DISSERTATION

DEFENSE RESPONSE SIGNALING FOR DISEASE RESISTANCE IN RICE

Submitted by

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ABSTRACT

DEFENSE RESPONSE SIGNALING FOR DISEASE RESISTANCE IN RICE

Plant disease resistance is often associated with a type of programmed cell death (PCD) called the hypersensitive response (HR). Upon recognition of pathogen proteins or their activity, the affected cell and surrounding cells commit to the HR to limit pathogen spread throughout the plant. This mechanism of plant disease resistance renders a pathogen avirulent on the host plant. Lesion mimics (LM) are a class of mutant or transgenic plants that spontaneously show lesions resembling the HR in the absence of biotic stress. Based on the association of the LM phenotype to cell death and its similarity to disease symptoms and the HR, this phenotype is a useful tool to dissect and understand the plant defense response.

To identify genes that when mutated result in the LM phenotype in rice, we used a microarray approach. By hybridizing labeled genomic DNA from an allelic series of deletion mutants to an oligonucleotide microarray, we identified candidate genes and genic regions that were deleted in a set of mutants. For one mutant, *spl1*, mutations in a cytochrome P450 gene were confirmed to confer the LM phenotype. A genome browser developed to handle these microarray data is a community resource that enables researchers to rapidly identify untagged deletion mutations in rice.

Members of the 14-3-3 protein family were recently shown to be positive regulators of cell death and the HR in Arabidopsis. In contrast, the work herein shows that a rice 14-3-3 protein is a negative regulator of cell death and resistance. Transgenic plants carrying a construct that silences the rice 14-3-3 gene *GF14e* exhibit a LM phenotype and enhanced resistance to two distinct rice pathogens, *Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia solani*. These *GF14e*-silenced plants also showed enhanced expression of genes associated with salicylic acid (SA) mediated defense responses, including members of the peroxidase gene family. The *GF14e*-silenced plants did not show enhanced expression of marker genes associated with the ethylene response pathway, indicating that *GF14e* may negatively regulate SA mediated defense responses, but does not affect ethylene regulated responses.

Silencing *GF14e* results in the upregulation of several defense responsive peroxidases. cDNA from *GF14e* silenced plants was used in quantitative PCR (qPCR) to assay expression of four peroxidase genes. Of these, three showed significant upregulation in 2 weeks after sowing (WAS) and 5 WAS *GF14e*-silenced plants. The promoters of the three upregulated genes (*PO-C1*, *Pox8.1*, *Pox22.3*) contain at least one W-box element. In contrast, the peroxidase (*Pox5.1*) that did not show upregulation and is not upregulated in *R* gene mediated responses had no W-box elements. W-box elements are binding sites for the WRKY class of transcription factors. This result, coupled with bioinformatic predictions of potential rice 14-3-3 clients, and the observation that some WRKY genes are upregulated in *GF14e* silenced lines indicates that *GF14e* may negatively regulate WRKY transcription factors related to cell death and defense responses.

Based on the implication that one 14-3-3 protein negatively regulates defense responses related cell death, provides a framework to develop a model for how this protein might function in the plant disease resistance repsonse.

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CHAPTER 1

DEFENSE RESPONSE SIGNALING FOR DISEASE RESISTANCE IN RICE

Plant disease resistance: a molecular arms race

Plants are constantly bombarded by pathogens in their natural environment and have been in constant competition with their pathogens throughout time. The evolutionary dynamics of host-pathogen systems has been likened to a molecular arms race [1, 2]. Adaptations of plants for resistance are overcome by changes in pathogen population structures, which in effect are alterations of the tools required for successful colonization and evasion of detection by the host plant. However, plants have continued the fight during their coevolution with their pathogens. The greatest difficulty for the host is the speed of evolution, as fast growing pathogens alter their armory at a much faster rate than plants can adapt.

Preformed defense - structural barriers, antimicrobials

The first lines of defense for plants are structural or constitutive defenses. For example, the leaf surface is covered in a waxy cuticle that impedes pathogen ingress [3]. To avoid this barrier, many pathogens utilize natural openings in the leaf surface, such as stomates or hydathodes [4]. Alternatively, some fungal pathogens use enzymes to breach this surface [5], while others use hydrostatic force to physically rupture the cuticle and

underlying epidermis [6]. Other preformed defenses include constitutively produced small antimicrobial secondary metabolites that may be present on or near the leaf surface, such as the phytoalexin sakuranetin, which constitutively produced in the leaves of black currant plants and has been shown to have antifungal properties [7]. These compounds prevent microbial growth or even kill potential pathogens indiscriminately [8]. If the pathogen breaches these barriers, inducible defense responses follow.

Induced defense - PAMP triggered immunity and pathogens fight back - effector triggered susceptibility

Plants have evolved the ability to recognize molecules that are conserved among classes of microbes. Pathogen associated molecular patterns (PAMPs) are recognized by plant cell surface receptors called pattern recognition receptors (PRRs) that serve as the next line of pathogen defense at the cell surface [9]. PAMPs small molecular motifs conserved within a class of microbes, and include bacterial flagellin subunits [10] and chitin derived from the fungal cell wall [11]. When PRRs bind a PAMP, the signal is relayed across the cell membrane into the cytoplasm. A signal cascade alerts the cell to the potential danger outside, activating a defense response that can stop the microbe from successfully colonizing the plant. This form of resistance is called PAMP triggered immunity (PTI).

PTI is not specific to any particular pathogen, rather it acts against classes of organisms, some of which may be pathogenic, such as fungi and bacteria [12]. There are interesting aspects of PTI that have been described. For example, plants have the ability to monitor

the integrity of their cell wall. Many mechanisms have been proposed for the molecular method of monitoring, including detection of cell wall degrading enzyme products, and perception of the mechanical integrity of the wall where it is in contact with the cell membrane [13]. Pathogens well-suited to their hosts have evolved ways around PTI. By injecting 'effector' proteins directly into the host cytoplasm, they can alter or block the PTI signal, preventing it from reaching the nucleus, and thereby deactivating PTI. As this can result in a successful colonization event, it is termed effector triggered susceptibility (ETS) [12].

Gene-for-gene resistance - effector triggered immunity

In the 1940s, H. H. Flor first described the 'gene-for-gene' model for plant disease resistance [14]. Simply stated, Flor's model evolved to suggest that if a plant gene product recognizes a pathogen gene product, then immunity is triggered in the plant. As research progressed, the plant genes, called resistance genes (*R* genes), were identified, and many were shown to encode proteins of a similar class, called nucleotide binding-leucine rich repeat proteins (NB-LRR). It was first suggested that the pathogen effector may bind the R protein directly through the LRR domain. However, only a few studies demonstrated direct interactions [15]; in most cases, attempts to show direct interactions were unsuccessful. Protein-protein interaction studies later showed that instead of binding the effector directly, usually the R protein monitored the status of plant proteins targeted by the effector. This led to the guard hypothesis of *R* gene function [16]. Since multiple effectors may target the same protein and a single effector may target multiple genes, monitoring or "guarding" the state of a single critical factor in PTI signaling is a

good approach to resistance. Other research suggests that R proteins may monitor the state of a decoy protein that is structurally similar to effector targets [17]. In either event, upon recognition of changes in targeted proteins, a signal cascade is activated that ultimately results in resistance. Often, this response includes a form of programmed cell death called the hypersensitive response [18].

