression of chr. 12 *OsOXL* genes did not affect disease. (b) Two weeks after seed emergence from the soil, the second youngest leaf of the T₁ transgenic and Kitaake and Nipponbare control plants was detached and spot inoculated with *Mg* Che86061. Symptoms were scored and photographed 7 days after inoculation.

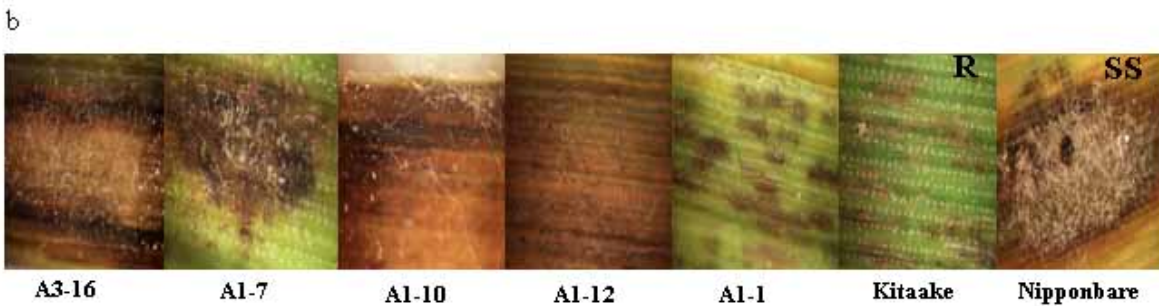
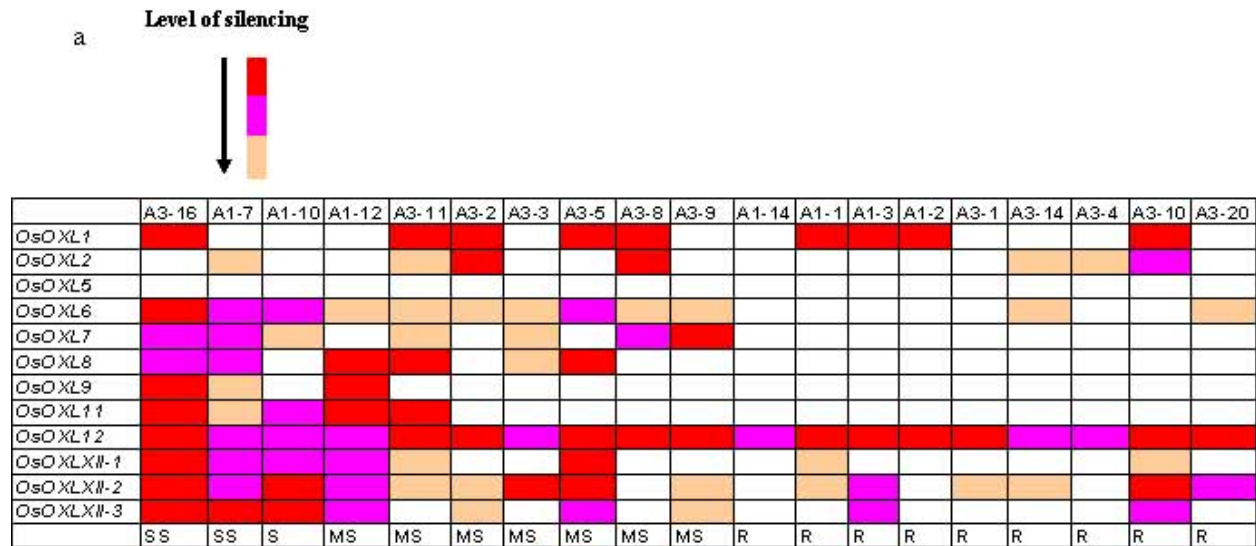


Table 3-1 Nucleotide sequence homology between the RNAi trigger region and the GLPs predicted on chromosome 8 and 12.

Gene	Nucleotide Homology %	Identity of maximum length of matching nucleotides
<i>OsOXL1</i>	76	26
<i>OsOXL2</i>	75	24 (1 mismatch)
<i>OsOXL3</i>	100	
<i>OsOXL4</i>	85	26
<i>OsOXL5</i>	74	24
<i>OsOXL6</i>	73	23 (1 mismatch)
<i>OsOXL7</i>	74	24 (2 mismatch)
<i>OsOXL8</i>	73	22 (1 mismatch)
<i>OsOXL9</i>	74	25 (1 mismatch)
<i>OsOXL10</i>	72	23
<i>OsOXL11</i>	73	26 (1 mismatch)
<i>OsOXL12</i>	85	26
<i>OsOXLXII-1</i>	72	25 (1 mismatch)
<i>OsOXLXII-2</i>	72	25 (1 mismatch)
<i>OsOXLXII-3</i>	69	25 (1 mismatch)
<i>OsOXLXII-4</i>	73	25 (1 mismatch)

Table 3-2 Gene specific primers for *OsOXL* genes on Chr.2 and control RT-PCR primers.

Gene	Primer name	Sequence 5-->3
<i>OsOXLXII-1</i>	Oxo12-14-F	ATCAACTATAGCTATACAAGAAT
<i>OsOXLXII-1</i>	Oxo12-14-R	GTAGTGTTATACATTATTGATGCGT
<i>OsOXLXII-2</i>	Oxo12-15-F	TAGACTACAGCTATACAAGAAGCAT
<i>OsOXLXII-2</i>	Oxo12-15-R	CTCTTTTTACTACCAATCACTAGTTTTG
<i>OsOXLXII-3</i>	Oxo12-16-F	CAAGCTAGCATCGAGTAATACTTC
<i>OsOXLXII-3</i>	Oxo12-16-R	GATAACTAATTTTCAACAGATAAGCATC
<i>OsOXLXII-4</i>	Oxo12-17-F	GACTACTTCTACAGGATCTGTAG
<i>OsOXLXII-4</i>	Oxo12-17-R	CCTTTTTATACCAATCACTAATTCTGA
<i>Ubiquitine</i>	Ubiqui-F	CCAGGACAAGATGATCTGCC
<i>Ubiquitine</i>	Ubiqui-R	AAGAAGCTGAAGCATCCAGC
<i>EF1-alpha</i>	EF1-alpha 1F	AGCCTCGTTCAAATGGTGGT
<i>EF1-alpha</i>	EF1-alpha 1R	TAGTGACATTGCGAGCAGA
<i>Hygromycin</i>	HygroF1	GAGCCTGACCTATTGCATCTCC
<i>Hygromycin</i>	HygroR1	GGCCTCCAGAAGAAGATGTTGG

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CHAPTER 4 - *GF14e* is a negative regulator of plant cell death and resistance against virulent strain of *Xanthomonas oryzae* pv. *oryzae*

Summary

Lesion mimic mutant plants develop spontaneous lesions that resemble disease symptoms in the absence of pathogen attack. Lesion mimic phenotypes indicate inappropriate activation of programmed cell death (PCD) and their presence is, in several cases, associated with enhanced resistance to multiple pathogens. In this study, we suppress the expression of *GF14-e*, one of eight members of the *14-3-3* gene family described in rice, by RNA interference. *GF14-e* suppression correlates with the appearance of spontaneous HR-like lesions (lesion mimic phenotype) in both T₀ and T₁ rice transgenic suppressed plants. The lesion mimic (LM) phenotype segregates 3:1 Mendelian ratios in the T₁ progeny and correlates with the presence of the transgene in these plants. LM T₁ lines exhibit enhanced resistance against a virulent strain of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) compared with the typical bacterial disease lesions observed in T₁ segregants not exhibiting LM and the nontransgenic wild type plants. Bacterial numbers in the T₁ segregants not exhibiting LM was two fold higher than the T₁ segregant showing the LM. T₁ segregant LM-*GF14-e* suppressed lines that are presumed homozygous for the silencing construct are stunted with low survival rates, and they do not produce seed, and do not survive. Because silencing of *GF14-e* allows the activation of the programmed cell death pathway and confers resistance to virulent isolates of rice pathogens, we conclude that *GF14-e* is a negative regulator of PCD and resistance in rice.

Introduction

In plants, programmed cell death (PCD) occurs during both normal development and in response to pathogen infection (Zeng et al., 2004). In plant-microbe interactions, PCD occurs during both plant hypersensitive response (HR) to avirulent pathogen infection and plant disease susceptibility under virulent pathogen attack (Greenberg, 1997). The HR is correlated with a transient burst of active oxygen species, activation of specific defense-related genes, accumulation of antimicrobial compounds, and alterations of the plant cell wall (Dangl et al., 1996). The HR is also associated with systemic acquired resistance (SAR) (Ryals et al., 1996). Considerable effort has been expended to understand the events and genes involved in the production of the HR. One avenue to understanding the basis of the HR is the identification of mutants with visible phenotypes that resemble the HR caused after pathogen infection (HR-type lesions).

A large number of mutants that exhibit abnormal regulation in the PCD pathways and phenotypes mimicking the HR have been identified in plants such as maize, *Arabidopsis*, barley, and rice (Walbot et al., 1983; Wolter et al., 1993; Johal et al., 1995; Dietrich et al., 1997; Yin et al., 2000; Yan et al., 2002). The constitutive activation of cell death and defense pathways in some of these mutants suggests that these mutations might define genes involved in the regulation of HR in wild-type plants (Zeng et al., 2004). These mutants are called lesion mimics based on their spontaneous HR-type lesion formation in the absence of pathogen infection. Several genes controlling lesion mimics have been isolated, the proteins encoded by these genes are membrane-associated proteins (Buschges et al., 1997), ion channel (Balague et al., 2003), Zing-finger protein (Dietrich et al., 1997), heat stress transcription factor (Yamanouchi et al., 2002); components involved in the metabolism of fatty acids/lipids, porphyrin, and phenolics (Gray et al., 1997; Hu et al., 1998; Kachroo et al., 2001), and a U-box/armadillo repeat protein with E3 ubiquitin ligase activity (Zeng et al., 2004).