Incorporating research on the different methods of plant disease resistance has led to the proposal of the 'zig-zag' model [12] (Figure 1). Gene expression studies have shown that many of the genes induced during PTI and ETI overlap, though there are differences in amplitude of induction [19]. Understanding how these two forms of immunity are similar and how they differ requires an understanding of the signaling components utilized during these resistance events.

Disease resistance signaling is a complex process that can begin at the cell surface (PTI) or within the cell (ETI). As ETI was the first type of disease resistance to be discovered, much of the research in plant disease resistance signaling is in this area. However, there is significant overlap between PTI and ETI signaling, with a majority of the difference being speed and amplitude of expression responses. Figure 2 illustrates part of the current understanding of ETI signaling [20]. Also important to the regulation and propagation of defense response signaling are the phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) [21]. SA primarily modulates defense responses to biotrophic and hemibiotrophic pathogens [22], while the JA and ET pathways generally mediate resistance responses to wounding and insects [23]. The SA signaling pathway is

generally antagonistic to JA and ET signaling [24]. Using this information, defense signaling through specific pathways can be elucidated. Below, components of disease signaling that are particularly relevant to this dissertation are discussed.

14-3-3 proteins

An interesting component of the defense signaling pathway (relative to this dissertation) are the 14-3-3 family of proteins. 14-3-3 proteins are ubiquitous in all eukaryotes. They function as homo- or heterodimers by binding phosphorylated client proteins, altering the client's activity, stability or subcellular localization [25] (Figure 3, [26]. One 14-3-3 protein, TFT7, was first described as a defense signaling component in tomato [27]. TFT7 positively regulates defense signaling by binding a bacterial elicitor protein and activating a component of a MAPK signaling cascade. The interaction was shown to be necessary for induction of the HR as mediated by R-gene complex *Pto* and *Prf*. Similarly, the interaction of an Arabidopsis 14-3-3, GF14lambda, with an ankyrin repeat is essential for the activity of an ascorbate peroxidase [28]. In this case, in the absence of any of the components, the defense response is partially compromised [28]. Of eight 14-3-3 isoforms in rice, named *GF14a-h* [29], function has been demonstrated only for one [30, 31]. *GF14c* regulates multiple metabolic processes, including flowering and phytohormone signaling [30, 31]. Intriguingly, GF14c binds two different transcription modulators, *OsBZR1* and *OsHd3a*, keeping them from entering the nucleus [30, 31], and limiting their ability to activate transcription. Upon upstream signaling specific to the responses controlled by these transcriptional modulators, they are dephosphorylated, allowed to dissociate from the GF14c complex and translocate to the nucleus, where

transcriptional activation of their specific target genes occurs [30, 31]. In other systems, induced expression of 14-3-3 genes has been correlated with resistance, but beyond the reports above, little is known about how these interesting proteins function in resistance. [29, 32]. Chapter 2 and 3 of this dissertation will address the role for a rice 14-3-3 (*GF14e*) in defense responses.

Transcription factors

Another interesting components of the defense signaling pathways are transcription factors [33, 34]. Transcription factors are proteins that bind to specific DNA sequences and thereby control gene transcription. Some of the upstream signaling (from the plant surface or within the cytoplasm) is aimed at the activation of transcription factors that modulate the transcriptional changes that must take place during a successful defense response. An example are the TGA family of transcription factors, which when disrupted result in compromised defense responses and failure to upregulate defense response genes following treatment with salicylic acid (SA) [35].

Absent from the model in Figure 2 are the plant specific WRKY class of transcription factors. WRKY transcription factors generally bind W-boxes (C/T-TGAC-C/T) in plant promoters to modulate transcription of genes related to hormone signaling and stress responses (cold, drought, salinity and biotic stresses) [36, 37]. In rice, there are over 100 members of the WRKY transcription factor family. Many WRKY transcription factors are involved in defense responses, as both positive and negative regulators of resistance-related genes [37]. Rice transgenic lines overexpressing some WRKY transcription

factors show enhanced resistance to pathogens [38]. These overexpression lines have been used to place WRKY transcription factors in specific defense response pathways, notably as regulators of SA mediated signaling [39]. In chapter 3, we present a model that proposes WRKY transcription factors as positive regulators of disease resistance, and provide evidence that the WRKY transcription factors may interact with a rice 14-3-3 protein.

Downstream defense responses: Peroxidases and reactive oxygen species (ROS)

Members of the peroxidase gene family are upregulated during both ETI and PTI defense responses. Defense signaling often results in an oxidative burst of reactive oxygen species (ROS). These reactive molecules, which include superoxide, hydroxyl radicals and hydrogen peroxide, can have antimicrobial activities, act as important signaling molecules and serve as substrates for peroxidases [40]. Peroxidases catalyze the oxidation of a variety of substrates, often using hydrogen peroxide as a substrate in the reaction. Peroxidases related to defense are often secreted into the apoplast, where they are the last enzymatic step in cell wall fortification by catalyzing the polymerization of lignin, a relatively strong and largely insoluble polymer that adds strength to the cell wall [40]. This may function to impede pathogen progress. Overexpression of some peroxidases enhances resistance to necrotrophic and biotrophic pathogens [41].

Activation of peroxidases during defense responses illustrates an output of defense signaling. That is, activation and signaling through ETI or PTI pathways results in the upregulation of proteins that cause some change in cell structure or function that impedes

pathogen progress, be it through cell wall fortification, production of other antimicrobials or in the case of the HR, cell death. By identifying downstream effects of defense signaling, upstream signaling components can be placed in relevant pathways. In Chapters 2 and 3, peroxidases and other downstream defense response genes are used to predict the signaling pathways regulated by the rice *GF14e.*

Genetic approaches to studying defense responses

Lesion mimic mutants as tools to study plant defense pathways

The lesion mimic (LM) phenotype describes mutant or transgenic plants exhibiting disease-like lesions absent any obvious causative agent [42]. Broadly, genes whose disruption result in a LM phenotype can be called negative regulators of cell death. Conversely, genes whose ovexpression show this phenotype are positive regulators of cell death [43]. Many lesion mimics show changes in their response to pathogens, including enhanced resistance to normally virulent pathogens [44]. For example, a collection of rice lesion mimics (Figure 4) was shown to exhibit varying levels of resistance to pathogens [45]. Lesion mimic mutants might be disrupted in various steps of cell death signaling pathways. For example, induction and suppression of defense-related genes that are markers of the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signaling pathways was observed for the rice lesion mimic mutants (Figure 5, Bruce, unpublished). The differences in responses to various pathogens, and the differences in activation of defense-related pathways suggest the value of lesion mimic mutants in examining defense response signaling to identify components of different signaling pathways.

The lesion mimic phenotype can be the result of disruption of many functional classes of genes, including signaling, metabolism, transcription, and protein stabilization [46-49]. Not all may have a direct effect on defense signaling, but the cell death phenotype and altered resistance to pathogens suggests a role for these genes in defense. For example, the Arabidopsis gene *LSD1* encodes a protein that negatively regulates a transcription factor that binds the promoters of defense response genes. In *lsd1* mutants, this regulatory mechanism is disrupted, leading to constitutive activation of the defense response and cell death [48]. In rice, lesion mimic *spl11* encodes an E3-ubiquitin ligase, demonstrating a role for protein degradation in the defense response [49]. Overexpression of genes can also lead to a lesion mimic phenotype. Constitutive overexpression of Arabidopsis gene *NPR1* results in a LM phenotype [50].

In Chapter 1, a technique to identify deleted genes (in particular, those responsible for lesion mimics), and the discovery of a gene responsible for a lesion mimic phenotype in rice are described.

Transgenic alteration of gene expression

Transgenic silencing or overexpression of genes that are predicted to function in defense signaling are also useful approaches to studying defense signaling pathways. By examining transcript profiles from plants undergoing defense reactions or examining genes that colocalize with disease resistance quantitative trait loci (DR-QTL), candidate genes can be identified that contribute to defense or are involved in defense signaling. By silencing or overexpressing these genes, clues to their function can be uncovered.

Transgenic alteration of gene expression can be especially useful in cases where full loss-of-function mutations are lethal.

One example of the use of transgenic alteration of gene expression was a study to determine the role of genes in disease resistance. The rice germin-like proteins (GLPs) colocalize with a disease resistance quantitative trait locus (QTL) [51]. By silencing these genes using RNAi, Manosalva et al [52] demonstrated that the GLPs contributed to resistance to different types of pathogens, including both necrotrophs and biotrophs. Other studies using a similar transgenic RNAi approach have demonstrated defense functions for other candidate disease resistance genes [53, 54]. Overexpression of a 14-3-3 gene in Arabidopis that had been shown to interact with an R-gene resulted in enhanced disease resistance [55]. 14-3-3 proteins, as described above, are predicted to play a major role in defense signaling [27, 29, 32]. In Chapters 2 and 3, RNAi silencing is used to study the roles of a 14-3-3 gene in rice defense responses.

Scope of dissertation

Because of the complexity of interacting pathways involved, dissecting the pathways leading to disease resistance requires a multipronged approach. In this work, two types of mutants were studied to understand rice defense response pathways, plant lesion mimic mutants derived by chemical and radiation mutagenesis, and plants silenced for a predicted regulator of plant defense.

Research goals

1. To develop a method for discovery of deleted genes in rice deletion mutants (Chapter 2). Deletion mutants are a resource of great value for understanding the contribution of genes to phenotypes. They are inexpensive and rapidly produced, however the deleted genes can be difficult to identify because, unlike insertion mutagenesis using transposons or *Agrobacterium* T-DNA, deletions mutations are not tagged. To add value to deletion collections and enable gene discovery, I explored a method using genomic DNA hybridizations to oligonucleotide microarrays to identify deleted genes and genomic regions.

2. To determine if the rice 14-3-3 gene *GF14e* contributes to the defense response (Chapter 3). Expression of *GF14e*, which encodes a rice 14-3-3 isoform, is enhanced during rice defense responses to *Xanthomonas oryzae* pv. *oryzae* (bacterial leaf blight). To evaluate if *GF14e* contributes to disease resistance, a transgenic RNA interference (RNAi) approach was used to silence the gene expression.

3. To understand how GF14e functions in the defense response (Chapter 4). Using a transgenic *Gf14e* -silenced line and rice undergoing ETI, the expression of genes characteristic of different defense response pathways was assessed. WRKY elements computationally predicted to interact with GF14e were evaluated in yeast two hybrid studies. The promoters of defense genes differentially regulated in *Gf14e*-silenced lines were scrutinized for WRKY regulatory motifs.