The 14-3-3 proteins are highly conserved proteins with diverse functions in eukaryotes (Finnie et al., 1999). The 14-3-3 family consists of acidic 30 kDa proteins which form homo- and

hetero-dimers and has been found in the cytosol, nuclei, and nuclear matrix (Szopa, 2002; Chen et al., 2006). In animals, some 14-3-3 regulates activities of some protein kinases and phosphatases, thereby modulating multiple signal transduction pathways (Aitken, 1996). In plants, 14-3-3 play roles in regulation of genes (Ferl, 1996) and enzyme activities such as nitrate reductase (Hubert, 1996) and plasma membrane H⁺-ATPase (Jahn et al., 1997).

The diversity of function associated with 14-3-3 proteins is due to their structural features. Most eukaryotic cells contain more than one 14-3-3 isoform (Aitken et al., 1992). Different isoforms and how they combine to form dimers may correlate with the diversity of proteins with which 14-3-3 proteins interact. Functional diversity of individual 14-3-3 proteins may result from sequence variation which is observed mainly at either the N-terminal or C-terminal end (Aitken et al., 1992). Others have suggested that the conservation of the primary structures of 14-3-3 proteins suggests that there might be a common function shared by all 14-3-3 proteins (Yan et al., 2002).

The members of the *Arabidopsis* 14-3-3 proteins are named based in sequence information rather than in their original biochemical properties. In addition to the three-letter gene name for *Arabidopsis* 14-3-3s is *GRF* (*general regulator factor*) (Rooney and Ferl, 1995), and *Arabidopsis* 14-3-3 gene and isoform designations which often include the name *GF14* (*G-box factor 14-3-3 homolog*) (Wu et al., 1997). In *Arabidopsis* there are 15 members of 14-3-3 gene family, bearing the name *GRF1* through *GRF15* (DeLille et al., 2001). These members share overall amino acid similarity of 50%, with large blocks of the central region absolutely conserved among all isoforms and high variation in the C and N termini (Wu et al., 1997). 14-3-3 proteins in *Arabidopsis* display a high cell and tissue-type specificity and are localized within the cytoplasm and organelles such as nuclei, plastid, and mitochondria. The expression specificity and subcellular compartment of isoforms contribute to their diverse interactions with partners and differential functions in cellular activities (Sehnke et al., 2002).

Several lines of evidence implicate 14-3-3 protein involvement in plant defense responses. First, 14-3-3 genes are differentially regulated in defense responses. A 14-3-3 gene was up regulated in the non-host hypersensitive response (HR) between barley and *Blumeria graminis* f.sp. *tritici* (Brandt et al., 1992). A 14-3-3 gene is induced during HR of soybean

inoculated with *P. syringae* (Seehaus and Tenhaken, 1998), and in cotton inoculated with the wilt pathogen *V. dahliae* (Hill et al., 1999). In tomato plants, *14-3-3* genes were found to be regulated in resistant or susceptible plants challenged with the avirulence effector *Avr9* (Roberts and Bowles, 1999).

Second, 14-3-3s have been shown to bind to and activate various enzymes involved in plant defense responses. 14-3-3s are thought to regulate barley epidermis-specific responses to the powdery mildew fungus by activation of the plasma membrane H⁺-ATPase, which, in turn, would stimulate the HR (Finni et al., 2002). *Arabidopsis* genes involved in oxidative stresses such *ascorbate peroxidase* also interacts with 14-3-3 in yeast two hybrid experiments (Zhang et al., 1997). Interestingly, another interactor of these 14-3-3 proteins in *Arabidopsis* is the AKR2 protein. AKR2 is an arkyrin-repeat containing protein and negatively regulates transcription factors that mediate defense responses. The AKR2 was identified as a putative GF14- λ -interacting protein from *Arabidopsis*. AKR2-antisense plants developed HR-like lesions and exhibited enhanced resistance against a bacterial pathogen (Yan et al., 2002).

Direct evidence for involvement of 14-3-3s in disease resistance or stress tolerance also comes from genetic studies. In mapping studies, *14-3-3* genes were co-localized with disease resistance QTLs. Faris et al., (1999) co-localized a *14-3-3* marker with a fungal disease resistance QTL in wheat. In rice, *14-3-3* genes have been co-localized with a QTL against rice blast on chromosome 2 (Liu et al., 2004).

The release of the rice genome sequence opens possibilities to study the diversity of the *14-3-3* gene family in rice and to study their specific roles in regulation of rice biological processes, particularly in biotic stresses including disease resistance against rice major pathogens. Rice 14-3-3 proteins are implicated to be involved in stress responses as well as plant development. For example, the 14-3-3 proteins GF14b, GF14c, GF14e, and GF14f (previously named SR14-3-3) interact with target proteins that are involved in stress responses (Cooper et al., 2003). GF14b, and GF14f may interact with mitogen-activated protein (MAP) kinase BIMPk, a component induced by rice blast fungus and known to be involved in signaling in SAR (Cooper et al., 2003). In large-scale microarray analysis for rice genes involved in defense responses, *14-*

3-3 genes were regulated by different interactions between rice and pathogens and by defense signaling molecules (Cooper et al., 2003).

Rice contains eight *14-3-3* members which are located on six of the 12 chromosomes (Chen et al., 2006). All eight predicted genes have matching ESTs in the databases, indicating that all are expressed in rice. The genes were named *GF14a* through *GF14h*. *GF14b*, *GF14c*, *GF14e*, and *GF14f* were induced by inoculation with the rice blast pathogen in both compatible and incompatible interactions, and were differentially induced by defense signaling compounds and abiotic stress stimuli. *GF14b*, *c*, and *e* were also induced by the bacterial blight pathogen. Chen et al (2006) also demonstrated that the rice *14-3-3* gene members exhibited a degree of tissue specificity, with some of the members like *GF14e* expressing in all tissues tested in their experiments.

In this study we determine if a rice *14-3-3* family member is involved in plant defense responses by suppressing the expression of the *GF14-e* using RNA interference. We chose to silence this member because markers corresponding to this gene member were co-localized with a sheath blight and rice blast QTL on chromosome 2. We demonstrated that suppression of this specific member in rice (*GF14-e*) induces spontaneous HR-like lesions and this phenotype correlates with enhancement of resistance against virulent strain of the bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We report that *GF14-e* is a negative regulator for both rice plant cell death pathway and for rice resistance against *Xoo*.

Material and Methods

Bioinformatics

The *Hordeum vulgare* mRNA sequence corresponding to *14-3-3* gene (accession no: Hv1433c) was used as a query for tblastx searches (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>) using The High Throughput Genomic Sequences (HTGS) database. The FGENESH program (<http://www.softberry.com/berry.phtml>) was used to predict the *14-3-3* gene members from the significant rice BAC hits. All sequences corresponding to different members predicted using this barley sequence were aligned using CLUSTAL W. 1.8 in the BCM Search Launcher Interface (Baylor College of Medicine HGSC, <http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>).

Construct for RNAi silencing and Agrobacterium mediated-transformation

A 350 bp region in the first exon of GF14-e was selected as the trigger for RNAi gene silencing. This fragment was amplified from IR64 genomic DNA using the following amplification primers: AP004003F1: 5'-CACCGTGAGGAGAATGTGTACATGGCT-3' and AP004003R1: 5'-CTTGGACTCTGGAGCAGTGGATGAA-3'. The PCR product was cloned in pENTR/D-TOPO vector (Invitrogen) and transformed in *E. coli*. LR clonase reaction using Gateway LR Clonase Enzyme Mix (Invitrogen) was done to recombine the entry clone (pENTR/GF14-e) with the pANDA vector (Miki and Shimamoto, 2004). The resulting pANDA-GF14-e vector was transformed into *E. coli*. The pANDA-GF14-e containing the sense-antisense RNA trigger region was used to transform *Agrobacterium tumefaciens* EHA105 strain. *Agrobacterium* transformed cells were cultured overnight at 28 °C in MGL medium containing kanamycin (50mg/ml). 1 ml aliquot of this overnight culture was added to 15 ml of TY medium containing 200 uM acetosyringone and the culture was incubated overnight at 28 °C. The overnight culture was diluted to 0.1 to 0.25 O.D at 600 nm and used to transform rice calli obtained from Kitaake seeds following the protocol for rice transformation described by Cheng et al., 1997 and modified by Patrick Canlas (UC Davis, *personal communication*).