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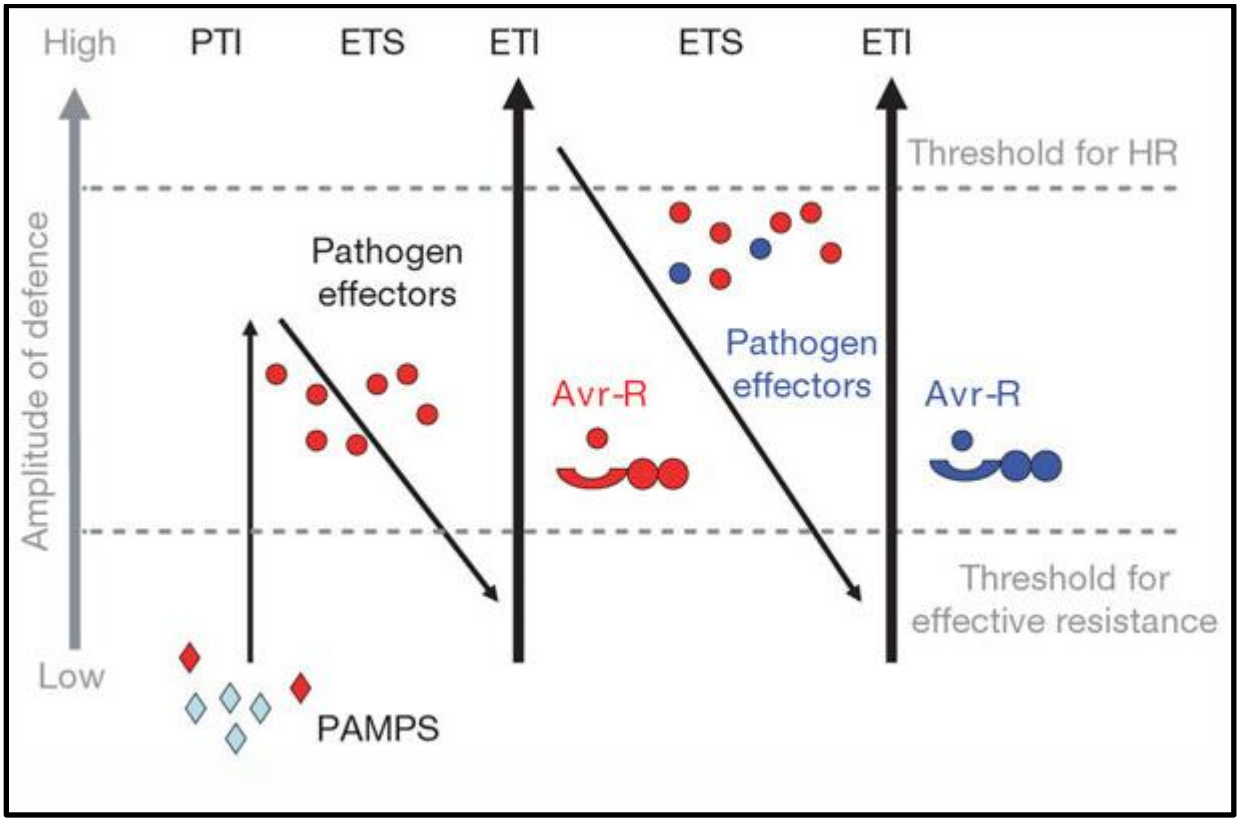
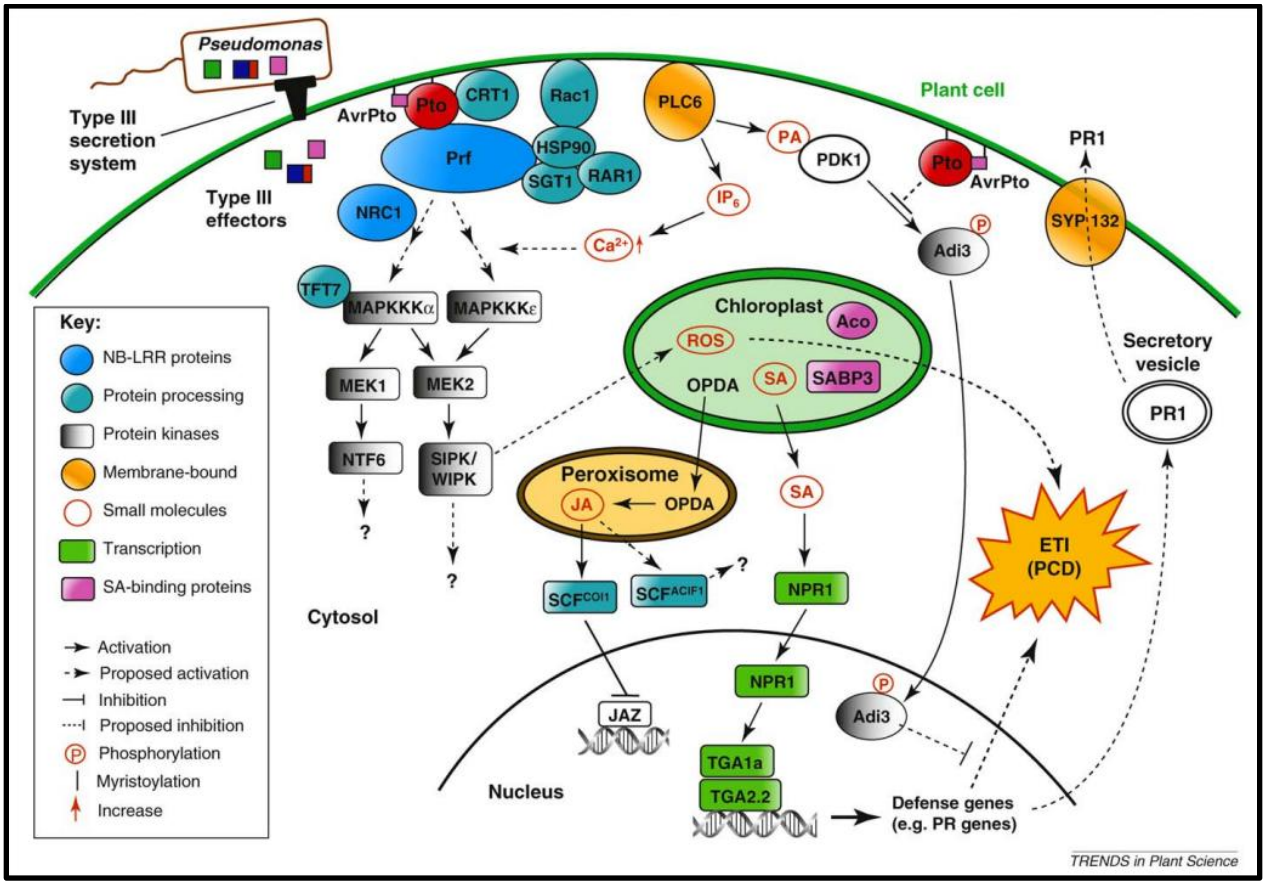
Figure 1. The 'zig-zag' model of plant immune responses



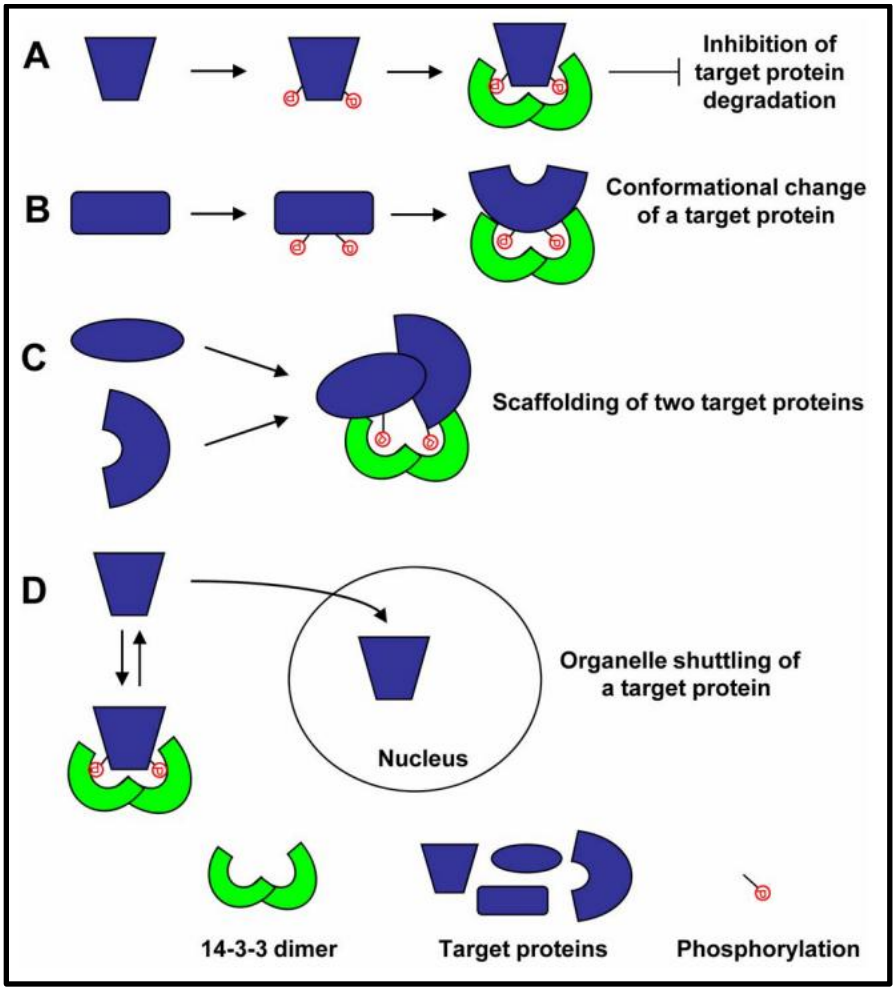
The model describes thresholds of induction and their relation to the amplitude of defense responses. Pathogen associated molecular patterns (PAMPS) are recognized by pattern recognition receptors (PRRs), activating PAMP triggered immunity (PTI). Pathogen effectors secreted or injected into the cell block PTI signaling, inhibiting defense signaling and resulting in effector triggered susceptibility (ETS). Effectors recognized by resistance proteins (R) activate signaling for effector triggered immunity (ETI) and often a hypersensitive response (HR). These signals can also be blocked by effectors, resulting again in ETS. Other R proteins (blue) may recognize effectors blocking signals from the red R protein, activating ETI and HR. From Jones and Dangl [12].