DNA and RNA analysis

Rice genomic DNA was isolated from leaf tissue using a modified CTAB procedure (Saghai-Maroo et al., 1984). Genomic DNA was quantified by UV absorbance using a NanoDrop ND-1000 spectrophotometer (Rockland, Denver). PCR reactions were performed using HotStar Taq DNA Polymerase (Qiagen). Trizol reagent (Invitrogen) was used to isolate total RNA from the flag leaf of T₀ transgenic plants (show the HR-type lesions) and from the flag leaf of wild type Kitaake plants (same developmental stage as the transgenic plants) using the manufacturer's protocol. RNA concentrations were determined by UV absorbance using the NanoDrop spectrophotometer. Total RNA was treated with one unit of DNase (Promega) per ug total RNA. cDNA was synthesized from total RNA (500 ng) using Superscript III reverse transcriptase kit (Invitrogen). The cDNA was amplified with a pair of gene specific primers (10 pmol of each primer) developed for *GF14-e* gene on chromosome 2 and their closest members *GF14-c*, *GF14-a* using HotStar Taq DNA Polymerase (Qiagen). Gene specific primers were designed in the 5' and 3' untranslated regions (UTR) of the *14-3-3* gene family members. *GF14-e*, AP004003 F1: 5'- GGATTCCTTTATCTGGCACTATTGAAG-3' and AP004003 R1: 5-GTGAATCAAGAA CCATCGTGACAC-3'; *GF14-c*, AP003881-F2: 5'- TTCTGGAGAGACCCCATGGCTTTC and AP003881-R2: 5'- GACAGCAGCATGAGGCATCTAAATC; *GF14-a*, AP004643-F2: 5'- CTCAACTCAACTCCACCCGAATC-3' and AP004643-R2: 5'- GTAAGCAAAGACCCG ACGAAAGG-3'. *EF-1alpha* gene and *hygromycin* genes were amplified as internal controls for each cDNA samples and as in chapter 3 (Table 3.2).

Lesion mimic segregation

A total of 97 T₁ progeny from a T₀ *GF14-e* suppressed plant expressing the lesion mimic phenotype and the Kitaake wild type line were grown in the growth chamber. The presence or absence of the lesion mimic phenotype was recorded in all the individuals of the progeny. The lesion mimic phenotype was correlated with the presence of the transgene by PCR analysis using the *hygromycin* primers. 3:1 Mendelian segregation ratios of the lesion mimic phenotype in the T₁ progeny were tested by Chi Square (X^2) test.

Plant and Fungal Growth and Rice Blast inoculation

T₀ transgenic plants that had been transferred to soil and T₁ lines germinated from seed were grown with a photoperiod of 16 h light/8 h dark in a growth chamber with photon flux of 135 $\mu\text{molm}^{-2}\text{s}^{-1}$ and day/night temperatures of 28°/26°C. Two weeks after transfer to soil or after seed soil emergence, T₀ plants and T₁ lines, respectively, were inoculated with 5×10^4 spores/ml suspension of *Magnaporthe grisea* strain Che86061 using the detached leaf spot inoculation method described in chapter 3.

Bacterial Blight Inoculation and Bacterial Counting

A virulent strain of *Xanthomonas oryzae* pv. *oryzae* (Xoo) PXO99 was used to inoculate 67 segregating T₁ progeny lines in the growth chamber. The second youngest and third youngest fully expanded leaves from the main tiller (M) and lateral tiller (L) of 7 week-old rice plants were inoculated with a bacterial suspension (5×10^8 CFU/ml) using the leaf clipping method (Kauffman et al., 1973). Disease was assessed at 2 weeks after inoculation by measuring the lesion lengths. Differences between means were tested using the SAS program (SAS Institute 2004). Bacterial counts were done in 20 T₁ lines including 10 T₁ segregant lines showing lesion mimic and that were resistance to PXO99 and 10 T₁ segregant lines (no transgene) with no lesion phenotype and susceptible to PXO99. The second youngest inoculated leaf in the lateral tiller (L2) was collected at two weeks after inoculation and macerated in 5 ml sterile water using extraction bags (BIOREBA) and a hand model homogenizer (BIOREBA). 1:10 dilutions were made for each sample (7 dilutions) and 20 μl of each dilution was spotted three times in nutrient agar plates containing 20 $\mu\text{g/ml}$ of cephalexin and 50 $\mu\text{g/ml}$ cycloheximide. Plates were incubated at 28°C and bacterial colonies were counted at 48 hr after incubation.

Results

RNAi silencing of GF14-e member on rice chromosome 2

The *Hv1433* cDNA was used to scan the rice genome sequence for *14-3-3* gene sequences. We predicted eight *14-3-3* gene members scattered on rice chromosomes 1, 2, 3, 4, 8, and 11. These genes are the same as those described recently by Chen et al. (2006). Chen et al. (2006) named the eight members *GF14-a* through *GF14-h*. We used the 350 bp in the first exon of *GF14-e* family member as trigger for RNAi (Figure 4.1). The relative nucleotide identity exhibited between this 350 bp region (PM6) and the next closely related *14-3-3* gene member (*GF14-b*) is 85% (Figure 4.2). Reverse transcription (RT)-PCR analysis of the *GF-14e* member on chr.2 in transgenic T₀ Kitaake rice plants showed that this gene member was efficiently suppressed using the pANDA silencing vector (Figure 4.3). Expression of the *14-3-3* gene members with close sequence identity to *GF14-e* (*GF14-c* and *GF14-a*) was not suppressed by the construct, as determined by RT-PCR (Figure 4.3). Although the silencing was specific to *GF14-e* and resulted in strong reduction in expression, the gene suppression was not complete (Figure 4.3). These results demonstrated that the pANDA RNAi rice vector was efficient in specific silencing the *GF14-e* gene family member in rice.

RNAi silencing of GF14-e induced HR-type lesions in T₀ transgenic Kitaake lines

T₀ *GF14-e* suppressed lines at the flowering stage showed spontaneous HR-type lesions in the flag leaf (Figure 4.4). Wild type Kitaake lines and other transgenic lines suppressing other genes such *OsOXL* did not show this phenotype. The presence and intensity of the lesion mimic phenotype correlated with the level of *GF14-e* suppression in T₀ plants (Figure 4.5). Sixty seven out of 97 T₁ progeny originating from the selfing of a T₀ lesion mimic *GF14-e* suppressed plant showed the lesion mimic phenotype which indicates a 3:1 Mendelian segregation ratio ($X^2= 1.8$). The phenotype of the plants was similar to the wild type until about 14 days, which is before flowering, when plants began showing lesion mimics. In the T₀ plants, the lesions occurred first in the flag leaf. Among the T₁ segregants, a small group of lesion mimic T₁ lines was observed to be stunted, with poor survival, and poor seed set (Figure 4.6). Other T₁ lesion mimic segregants (presumably the heterozygous) were phenotypically similar to the Kitaake wild type plants and

the T₁ segregants that did not have lesion mimics (transgene not present) except that the lesion mimic plants produced less seed (data not shown).

RNAi silencing of GF14-e induced resistance against bacterial blight pathogen

14-3-3 genes have been co-localized with fungal disease resistance QTLs in wheat and rice (Faris et al., 1999; Liu et al., 2004). In rice *GF14-e* was co-localized with a QTL for rice blast DLA (LOD 1.6) on chromosome 2 explaining 5.5 % of the phenotypic variance (Liu et al., 2004). We tested two week-old T₀ plants and T₁ *GF14-e* Kitaake suppressed lines for increased susceptibility to *M. grisea* (Che86061) using detached leaf inoculation assays. Neither the T₀ or T₁ suppressed line exhibited increased susceptibility to this blast isolate, and all transgenic plants behaved as the wild type (data not shown).

Many lesion mimic plants, including rice lesion mimics, exhibit enhanced resistance to pathogens (Dietrich et al., 1997; Yin et al., 2000; Yan et al., 2002; Zeng et al., 2004). We tested this hypothesis by inoculating 67 T₁ *GF14-e* Kitaake suppressed lines segregating for the lesion mimic phenotype with a virulent strain of *X. oryzae* pv. *oryzae* (PXO99). We inoculated four leaves in the rice plants corresponding to the second and third youngest fully expanded leaves in the main (M2, M3) and lateral tillers (L2, and L3). The lesion mimic *GF14-e* suppressed lines did not develop typical bacterial blight lesions to *X. oryzae* pv. *oryzae* PXO99, and were thus considered highly resistant (Figure 4.7). Kitaake wild type and the transgenic segregants that did not exhibit lesion mimics showed longer and typical bacterial blight lesions (Figure 4.7). The mean lesion lengths in each of the leaves inoculated (M2, M3, L2, and L3) in the lesion mimic plants were significantly lower when compared with the mean lesion lengths in the non-suppressed, lesionless segregants ($P < 0.0001$) (Figure 4.8).

The resistance observed in T₁ lesion mimic plants correlates with the amount of bacteria present in the inoculated leaves. Bacterial numbers in the L2 inoculated leaves of the lesion mimic *GF14-e* suppressed lines (0.9×10^7 cfu/ml) were significantly lower ($P < 0.0001$) than the numbers of bacteria found in the T₁ lesionless segregant lines (1.2×10^9 cfu/ml) (Figure 4.9).

Discussion

14-3-3 proteins acting as ubiquitous regulators (adapters, chaperons, activators or repressors) have been associated with several biological processes in plants (Sehnke et al., 2002). However, their involvement in abiotic and biotic stresses has not been well studied. 14-3-3 proteins have been associated with defense responses against pathogens in several pathosystems including rice (Dangl et al., 1996; Dietrich et al., 1997; Yin et al., 2000; Yan et al., 2002; Zeng et al., 2004). In rice, *14-3-3* genes (*GF14-e*) co-localize with QTL for resistance against rice blast (Liu et al., 2004). This localization on chromosome 2 corresponds to a region that was identified also as sheath blight QTL (Wang et al., 2001). Chen et al. (2006) described eight *14-3-3* gene family members in rice and determined that *GF14-b*, *c*, *e*, and *f* were differentially expressed by rice fungal and bacterial pathogens. In this study we determined the contribution to disease resistance of the *14-3-3* gene member on chromosome 2 (*GF14-e*) by gene suppression analysis using RNAi gene silencing.

We specifically suppressed the expression of *GF14-e* in Kitaake transgenic lines expressing a sense-antisense RNAi vector (pANDA). We did not find increased susceptibility against rice blast in inoculation studies of the T₀ plants and T₁ suppressed transgenic lines suggesting that the *GF14-e* gene does not contribute to QTL derived resistance. However, late in development of the transgenic lines, T₀ plants and T₁ *GF14-e* suppressed lines exhibited spontaneous HR-like lesions on the leaves. This phenotype correlated with the level of *GF14-e* gene silencing. The development of the lesion mimic phenotype in several T₀ transgenic plants was not likely due to disruption of a gene by transgene integration because all of the T₀ suppressed lines showing the lesion mimic phenotype came from independent transformation events. The 3:1 Mendelian segregation ratios in the T₁ progeny of a T₀ *GF14-e* suppressed lesion mimic plant indicate that there is a single insertion of the transgene in the T₀ parent and T₁ segregants.

The lesion mimic phenotype in the *GF14-e* suppressed line is in agreement with the involvement of 14-3-3 proteins in apoptosis in mammals. Masters et al., (2002) studied the survival-promoting functions of 14-3-3 proteins. They demonstrated that one role of these

proteins is to support cell survival in mammals. 14-3-3 proteins promote survival in part by antagonizing the activity of associated proapoptotic proteins, including Bad and apoptosis signal-regulating kinase 1 (ASK1). Using 14-3-3 inhibitor peptides in cells is sufficient to induce apoptosis. Masters et al (2002) demonstrated that the blockage of 14-3-3-ligand binding induced apoptosis in mammal cells. Other evidence of the role of the 14-3-3 proteins in the programmed cell death pathway is the identification of a 14-3-3 client in *Arabidopsis* (Yan et al., 2002). The GF14 λ (14-3-3)-interacting protein in *Arabidopsis* called AKR2 was found to contain four ankyrin repeats at the C-terminal end. Antisense AKR2 *Arabidopsis* plants showed lesion mimic phenotypes and this phenotype was correlated with the stronger antisense suppression.

We did not find increased susceptibility to rice blast fungi in the *GF14-e* silenced plants after inoculation with an *M. grisea* strain that produced a resistance reaction in the Kitaake wild type plants used to produce the silenced lines. Many lesion mimic mutants in plants are correlated with disruption in the cell death pathway leading to the constitutive production of HR-type lesions and expression of some defense response genes (Takahashi et al., 1999; Yin et al., 2000). Often these plants exhibit enhancement of disease resistance (Takahashi et al., 1999; Yin et al., 2000). Since the *GF-14-e* suppression results in induction of HR-like lesions, we asked if, rather than enhanced susceptibility, the transgenic suppressed lines would exhibit enhanced resistance against rice pathogens. We used a virulent strain of *X. oryzae* pv. *oryzae* to inoculate T₁ segregants originating from selfing of a T₀ *GF14-e* suppressed line that showed lesion mimics and 3:1 Mendelian ratios. As predicted, lines showing the lesion mimic phenotype in the T₁ progeny were highly resistant to the virulent *X. oryzae* pv. *oryzae* isolate in comparison with the T₁ segregants with no lesion mimics or silencing and the Kitaake untransformed line (Figure 4.7, 4.8, and 4.9). The silenced lines with lesions exhibited significantly lower bacterial numbers and no lesion development. These findings clearly indicate that the down regulation of *GF14-e* slowed pathogen multiplication and hindered development of typical bacterial blight lesions in these silenced plants.

Several lesion mimic mutants have been identified in different plants, with some being dominant and some recessive (Richberg et al., 1998). Cell death in plants can be produced by both misregulation of genes involved in PCD and disruption of cellular homeostasis by a wide

range of metabolic perturbations (Yin et al., 2000). In some plants, lesion formation activates the expression of cytological and biochemical markers associated with disease resistance and leads to local and systemic resistance (Dangl et al., 1996). Negative control of both cell death and the disease resistance response was attributed to the genes defined by the *Arabidopsis lsd1* and *acd2* mutations (Dietrich et al., 1994) and alleles of recessive barley *ml-o* (Wolter et al., 1993). *ml-o* alleles condition resistance to powdery mildew and exhibit variable levels of spontaneous cell death lesions. The null *lsd1* phenotype suggests that the wild type gene is a negative regulator of cell death; this mutant reacts to both virulent pathogens and to chemicals that trigger SAR with the HR-like response (Dietrich et al., 1994).

In *Arabidopsis* the 14-3-3 interactor protein AKR2 is a negative regulator of disease resistance against a bacterial pathogen (Yan et al., 2002). Reduced expression of AKR2 using antisense technology results in an overproduction of H₂O₂ and HR-type lesions in the silenced leaves. The transcripts of two genes, *PR-1* and *Glutathione S-transferase* that are involved in disease resistance and stress responses were increased, and pathogen growth was inhibited in antisensed AKR2 plants. AKR2 and ascorbate peroxidase (APX3), another interactor of the same 14-3-3 protein member, can interact with each other, suggesting that AKR2 can regulate the expression of APX3. APX3 is involved in H₂O₂ scavenging in peroxisomes (Wang et al., 1999). In the antisense AKR2 lines the activity of APX3 is reduced resulting in higher concentrations of H₂O₂ in localized areas that may induce HR-type lesions and constitute expression of defense response genes. This data from *Arabidopsis* suggested that AKR2 regulated by 14-3-3 proteins plays an important role in the oxidative metabolism of disease resistance and stress responses in plants, probably by regulating the activity of APX3.

In this report we identify a lesion mimic rice line as a result of the down regulation in the expression of *GF14-e* gene family member and show that the suppressed lines exhibit enhanced disease resistance against a bacterial pathogen. Many lesion mimics mutants have been identified in rice and some of these mutants display altered early defense signaling or disease resistance (Takahashi et al., 1999; Yin et al., 2000). Disruption of a heat stress transcription factor was found to be responsible for the *spl7* lesion mimic (Yamanouchi et al., 2002). Yin et al. (2000) identified nine rice mutants exhibiting lesion mimic phenotype, four mutants, *spl1*, *spl5*, *spl9*,

and *spl11*, conferred enhanced resistance to rice blast. *PR1* and *PBZ1 (PR-10)* were activated in those mutants. Only *spl11* showed enhanced resistance to multiple isolates of both rice blast and bacterial blight. The lesion mimic phenotype in the *spl11* mutant is due to a U-Box/Armadillo repeat protein with E3 ubiquitin ligase activity (Zeng et al., 2004). Yin et al. (2000) found correlation between the lesion development on leaves and the activation of several defense-related genes and enhanced resistance of the *spl11* mutant to pathogens.

This study reports the third gene (*GF14-e*) responsible for a lesion mimic phenotype that showed correlation with enhanced resistance against a virulent strain of *X. o pv. oryzae*. The *GF14-e* silenced plants are a good resource to understand cell death regulation in rice. Future studies will involve the discovery of potential GF14-e protein-interactors in rice as well as testing those silenced lines for broad-spectrum resistance. We will test the hypothesis that the *Arabidopsis* AKR2 ortholog in rice is one of the interactors of this GF14-e protein and whether this interaction is important in regulation of genes involved in oxidative stresses such as APX3 in rice. The presumed homozygous lesion mimic *GF14-e* silenced T₁ transgenics in rice are stunted with low survival rates (Figure 4.6) which may result from the overproduction of toxic components such ROS- (H_2O_2) and phenolic compounds. This accumulation may reflect the down regulation in the enzymatic activities of genes associated with scavenging of reactive oxygen species such genes encoding *ascorbate peroxidases*, *catalases*, and *glutathione S-transferases*. Because the accumulation of ROS- (such as H_2O_2) is known to induce genes involved in SAR, such as *PAL* encoding for a key enzymes in the secondary metabolism of phenolic compounds, these silencing *GF14-e* lines may overexpress genes in this pathway that will lead to a constitutive production of toxic phenolic compounds such as phytoalexins. The accumulation of ROS- and phenolic compounds may be responsible for the necrotic lesions in the rice leaves (lesion mimic phenotype) in these silencing *GF14-e* lines.