Figure 2. ETI signaling can result in programmed cell death



Receptors and signaling components in the Pto/Prf-mediated tomato (*Solanum lycopersicon*) resistance response to *Pseudomonas syringae* pv. *tomato* bacterial pathogen. Effector activities recognized by the Pto/Prf complex activate signaling through mitogen activated protein kinase (MAPK) cascades, though the downstream targets have not been recognized. Of relevance to this dissertation is TFT7, a 14-3-3 protein required for activation of MAPKKKα. Eventual signaling consequences include activation of defense response gene expression by the TGA family of transcription factors and eventual effector triggered immunity (ETI) and programmed cell death (PCD). From Oh and Martin [20].

Figure 3. Influence of 14-3-3 proteins on their phosphorylated client proteins

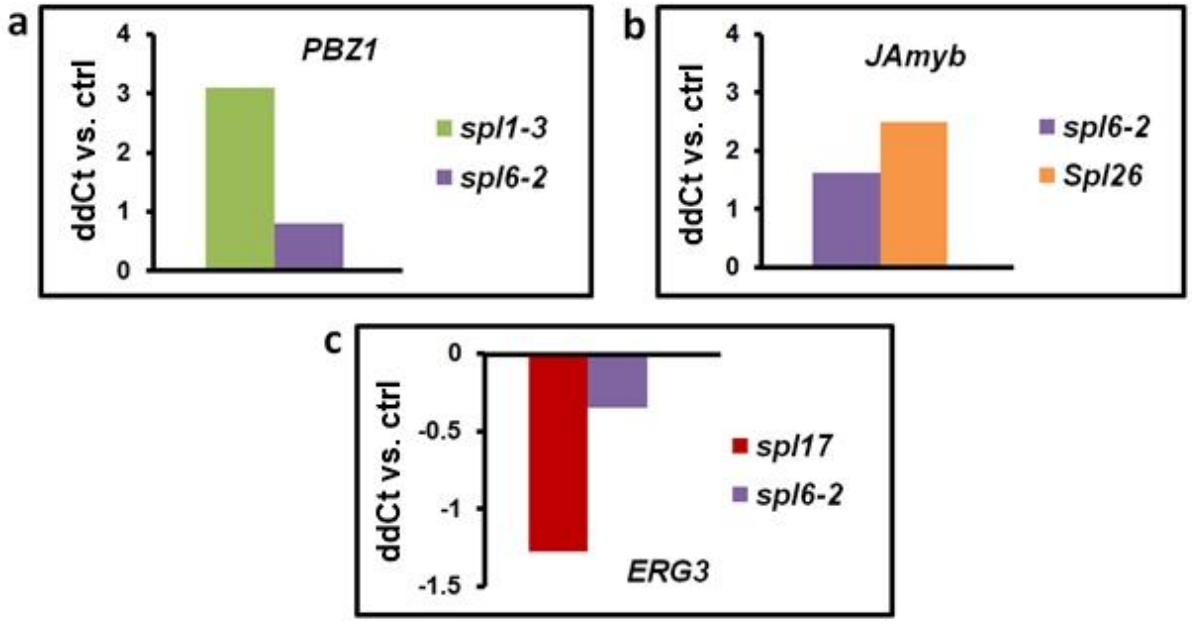


A versatile class of proteins, the 14-3-3 family has a range of effects on its bound phosphorylated client protein. These functions include but are not limited to a) preventing a client protein from being degraded when in the phosphorylated state, b) causing a conformational change in the client, exposing buried domains, c) acting as a scaffold for two different proteins, bringing interacting domains into close proximity and d) changing the subcellular localization of a client protein. From Oh [26].

Figure 4. Rice lesion mimic mutants show various cell death phenotypes

Rice lesion mimic mutants were generated from rice cultivar IR64 by chemical (diepoxybutane) and physical (fast neutron, gamma ray) mutagenic treatments. a) The mutants exhibit a range of cell death phenotypes and varied responses to pathogens. b) *spl6-2* x *spl17* double mutants display a more severe lesion mimic phenotype than the single mutants alone. From Wu et al. [45].

Figure 5. Lesion mimics show induction and suppression of defense signaling genes



RNA was from 45 day old lesion mimic and wild type plants and used to generate cDNA. qPCR was performed using gene-specific primers for a) *PBZ1*, a gene induced during SA-mediated responses, b) *JAmyb*, a jasmonic acid-related myb-family transcription factor and c) *ERG3*, a rice gene responsive to ethylene production and signaling. Delta-delta Ct (ddCt) was calculated as described in chapters 3 and 4 using the internal control *EF1alpha* and wild type IR64 (Bruce, unpublished).

CHAPTER 2

DETECTION OF GENOMIC DELETIONS IN RICE USING OLIGONUCLEOTIDE MICROARRAYS

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Author's contributions

MB, JB, GLW, HL, and JEL conceived, designed, and supervised various aspects of the study. MB, JB, and MGD performed the DNA preparations and microarray processing, and MB carried out all PCR validations. AB performed genetic analysis of mutants and NS performed TILLING analyses. MB, AH, RM, HL and JEL contributed to the analysis

and interpretation of the data. MB, HL and JEL drafted the manuscript. All authors read and approved the final manuscript.

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Abstract

Background: The induction of genomic deletions by physical- or chemical- agents is an easy and inexpensive means to generate a genome-saturating collection of mutations. Different mutagens can be selected to ensure a mutant collection with a range of deletion sizes. This would allow identification of mutations in single genes or, alternatively, a deleted group of genes that might collectively govern a trait (e.g., quantitative trait loci, QTL). However, deletion mutants have not been widely used in functional genomics, because the mutated genes are not tagged and therefore, difficult to identify. Here, we present a microarray-based approach to identify deleted genomic regions in rice mutants selected from a large collection generated by gamma ray or fast neutron treatment. Our study focuses not only on the utility of this method for forward genetics, but also its potential as a reverse genetics tool through accumulation of hybridization data for a collection of deletion mutants harboring multiple genetic lesions.

Results: We demonstrate that hybridization of labeled genomic DNA directly onto the Affymetrix Rice GeneChip® allows rapid localization of deleted regions in rice mutants. Deletions ranged in size from one gene model to ~500 kb and were predicted on all 12 rice chromosomes. The utility of the technique as a tool in forward genetics was demonstrated in combination with an allelic series of mutants to rapidly narrow the genomic region, and eventually identify a candidate gene responsible for a lesion mimic phenotype. Finally, the positions of mutations in 14 mutants were aligned onto the rice pseudomolecules in a user-friendly genome browser to allow for rapid identification of untagged mutations http://irfgc.irri.org/cgi-bin/gbrowse/IR64_deletion_mutants/.