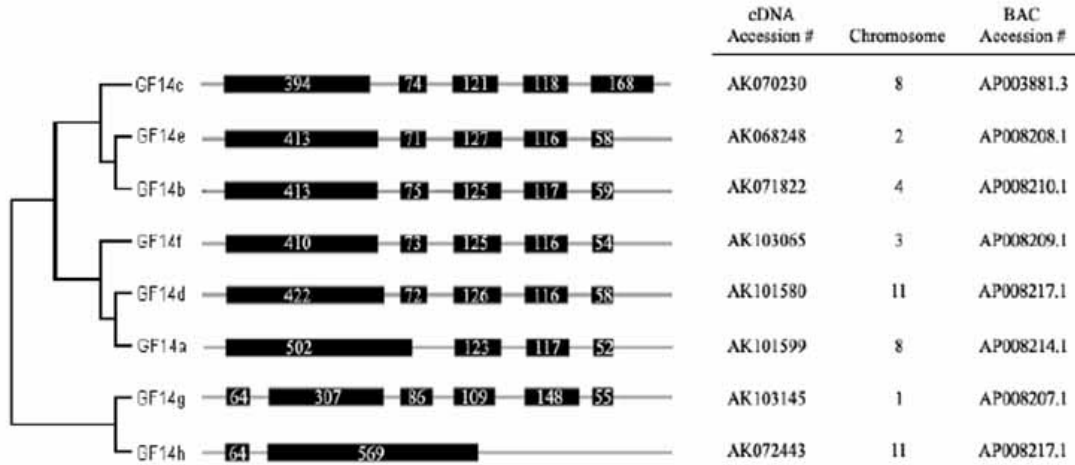
The practical question is whether the resistance conferred by a rice lesion mimic line such *GF14-e* could be used to improved resistance against multiple diseases. These *GF14-e* lesion mimic lines showed complete resistance against the virulent strain of bacterial blight pathogen different from the partial resistance produced for the other rice lesion mimics reported in rice (Yin et al., 2000). One problem of using this lesion mimic will be related to the cost of

constitutive expression of defense response genes. For example, the stunted and poor-surviving phenotype of the putative homozygous *GF14-e* suppressed lines may be a reflection of this loss of fitness. Yin et al. (2000) showed that relative to the parent IR68, near-isogenic *sp111* mutant lines grew slower, flowered about 1 week later, and had smaller panicles. Thus, the practical use of the *GF14-e*-suppressed lesion mimic lines identified in this study will depend on whether the undesirable pleiotropic effects of lesion mimic can be uncoupled from enhanced disease resistance by recombination or genetic engineering. If not possible, this suppressed line will still be a valuable tool to understand the cell death pathway in rice.

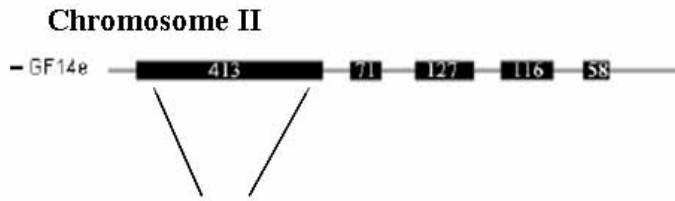
Tables and Figures Chapter 4

Figure 4.1 *GF14-e* selected to RNAi is a member of a family of eight gene members.

350 bp corresponding to the first exon of *GF14-e* was selected as trigger for RNAi using pANDA RNAi vector for rice *Agrobacterium* transformation.



(Chen *et al.*, 2006)



350 bp trigger region in pANDA RNAi vector for rice (Shimamoto, *et al* 2003)

Figure 4.2 Multiple sequence alignment of the GF14-e sequence that was used as an RNAi trigger with the sequences corresponding to the other GF14 gene members

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PM6      1  -CACGGTGAGGAGAATGTGTACATGGCAAGCTT---GCAGAGCAGGCCGAGAGGTATGAGGAGATGGTT
GF14-e   1  ---CGGTGAGGAGAATGTGTACATGGCAAGCTT---GCAGAGCAGGCCGAGAGGTATGAGGAGATGGTT
GF14-b   1  -TCCCGGTGAGGAGAATGTGTACATGGCAAGCTC---GCTGAGCAAGCCGAGAGGTATGAGGAGATGGTT
GF14-c   1  GTCCTCGGTGAGGAGAATGTGTACATGGCAAGCTG---GCCGAGCAGGCCGAGAGGTATGAGGAGATGGTT
GF14-f   1  -TCCCGGTGAGGAGAATGTGTACATGGCAAGCTT---GCCGAGCAGGCCGAGAGGTATGAGGAGATGGTT
GF14-d   1  GACGAGCGGAGGAGAATGTGTACATGGCAAGCTG---GCCGAGCAGGCCGAGAGGTATGAGGAGATGGTT
GF14-a   1  -ACGAGCGGAGGAGAATGTGTACATGGCAAGCTG---GCCGAGCAGGCCGAGAGGTATGAGGAGATGGTT
GF14-g   1  -ATGCAATTTGATCAAC-TGTCCATTCTTGTGGA---GATTTTTCATCCATGT-TCCAGAGATGGTTG
GF14-h   1  -----ACAAACATGTTGACTTTGCTCTGCTTTAAACITTTGACATCAACCAGGTTTTACCAATGATG

PM6      67  ---GAGTTCATGGAG--AAGGTGGC---TAAGACACT-TGACTC-----T---GAGGAGCTCA
GF14-e   65  ---GAGTTCATGGAG--AAGGTGGC---TAAGACACT-TGACTC-----T---GAGGAGCTCA
GF14-b   67  ---GAGTTCATGGAG--AAGGTGGC---CAAGACACT-TGACTC-----T---GAGGAGCTCA
GF14-c   68  ---GAGTTCATGGAG--AAGGTGGC---AAAGACACT-AGATCT-----G---GAGGAGCTCA
GF14-f   67  ---GAGTTCATGGAG--AAGGTGGC---AAAGACACT-TGACTC-----T---GAGGAGCTCA
GF14-d   68  ---GAGTTCATGGAG--CGGTGGCCCGCCGCGGG---GGCCGCTCCGGCGGG---GAGGAGCTCA
GF14-a   67  ---GAGTTCATGGAG--AAGGTGGT---GAGCCGCG---CGCCGGGGCGGGGGT---GAGGAGCTCA
GF14-g   66  ---GAGGCAATGAAT--AGTGTGGC---AAAG---TAGAC-----T---GAGGAGCTCA
GF14-h   63  CTAAGCTTTTCATCAATCAAGATTAAACATACCCAGCCATGACTCATGTGTCAATCCTCCAGGCACTAA

PM6      113  CCGTTCGAGGAGCCGAAACCTTCTATCCGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-e   111  CCGTTCGAGGAGCCGAAACCTTCTATCCGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-b   113  CCGTTCGAGGAGCCGAAACCTCCTCTCTGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-c   114  CCGTTCGAGGAGCCGAAACCTCTTCTCTGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-f   113  CCGTTCGAGGAGCCGAAACCTGCTTCTCTGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-d   126  CCGTTCGAGGAGCCGAAACCTGCTTCTCTGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-a   122  CCGTTCGAGGAGCCGAAACCTGCTTCTCTGTTGCCATACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-g   106  CAAAGGAGGAGAGGAAACCTCCTCTCAGTTGGCTACAGAATGTTATTGGTGCCTCCCGTCCGCTCATG---
GF14-h   133  CTAATGCTGACCTTTTTT-TGCTCACAGT---ATCCAAAAC---TTATAGGTACCCTCATGACTATAGTTT

PM6      180  -CGGCATCATATCATCCA--TTGAACAGAAGGAGAGAGC--CGTGGT--AATGAGGATCCTTCACACT
GF14-e   178  -CGGCATCATATCATCCA--TTGAACAGAAGGAGAGAGC--CGTGGT--AATGAGGATCCTTCACACT
GF14-b   180  -CGCATTTATCTCTCCA--TTGAGCAGAAGGAGGAAAGC--CGTGGT--AACGAGGACCGTTCACACT
GF14-c   181  -GCTATTCTCTCATCCA--TTGAACAGAAGGAGAGAGC--CGTGGC--AATGAGGACATGTTACTCT
GF14-f   180  -GAGGATCATCTCTCTCA--TTGAGCAGAAGGAGGAGAGC--CGTGGG--AATGAGGATATGTTACTCT
GF14-d   193  -CGGCATCATCTCTCCA--TTGAGCAGAAGGAGAGAGC--CGCGGG--AACGAGCCCAAGCCGCCAC
GF14-a   189  -CGGCATCTCTCTCCA--TTGAGCAGAAGGAGGAAAGC--CGCGGC--GCGCGGCAAGCCGCCCGCC
GF14-g   173  -GCTATCATATGATCCA--TTGAGCTAAGGAGGAGAGCA--AAGGGC--AAGGAGATCATGTAAGGCA
GF14-h   197  TGCATCAATGCTCCAAAAGTTTCAGAAATAAGACTAATTATCATATTTAAAGGCAATATGATACATA

PM6      243  CATCAAGGAATACAG---GGGAAGATTGAAACTGAGCTCTCCAAGATCTCTGATGGCATCCTCAAGCTT
GF14-e   241  CATCAAGGAATACAG---GGGAAGATTGAAACTGAGCTCTCCAAGATCTCTGATGGCATCCTCAAGCTT
GF14-b   243  CATCAAGGAATACCG---TGGCAAGATGAGACTGAGCTCACCAAGATTGCGGACCGCATCTCAAGCTG
GF14-c   244  GATCAAGGAATACCG---TGGCAAGATGAGACTGAGCTGAGCAAGATTGCGGATGGTATCCTGAAGTTG
GF14-f   243  AATTAAAGGAATACCG---TAGCAAGATTGAAACTGAGCTCAGCAAGATCTCTGATGGTATCCTGAAGTTG
GF14-d   256  CATCCCTCTCTACAG---GGGCAAGATTGAGGCTGAGCTCCCCATCTGCGAGGATCCTGCCCCCTG
GF14-a   252  CCGCCCTCTCTACCG---CGCCCTCTGAGGCTGAGCTCTCCAAGATCTGCGGGGATCTCCCTCTG
GF14-g   236  GACTCTCTGATATCG---TCCGAAAGTTGAGGCTGAGATGATAAATCTGCTGTGATTCATAAACAT
GF14-h   267  TATATCTGATAAATAAAGGGAAGGCTTCTAATAAGCA--AGGATTTGAAAGCTGATCTGTGCAAGCAC

PM6      310  CTTGACTCCCACCTTGTGCCCTTGCATCCACTGCTCCAGAG---TCCAAG
GF14-e   308  CTTGACTCCCACCTTGTGCCCTTGCATCCACTGCTCCAGAG---TCCAAG
GF14-b   310  CTTGAAATCCCACCTTGTGCCCTTTCCTACTGCCCTTGCAG---TCCAAG
GF14-c   311  CTTGACTCCACCTTGTGCCCTTGCATCTACTGCTGCAGAA---TCTAAG
GF14-f   310  CTGGATTCCCACCTTGTGCCATCTGCCACTGCTGCAGAG---TCCAAG
GF14-d   323  CTCGACTCCCACCTGTCGCCCTCGGCCGGCGCCGCGAG---TCCAAG
GF14-a   319  CTCGACGAGCCCTCTGTCGCCCGCGCCGCGCGCTGAC---GCCAAG
GF14-g   303  AATTGACAACTACTTTTCCACATCTCTCTGAGGCTCAG---AGTCA
GF14-h   336  AATTGACT--TAACTGTATTATTATCTAATCTGCAACAGAAAAGCAAG

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Figure 4.3 RNAi silencing of *GF14-e, c, and a* genes in T₀ Kitaake transgenic lines was detected using RT-PCR with gene specific primers for each family member.

RT-PCR results from uninoculated plants were compared with an untransformed Kitaake line (W). Amplification of the hygromycin gene indicated the presence of the transgene. *EF1-alpha* and ubiquitin genes were amplified as internal controls.

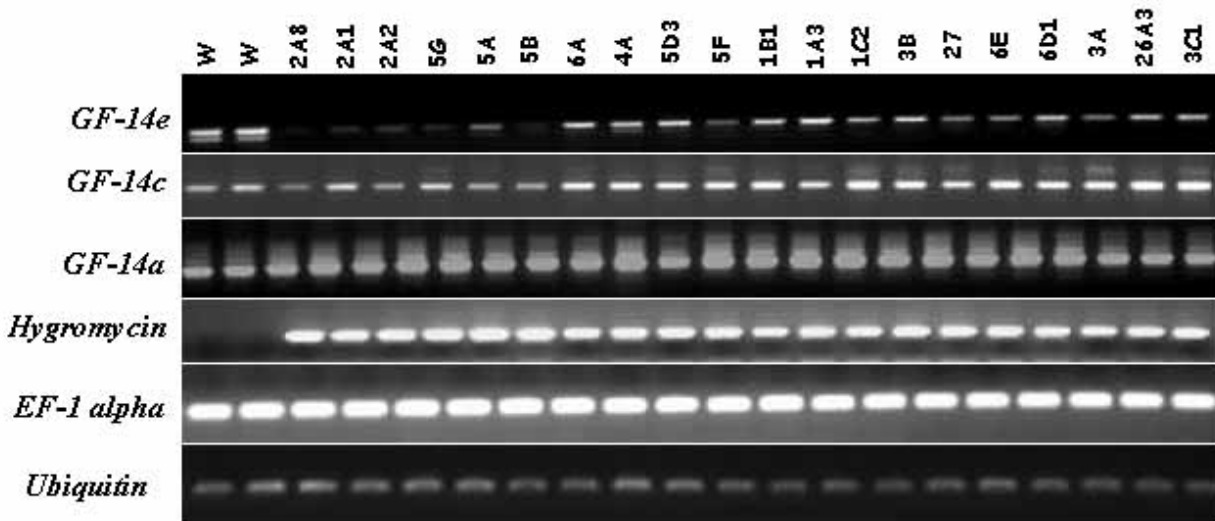


Figure 4.4 Lesion mimics (LM) phenotype in T₀ and T₁ *GF14-e* suppressed lines.

Spontaneous lesion mimic appeared in the T₀ *GF14-e* suppressed line (*Tt*) and in its T₁ *GF14-e* suppressed segregant progeny lines. This phenotype did not appear in wild type Kitaake lines, in T₀ *OsOXL* suppressed lines, and in the segregant T₀ lesion mimic that did not have the transgene (*TT*)

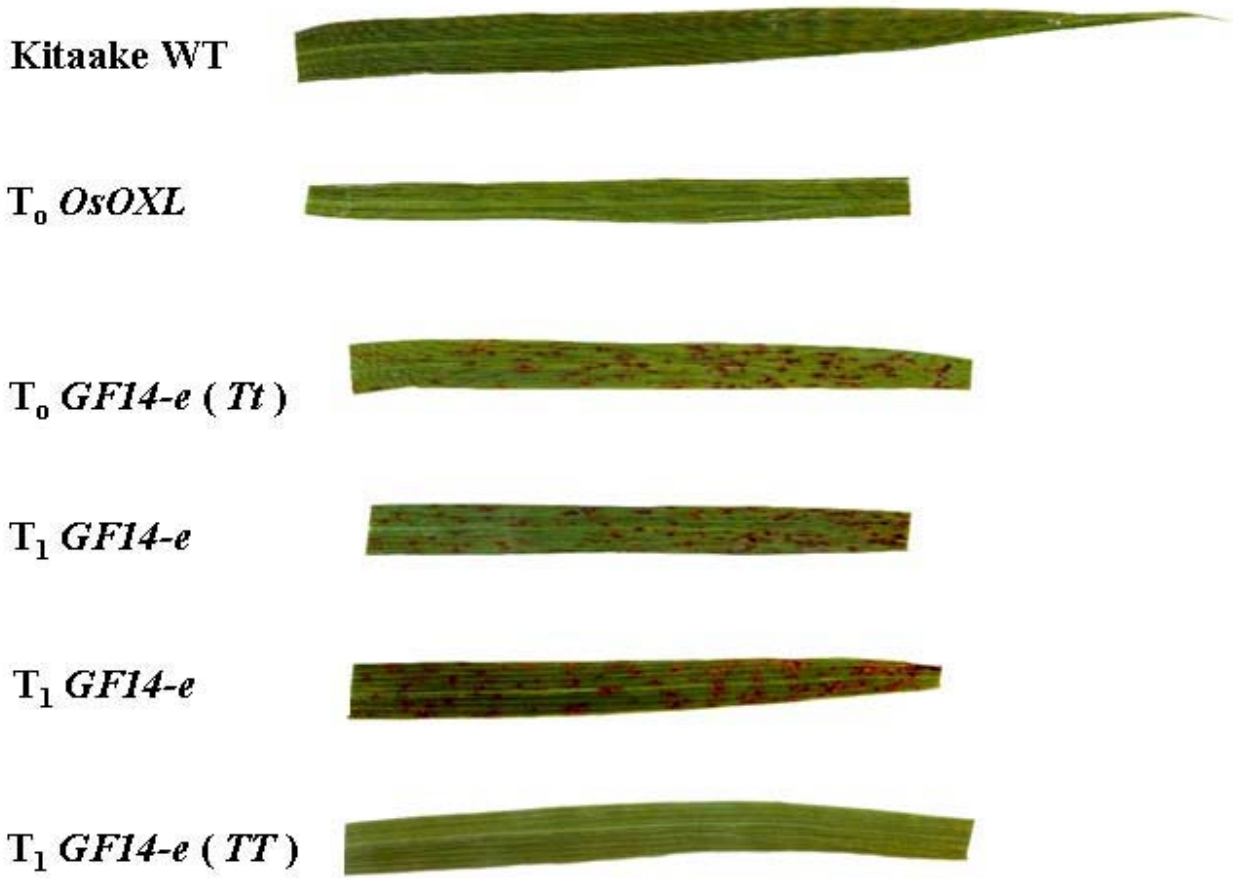


Figure 4.5 Intensity of lesion mimic phenotype in T₀ transgenic lines correlates with the level *GF14-e* silencing.

Level of silencing of *GF14-e* gene member in several T₀ transgenic lines were compared with untransformed Kitaake plant (W). Lesion mimic phenotype (LM) was score as Y (lots of lesion distributed whole leaf), +/- (lesion but not many), and N (no lesions). *EF1-alpha* was used as an internal control for RT-PCR.