Conclusions

We demonstrate the utility of oligonucleotide arrays to discover deleted genes in rice. The density and distribution of deletions suggests the feasibility of a database saturated with deletions across the rice genome. This community resource can continue to grow with further hybridizations, allowing researchers to quickly identify mutants that harbor deletions in candidate genomic regions, for example, regions containing QTL of interest.

Background

Mutants are critical tools for forward and reverse genetic approaches to dissect biochemical and metabolic pathways, and to determine gene function in plants. In the past few years, several strategies have been used to develop different rice mutant collections [1]. Although large collections of mutant lines were generated using T-DNA, *Ac*/*Ds*, and transposon insertions [1-3], they are limited to *japonica* rice varieties which are more amenable to transformation and regeneration than *indica* varieties. This is unfortunate, as *indica* varieties represent the predominant rice type grown in the world (~80%) and harbor many interesting traits important for rice production [4].

Genomic deletions induced by chemical and irradiation mutagens provide a rapid method to obtain a large mutant pool [5]. Advantages to these types of mutants are that they are relatively inexpensive to produce, any genotype can be used because there is no need for transformation, and the density of mutations generated allows for genome-wide saturation with relatively small populations. In rice, a collection of over 40,000 mutants induced by various chemical and irradiation strategies was developed in the *indica* rice cultivar IR64 [6]. IR64 was chosen because it is the most widely grown *indica* rice in Southeast Asia and because it contains a large number of valuable agronomic characteristics. The variety of mutagens was selected to ensure a collection with a range of deletion sizes, providing the opportunity to identify a mutation in a single gene or a deleted group of genes that might collectively govern a trait (e.g., quantitative trait loci, QTL). However, as the mutations in this collection are not tagged, time and labor intensive mapping strategies are needed to identify genes conferring interesting phenotypes. Alternative strategies for identifying untagged mutations have evolved in

rice, with varying levels of technological difficulty and efficiency [7-12]. PCR-based strategies for reverse genetics use complex pools of mutant genomic DNA and PCR to detect deletions in genes of interest [7, 8, 11, 12]. An example in rice is the 'deletagene' approach [8]. This approach requires an *a priori* hypothesis of what gene might be deleted. Further, it requires the design of flanking PCR primers that would amplify across a range of deletion sizes, because the size of the deletion and the number of genes in the deleted region would not be known. Targeting induced local lesions in genomes (TILLING) provides a reverse genetics technique to detect point mutations in genes of interest [9, 10], but the detection and characterization of moderate to large deletions in rice remains tedious. None of these techniques are suitable for forward genetic screens.

With the completion of the rice genome sequencing projects and advances in microarray technology, comprehensive oligonucleotide microarrays are now available that can be used to discover genetic polymorphisms and deleted genes. Hybridization of genomic DNA to Affymetrix arrays has been used to discover single feature polymorphisms in Arabidopsis [13], rice [14], and barley [15]. Solid-support DNA arrays have been used for detection of deletions in the genome of Arabidopsis [16]. In addition, genomic DNA was hybridized to citrus spotted cDNA expression arrays to detect two hemizygous deletions induced by fast neutron in citrus [17]. Successful use of arrays for discovery of mutated genes is dependent on the proportion of the genome covered by the array, the size of the deletion (relative to the amount of coverage of an individual gene on the array), the complexity of the target genome. A key advantage of array hybridization is their potential for use in both forward and reverse genetics.

Our goal was to determine if oligonucleotide microarrays could be used to detect deletions mutations in rice, which has a genome size of 389 Mb [18], about three times the size of Arabidopsis. In a preliminary study, we used a proprietary custom Affymetrix oligonucleotide array [19] based on the Syngenta draft sequence of *Oryza sativa* ssp. *japonica* cv. Nipponbare [20], to show that hybridizing genomic DNA from mutants to oligonucleotide arrays could be used to identify known deleted regions in IR64, and therefore facilitate gene discovery (unpublished data). Although the chip was originally designed for use in expression-based experiments, the design was also ideal for genomic deletion detection because of the density of oligonucleotide probes for a given gene model (~11 probe pairs per gene model). The release of the Affymetrix Rice GeneChip®, which contains probe sets representing more than 50,000 transcripts (http://www.affymetrix.com/support/technical/datasheets/rice_datasheet.pdf) now provides a publicly available platform for hybridization-based deletion discovery.

In this study, we demonstrate the utility of the Affymetrix Rice GeneChip® to discover deleted genes in rice. We describe a proof-of-concept experiment wherein we used hybridization intensity changes relative to wild type on a probe-by-probe basis to detect a known deletion on chromosome 5 in an IR64 mutant [6]. We demonstrate the utility of the technique as a tool in forward genetics in combination with an allelic series of mutants to rapidly narrow the genomic region and eventually identify a candidate gene responsible for a lesion mimic phenotype *spl1* (spotted leaf 1). Finally, we align the positions of deletions in a total of 14 mutants onto the rice pseudomolecules in a user-friendly browser. The density and distribution of the deletions suggests the feasibility of creating a database describing a collection of available deletions in the genome. This

community resource can continue to grow with further hybridizations, allowing researchers to quickly identify mutants that harbor deletions in candidate genomic regions containing QTL of interest. Previously reported array hybridization methods have focused on characterizing single feature polymorphism [13-15] or to identify deletions in forward genetics approaches [16, 17, 21]. We focus not only on the utility of this method for forward genetics, but also its potential as a reverse genetics tool through accumulation of hybridization data for a collection of deletion mutants harboring multiple genetic lesions.

Results and Discussion

Oligonucleotide microarray-based identification of deleted gene regions

The Affymetrix Rice GeneChip® contains more than 55,000 probe sets representing 48,564 gene models based primarily on version two of the *japonica* cv. Nipponbare rice annotation provided by The Institute for Genomic Research (TIGR) and 1,260 *indica* transcripts (http://www.affymetrix.com/support/technical/datasheets/rice_datasheet.pdf). Although the oligonucleotides for the arrays were designed based primarily on *japonica* sequences and the IR64 mutant collection used in this study is an *indica* variety, all of our comparisons are based on changes in hybridization signals relative to the wild type IR64. Thus, differences in hybridization of *indica* rice DNA to *japonica* rice arrays are masked in the comparison.

To determine the efficiency of Rice GeneChip® arrays to identify genomic deletions, we first analyzed the distribution of probes along the coding sequences. The data show a 3'

bias in coding sequence representation (Figure 1). This is not unexpected as the array is designed to query expression data. However, promoters, introns and 5' genic regions are not or are less frequently queried in genomic DNA hybridizations as a result of the chip design, and deletions in these areas are thus less likely detected. Tiling arrays will likely provide better coverage of these regions.

Array diagnostics and normalization

Prior to data analysis, the hybridization data was subjected to several diagnostic analyses. These include examination of variation in signal intensity, proportion of "present" calls, and any spatial anomalies (smudges, streaks, patches of extremely high or low signal) among the arrays [22]. Any arrays with a strong deviation from the wild type were discarded from the analysis. After passing diagnostics, a scale normalization of the data was performed. The $\log_2$ perfect match (PM) probe signals for each array were scaled such that the average for each array was the same as that for wild type. A benefit of this normalization method is that adding arrays to the analysis does not affect other arrays in the normalization scheme.