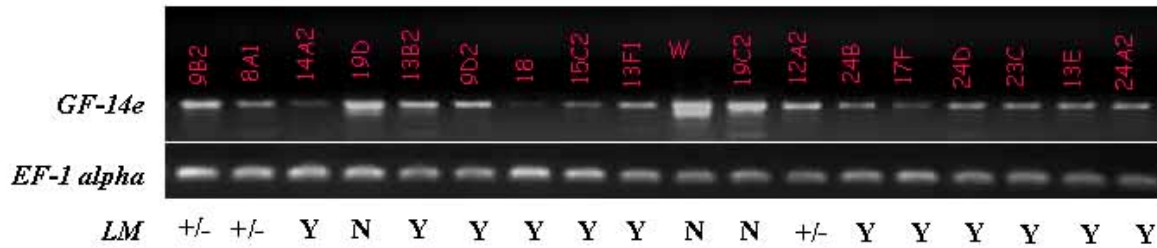
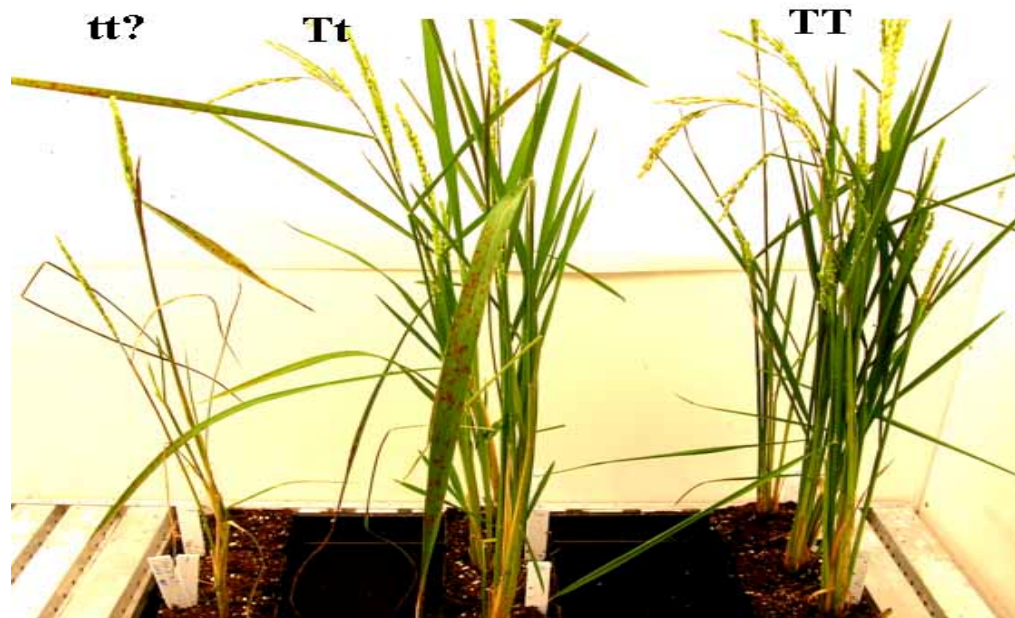


Figure 4.6 Lesion mimic phenotype in T₁ *GF14-e* suppressed segregant lines.

This figure shows three phenotypic classes among these T₁ lines. *TT* lines do not have lesion mimic and do not amplify the transgene, *Tt* lines that have the transgene and have lesion mimics, and a small group of plants that have the transgene and lesion mimics but are stunted, wimpy and did not produce seeds (putative *tt*).



T₁ *GF14-e* suppressed segregant lines

Figure 4.7 Bacterial blight lesions between lesion mimics and no lesion mimic T₁ segregants.

Typical bacterial blight lesions developed in the second youngest leaf in the main (M2) and lateral (L2) tillers inoculated with PXO99 from T₁ transgenic lines that did not segregate for lesion mimic phenotype (NLM). T₁ segregants showing lesion mimics (LM) did not develop bacterial blight symptoms in any of the inoculated leaves.

Bacterial blight lesions in lesion mimic and no lesion mimic T₁ *GF14-e* lines



Figure 4.8 *GF14-e* gene is a negative regulator of resistance against bacterial blight.

T₁ segregants were inoculated with an avirulent strain of *Xanthomonas oryzae* pv. *oryzae*. Four leaves were inoculated in each plant: The second youngest leaf from the main (M2) and lateral (L2) tillers and the third youngest leaf from the main (M3) and lateral (L3) tillers. Statistical analysis were performed using SAS to determine significant differences between means in lesion lengths between lesion mimics (LM) segregants and no lesion mimic (NLM) segregants; the probabilities are showing in the graphic.

T₁ *GF14-e* suppressed segregant lines inoculated with PXO99

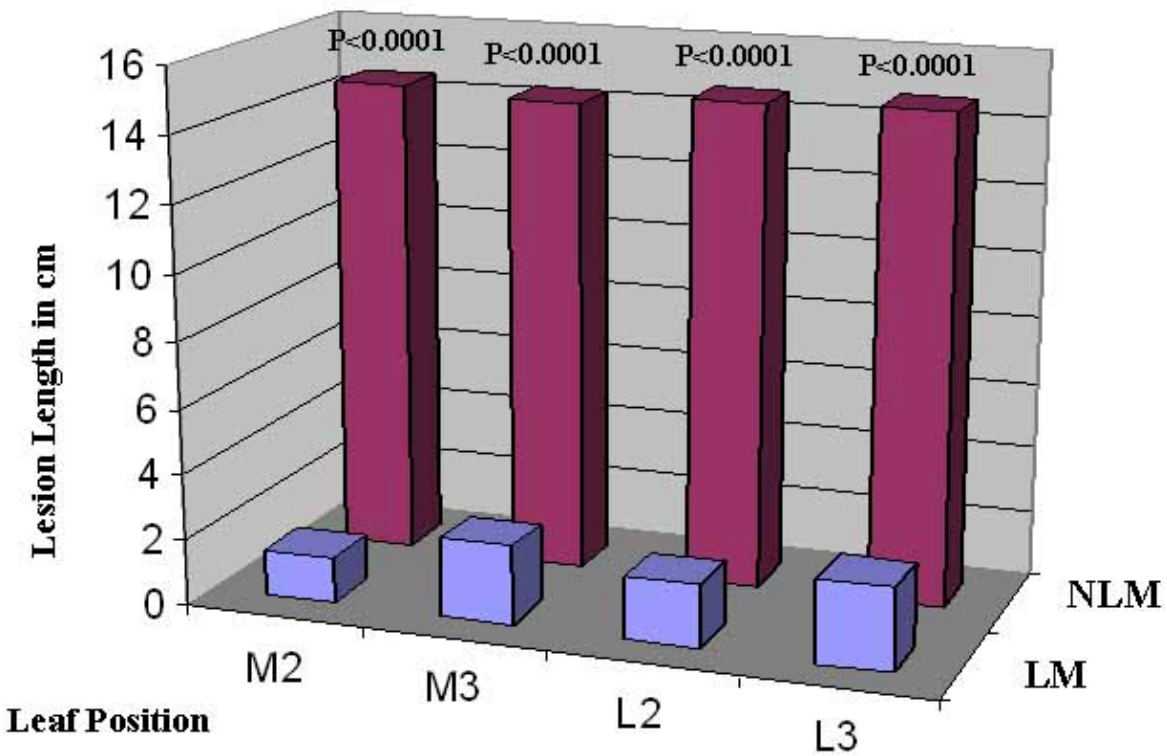
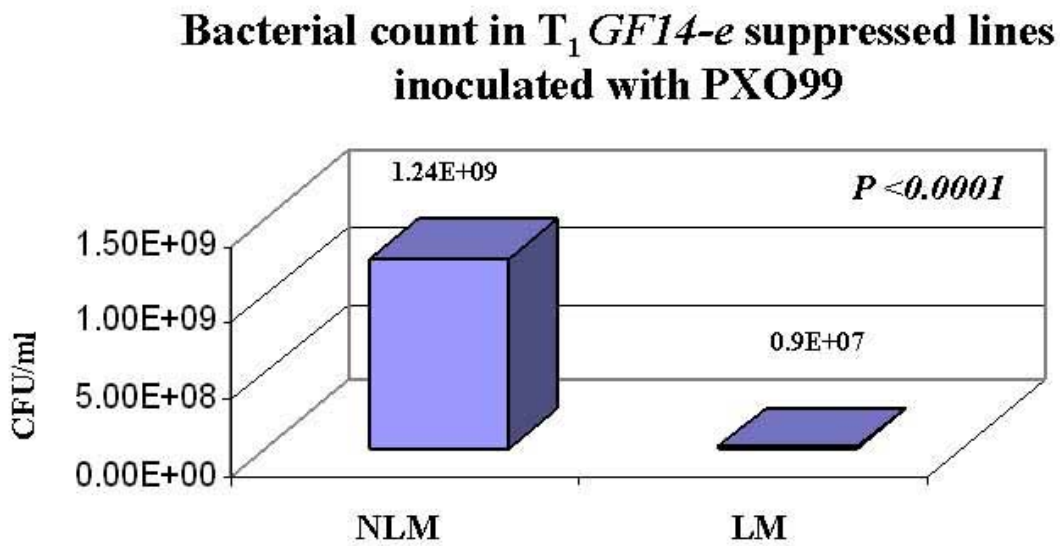


Figure 4.9 *GF14-e* suppression slow down the growth of *Xanthomonas oryzae* pv. *oryzae*.

Bacteria were counted in the second leaves inoculated (L2) in 10 T₁ lines showing resistance and lesion mimic phenotype, and 10 T₁ line showing susceptibility and no lesion mimics. Statistical analysis were performed using SAS to determine significant differences between means in the number of colonies counts between lesion mimics (LM) segregants and no lesion mimic (NLM) segregants; the probability are shown in the graphic.



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CONCLUSIONS AND REMARKS

In this study, I assessed the contribution of three candidate DR genes that were previously shown to co-localize with disease resistance QTL regions. Accumulation of QTL for disease resistance is a particularly appealing strategy, because this resistance is predicted to be durable and broad spectrum. The QTL focused on in this study governed resistance to three major rice diseases, including rice blast (chromosome 2 and 8), bacterial blight (chromosome 2) and sheath blight (chromosome 2). Given the demonstration here that *OsPAL4* and several *OsOXL* genes contribute to disease resistance, these genes will provide good markers for use in the accumulation of QTL through a breeding program.

DR genes frequently exist as gene families, and often the genes are arranged in clusters. There are 12 *OsOXL* clustered on chromosome 8 that co-localize with a disease QTL. Our findings show that no single member of the *OsOXL* family accounts for the resistance; rather multiple members likely function together to inhibit disease. *OsOXL3*, 6, 7, 8, and 9 are the most important contributors. Four *OsPAL* gene members are clustered on chromosome 2 in a region where a disease QTL has been mapped. Including this study, there are now several lines of evidence that implicate the contribution of at least two *OsPAL* members in this cluster with disease resistance. This information is very valuable for breeders, since they need to select for specific gene members in their programs. Usually these gene members are found in cluster arrangements which would facilitate the introgression of the whole cassette into their favorite susceptible rice lines to improve resistance against these major rice diseases to sustain rice production. Our studies also indicated that different rice varieties use different gene family members; however, it appears that gene members with the highest contribution are used by several rice varieties.

Although I have shown that the genes function in disease resistance, in these and prior studies we have not determined what the difference is between the ‘good’ alleles contributed by resistance lines such as IR64 and SHZ-2 and the ‘bad’ alleles present in the susceptible lines Azucena and LTH. We do not know if it is earlier or enhanced expression of a particular gene

family member or combination of members, if there is a critical difference in the cellular location or function of the encoded enzymes, or if the expression differences are due to differences in their promoter regions. Analysis of the sequences of the genes and promoter regions from IR64, SHZ-2, Azucena, and LTH are in progress. This analysis will identify single nucleotide polymorphism (SNP) signatures or promoter differences that will help us to discriminate between “good” and “bad” alleles among gene family members. Thus, at this point in time, the recommendations to breeders for accumulation of the genes are to focus on introduction of the clusters of *OsPAL* and *OsOXL3-11* genes from resistant donor parents such as SHZ-2 and IR64 rather than to focus on particular family members.

The *OsOXL* genes are likely major contributors to the QTL on chromosome 8 for rice blast resistance. However, it is also possible that other genes underlying the QTL also contribute to the resistance measured for the QTL. One of the hypotheses about the candidate genes controlling a broad spectrum disease resistance QTLs is that those genes are presumably defense response genes involved in the general basal defense response and are downstream of pathogen recognition. Several lines of evidence, including the results of this study, support this hypothesis. First, *PAL* and *OsOXL* genes are involved in general basal defense responses such as reinforcement of the cell wall, the oxidative burst, production of antimicrobial compounds, and induction of SAR. These defense responses are involved in both compatible and incompatible interactions, and SAR is a broad spectrum type of resistance. Thus, genes involved in these processes are good candidates to explain disease resistance QTLs. Our hypothesis would be that the cluster of alleles in SHZ or IR64 may function more effectively, but that the clusters in the susceptible lines do have some function in basal resistance.

Second, if genes such as *OsOXL* play an important role in basal defense responses, it would not matter which rice variety we used to determine their contribution to resistance. We used Kitaake, a japonica rice variety, as a host for suppression of the *OsOXL* gene members. The *OsOXL* genes are arranged in the same fashion in Kitaake as in the resistance QTL lines (SHZ, IR64). Although we had not previously mapped disease resistance QTL in Kitaake, because the *OsOXL* genes contribute to basal resistance, suppression of gene members in Kitaake also resulted in enhanced susceptibility to *Mg*.

Third, we demonstrated that most of the gene members of a specific rice gene family have functional redundancy and are tightly linked in their chromosomal positions, this is in agreement with the concept of the genes controlling QTL traits, i.e., that QTLs are controlled by several genes that have small additive effects. The prediction is that QTLs provide durable resistance because it is difficult for the pathogens to overcome this resistance since they have to overcome several gene members (at least four for *OsPAL* and eight for *OsOXL*) at the same time. It is hard to prove that only these genes are responsible for the QTL governed resistance. However, our work indicates clearly that minor genes like *OsOXL* do contribute to disease resistance QTL in plants.

One of the appeals of incorporation of disease resistance QTL into rice cultivars is the prediction that the QTL will confer broad spectrum resistance. The evidence provided here and elsewhere with *OsOXL* genes is consistent with QTL containing these genes as being broad spectrum. First, the genes are involved in basal defense responses, which are effective against several types of pathogens and pest. Second, there is increasing literature that correlates these GLPs with resistance against fungi, bacteria, viruses, and insects in several pathosystems. Finally, in our preliminary data, the *OsOXL* suppressed lines showed increased susceptibility to sheath blight (*Rhizoctonia solani*) and aphids, which indicates that those genes indeed confer broad spectrum disease resistance.

A DEB mutant line harboring a deletion in an *OsPAL* gene family member (*OsPAL4* on chromosome 2) was discovered using a PCR-based screening technique. This DNA-pooling, PCR-based screening technique allows the rice deletion collections to be used efficiently for reverse genetics. The pooling strategy decreases the amount of PCR that needs to be done and increasing the pool size will increase the efficiency of this PCR-pooling strategy. This will be true considering the number of deletion rice lines that we need to screen to detect deletions in all the target genes with at least 95% of confidence. One of the weaknesses of this technique is the fact that we needed to run polyacrylamide gels to detect polymorphisms associated with the deletion mutations. New strategies for mutant detection need to be tested to be able to screen a larger number of rice deletion mutants.

The *ospal4* mutant line identified here will be a valuable resource to study the defense related pathways associated with phenylpropanoid pathways. Expression analysis of other genes in the same pathway and closely related defense response pathways will be done to determine the effect of the mutation in *OsPAL* on other defense response genes. Also if the mutant line accumulates lower levels of salicylic acid (SA) relative to the wild type lines, since *PAL* is the first enzyme in the SA biosynthesis, the *ospal4* line would be a good resource to study SAR in rice. Rice has constitutively high levels of SA, so this mutant will be useful to determine the SA requirements in rice to induce SAR.

One of the problems encountered during these studies was the fact that in several cases homozygous mutant lines could not be recovered. For example, the homozygous recessive *ospal4* is likely lethal because no homozygous recessive line was found in the M3 and M4 generations. Transmission of the mutation to the progeny is impaired (1:1 ratio). Mutant pollen transmits the mutation, ruling out male gametophytic lethality. However, female gametophytic lethality has not been ruled out, and needs to be tested. Lines suppressing the expression of *OsOXL* and *GF14-e*, which are involved in general processes, also exhibited fitness costs such as low seed production, stunting, weakness, and low survival. In spite of the fitness costs, all of the variants, including the *ospal4* mutant lines and the *OsOXL* and *GF14-e* suppressed lines, were maintained as heterozygotes.

According to our results involving RNAi gene suppression, a highly conserved region of *OsOXL* used as trigger dsRNA could silence most of the *OsOXL* gene family members, but with different efficiencies. The silencing was conserved through the T₁ generation, but is reduced in the T₂ generation. The gene suppression is correlated with sequence homology, but other unknown factors also influence the process of RNAi gene silencing of gene family members. In this study we used two different silencing vectors, one based on 5' transitive RNAi (pTSi-1) used in the *OsOXL* gene suppression, and the pANDA vector based on a sense-antisense DNA trigger region used in the *GF14-e* suppression. Our results were in agreement with the fact that transitive RNAi silencing plays a role in the non-specific silencing of mRNA targets, because we were able to silence most of the gene members on chromosomes 8 and 12, but not the members on

chromosome 3. By using the pANDA vector we silenced specifically and only the *GF14-e* gene, which is the member for which we designed the target DNA trigger region. However, unlike the *OsOXL* family suppression, using this pANDA vector we did not silence *GF14-c*, which is the most closely related gene family member of *GF14-e*. These results suggest that some vectors can silence closely related gene family members, but that the efficiency varies. This is a valuable observation for scientists that want to silence gene specific members or the whole gene family members.

Suppression of the *14-3-3* gene *GF-14e* results in lesion mimic phenotype in T₀ and T₁ transgenic lines and the degree of silencing is correlated with the number of lesions in the T₀ suppressed lines. This *GF14-e* suppression did not result in enhanced susceptibility to rice blast, but rather enhances resistance against a virulent strain of *Xoo*. In *Arabidopsis 14-3-3* genes are involved in the regulation of oxidative bursts by controlling genes that encode ascorbate peroxidases and proton pumps. In rice, it is possible that *GF-14e* is also involved in the regulation of oxidative genes that are involved in general defense responses. Experiments are underway to test this hypothesis by looking for putative interactor proteins and analyzing the expression of genes involved in oxidative stress such as genes encoding peroxidases, ascorbate oxidases, glutathione S-transferases, and catalases in the *GF14-e* suppressed lines. The fact that these suppressed lines show lesion mimic phenotypes indicates a local accumulation of toxic compounds such as H₂O₂ that results from the downregulation of genes that encode H₂O₂ scavengers. We will also test if these resistant lesions mimic transgenic lines constitutively express defense response genes such *PAL*, *PR-1* and *OsOXL*. The *GF14-e*-suppressed lesion mimic line will be a good resource to test for broad spectrum resistance against the major rice diseases.

As a general conclusion, all of the candidate DR genes studied here have a role in disease resistance. The *phenylalanine ammonia lyase (OsPAL4)* on chr.2 and *oxalate oxidase-like (OsOXL)* gene members on chr. 8 contribute to quantitative resistance against bacterial blight and rice blast, respectively. In contrast, *GF14-e* is a negative regulator for programmed cell death (PCD) and for resistance against bacterial blight.