In preliminary studies, we tested the application of background correction to the data. However, while the background correction led to a higher power of detection, it also resulted in a higher false positive rate. This is because the background correction exaggerates probe level differences. While this is not a problem in "standard" microarray analysis, where probe values are summarized into a probe set summary statistic representing gene expression, it is a problem where probes are treated individually as in

our analysis. For example, after background correction, roughly 3% of probes have a $\log_2$ ratio of -1 or less, but without background correction, less than 1% of probes have a $\log_2$ ratio of -1 or less. Thus, a background correction was not applied.

Array hybridizations for detection of deleted regions

To reduce the costs associated with array-based deletion discovery, we explored the use of unreplicated hybridization data. The proposed analysis makes use of the multiple probes contained in each probe set. During the development phase, we performed replicate hybridizations of DNA from two different rice mutants (G650 and F1856), and determined that the use of a stringent log ratio [=$\log_2$ (mutant PM probe signal intensity/wild type PM probe signal intensity)] cutoff for a high proportion of probes within a probe set was almost equivalent to the use of a p-value cutoff. In addition, False Positive Rates (FPR1 and FPR2, based on two different methods of estimation described in Table 1) and True Positive Rates (TPR) were calculated to establish the parameters for calling deletions (Table 1). The FPR and TPR were determined from PCR-confirmation of deletions and non-deletions predicted from 14 array hybridization experiments. For example, using a log ratio cutoff $\leq$ -0.8 for at least 50% of probes in a probe set, we observed an FPR1 of 0, an FPR2 of <0.0001, and a TPR of 0.767. While this information indicates that it is suitable to use a single array hybridization per mutant, replicates are recommended for the wild type line to ensure data consistency and allow for error rate examination based on wild type by wild type comparisons.

The analysis used for deletion discovery allowed flexibility, depending on the end-user's tolerance for false positives or negatives. In this study, the specific parameters (log ratio and proportion) were selected using Table 1 as a guide. The log ratio [=$\log_2$(mutant probe signal intensity/wild type probe signal intensity)] for each probe was first determined and log ratios for flagging probes were selected at less than or equal to -0.6 or -0.8. Probe sets that had more than a defined proportion of probes (0.4-0.5), i.e., those with a log ratio ≤ -0.6 or -0.8, were called as potential gene model deletions.

As an example of the process for detecting deletions, we hybridized genomic DNA from a rice dwarf mutant *d1* with a known deletion in the single copy *RGA1* gene, previously shown to be responsible for the dwarf phenotype [23], to a single array. The mutation was induced by gamma radiation and confirmed using PCR and DNA blot analysis (data not shown). We predicted a deletion on chromosome 5 that contains the gene model Os05g26890, the *RGA1* gene (Figure 2). Nine of eleven probes in the Os05g26890 probe set showed a log ratio ≤ -0.8, or a proportion of 0.82, identifying *RGA1* as deleted.

In addition to the deletion of *RGA1*, 44 other gene models were predicted to be deleted in the *d1* mutant line at log ratio ≤-0.8 for 50% or more of probes. An aggregation analysis was used to automate identification of genomic regions with an overrepresentation of gene models predicted to be deleted, and the models were mapped to a genome browser (http://irfgc.irri.org/cgi-bin/gbrowse/IR64_deletion_mutants/). The analysis revealed a large deletion in the *d1* line on chromosome 5 spanning 30 gene models including the *RGA1* gene (Figure 2). One end of the *d1* chromosome 5 deletion is predicted to fall between the two TIGR v5 loci Os05g26926 and Os05g27050. The other end of the

deletion could not be reliably predicted because of the presence of multiple adjacent repetitive elements. A second large deletion was detected in the *d1* mutant line on chromosome 2 (see browser).

In total, 14 rice mutants were screened using single array hybridizations and the putative deletions were mapped in a chromosome-by-chromosome display that shows the distribution of mutations across the 12 rice chromosomes (http://irfgc.irri.org/cgi-bin/gbrowse/IR64_deletion_mutants). The browser allows for selection of different log ratio cutoffs, providing flexibility in data analysis. For example, in the total set of mutants, for probe sets with 50% or more probes showing a log ratio less than or equal to -0.6, the number of putatively deleted gene models ranged from 2 to 359 (Table 2). At this stringency, putative deletions were detected in all mutant lines, though some lines had many more than others. In mutant (G282), a high number of deletions were detected (Table 3). Increasing the stringency to log ratio -0.8 for 50% or more probes in a probe set revealed 89 deleted gene models in G282, with 43 deleted gene models on chromosome 7, suggesting a large deletion. The large deletion on chromosome 7 in G282 was also predicted by aggregation analysis to contain 46 gene models (Figure 3). This number is greater that the total number of deletions detected on chromosome 7 because not all probe sets within the putative deletion showed log ratio and proportions above the threshold. The deletion was confirmed by PCR. Large deletions detected in other mutants are shown in the browser.

Identification of overlapping mutated regions to target gene discovery

The rice mutant lines induced by chemical or irradiation strategies likely contained multiple deletions in the genome. We tested if hybridization of DNA from multiple mutant lines that exhibit a phenotype of interest could provide convergent data to identify the mutated region responsible for the phenotype and limit that region to the fewest gene models. Four mutants exhibiting the *spl1* lesion mimic phenotype were selected as proof of concept. Two mutants had been genetically confirmed by complementation testing to be allelic at the *Spl1* locus (G650 and F1856). Two additional mutants were included that displayed the *spl1* phenotype, but had not been genetically confirmed (G9799 and F2045). For the four mutants, we predicted a total of 242 gene models to be deleted throughout the genome (Table 2). However, the four mutants showed overlapping deletions only on chromosome 12, and these deletions were located within the region where the *spl1* mutation was previously mapped [24] (Figure 4). Selected candidate gene models predicted to be deleted in this region were validated by PCR. Thus, with a total of four hybridization experiments (one per mutant line), the location of the mutation conferring the phenotype was narrowed to a 70 kb region (21 gene models) on chromosome 12.

Due to their small size, the diepoxybutane-derived (DEB) mutations were not reliably detected by array hybridization. However, they and ethylmethanesulfonate-derived (EMS) mutants were useful for confirming the location of the *spl1* gene after delimiting the mutation to a few gene models by array hybridization. TILLING experiments using E16923 (shows *spl1* phenotype) focused on gene models within the predicted deletion,

and, after sequencing, revealed a point mutation resulting in an in-frame, premature stop codon in the first exon of Os12g16720, a member of the cytochrome P450 gene family (Figure 5). Sequencing of the entire gene from two DEB-derived mutants D1137 and D2943 (confirmed to be *spl1* alleles by genetic complementation [25]) also showed single nucleotide polymorphisms (SNPs) in the Os12g16720 gene model. These SNPs were predicted to result in amino acid changes within the gene product that could cause the *spl1* lesion mimic phenotype (Figure 5).

Prediction of deletion sizes

We estimate that gamma ray and fast neutron produce both large (70 to 500 kb, Figures 2, 3, 5) and small deletions (see browser) within a single gene model. Sometimes, in what appeared to be large deletions, we observed apparently undeleted probe sets bracketed by deleted probe sets (e.g., in Figure 2 note the break in the deleted region in mutant *d1*). On closer inspection, several of these probe sets were found to be improperly mapped, gene family members or repetitive elements.

Limitations

Though array hybridization and analysis proved a powerful tool for identifying deletions in rice, a limitation to the use of this method is the difficulty in detecting deletions in gene family members and other repetitive elements. An advantage of using mutagens that result in large deletions is the possibility of detecting mutations knocking out tandem-duplicated gene family members – a difficult mutation to obtain by traditional mutagenesis methods. Additionally, during mutagenesis, it is possible for large

fragments of DNA to recombine in remote locations in the genome. Such a case has been demonstrated in the analysis of a gamma ray-induced mutation (G978), where a deletion event occurred on chromosome 12, followed by reintegration of part of the gene into a neighboring region of the same chromosome (N. Sugiyama, unpublished data). The hybridization technique reported here is not able to detect such rearrangements, as the genomic DNA is still physically present. Mapping strategies are better suited to detect these genomic rearrangements.

Smaller deletions were less reliably detected. Detection of small deletions is theoretically possible with reduced stringencies, but is limited by probe coverage of the gene models and deletion size. The Affymetrix Rice GeneChip® design is limited by the coverage of probes for a gene model (usually 11 25-mers) and the distribution of those probes over a gene (Figure 1). Using DEB to induce mutations at the *rosy* locus in *Drosophila*, Reardon, *et al.* [26] found that 43% of the mutants were deletions ranging from 50 bp to 8 kb. DEB has also been reported to induce point mutations [27]; we observed point mutations after DEB mutagenesis of rice (Figure 5).

Comparison with existing microarray detection methods

In other reports, expression data was used to detect genomic deletions [13]. We hybridized cDNA from the *spl1* mutant G650 to the Agilent Rice 22k Oligo expression array. This array represents approximately 22,000 rice genes with 60-mer oligonucleotides. Two genes, LOC_Os12g16540 and LOC_Os12g16720, were identified that were significantly down-regulated compared to wild type (see Additional file 3).

These two genes were also detected as deleted by hybridization of the genomic DNA to the Affymetrix arrays (Figure 4). Indeed, LOC_Os12g16720 is the gene model that we identified as *Spl1* (Figure 5). However, other genes shown to be deleted by hybridization of genomic DNA were not detected as deleted in the expression experiments. This is because relying on the absence of gene expression to detect a deletion assumes that the gene's expression would be detectable in wild type, which may not be the case. Using hybridization data from genomic DNA, all genes in wild type will be equally represented, regardless of mRNA expression level.

The experimental design reported here to detect deletions differs from previous studies, e.g., Gong et al [16], in several ways. First, our approach does not require development of advanced genetic populations. Second, because our goal was to develop a community resource, which maintains information on all deletions in genomes of each mutant, even those not contributing to a phenotype (see below), we did not use a pooling strategy to mask deletions unrelated to the phenotype. Preservation of information in a genome browser on all deletions in the same lines is important for researchers investigating the functions of other genes. Finally, we used a single hybridization per mutant to reliably detect deletions, and show the availability of an allelic series provides an advantage in quickly delimiting deleted regions responsible for a phenotype (Figure 4).

In addition to differences in the experimental design, the analysis reported here differs from other reports. Like Gong et al. [16], our calls are based on differences between individual perfect match probe intensities when comparing mutant to wild type arrays.

They relied on adjacent probes, while we relied on the proportion of probes within a probe set and an aggregation analysis. For a fixed log ratio threshold, if a proportion and number of adjacent probes are chosen such that both methods have the same TPR, the FPR level is frequently higher when using the adjacent probes criteria (Table 1 and Additional file 2). The higher FPR may occur because many of the probe sets on the array contain probes that overlap often by more than 10 bases. In a case where these overlapping probes represent a region of variable hybridization efficiency, a few overlapping (adjacent) probes may produce low signal, while the rest of the probe set does not. In this case, relying on the "adjacent probe" method for deletion detection increases the FPR. Finally, to accurately detect larger deletions, we also used an aggregation analysis to delimit the potential borders of deleted regions.

Feasibility of producing a deletion stock database for reverse genetics

Our long-term goal is to build a set of mutant lines with mapped deleted genomic regions that would serve the rice genetics community as a tool to study traits governed by multiple genes (QTL). Results from the analysis of the *d1* and *spl1* mutants demonstrate that deletions in multiple gene models are reliably detected by single array hybridizations. These non-target deletions detected in individual experiments, accumulated over time, can collectively provide a useful database for retrieving mutations in genes or regions of interest.

The data presented in this study suggest that it is feasible to develop a database of characterized mutants with deletions that span regions of interest in the genome.

Assuming a median of 38 deleted gene models predicted at 80% TPR based on the 14 mutants analyzed (616 deletions/14 mutants, Table 2) and an coverage of ~38,000 gene models using Affymetrix Rice GeneChip® (based on version 5 of the TIGR annotation, http://www.ricearray.org/matrix.search.shtml), there is a 91% probability of detecting a deletion in each gene model at least once using only 3,000 mutants. Currently, over 52,000 M_4 mutant lines are maintained at IRRI; of these, approximately 15,000 are gamma ray-induced and 8,000 are fast-neutron-induced [4]. Thus, the available mutant collection is sufficient for near-saturation deletion mapping, provided that resources are available for analysis. Since a single array hybridization produces reliable data, the high costs usually associated with array experiments are minimized. Additionally, this collection will allow researchers to identify deleted regions that have been associated with QTL, presenting the possibility of using the collection to analyze the contribution of genes to complex phenotypes.

Conclusions

This study demonstrates that deleted rice genes and genomic regions can be localized by hybridization of genomic DNA to oligonucleotide arrays. The approach is most reliable when used to detect mutations in single copy genes or large deletions, such as those produced by physical mutagens like gamma ray and fast neutron.

Materials and methods

Mutants used in the study

A total of 14 mutants were used in this study; all were from the populations of mutants induced by treatment of the *indica* variety IR64 with DEB-treatment, FN or gamma ray (GR) exposure and were advanced to M_4 or M_5 lines prior to the experiment. Mutant *d1* resulted from gamma ray mutagenesis and was confirmed to be deleted for the *RGA1* gene by DNA blot analysis. The lesion mimic mutants, *spl1*, also known as *sl* (Sekiguchi lesion) [28], included six mutant lines, four of which had been confirmed by complementation tests to be allelic at the *spl1* locus (D1137, D2943, G650 and F1856, DEB, GR and FN generated) and two genetically unconfirmed mutants (G9799 and F2045).

Plant genomic DNA extraction and labeling

Genomic DNA was extracted from leaves of 45 day-old greenhouse-grown plants by CTAB extraction [29] and purified by cesium chloride gradient centrifugation [30]. The genomic DNA samples were assayed and quantified by spectrophotometry. Each sample was biotin labeled using the random priming method with BioPrime® Array CGH Genomic Labeling System (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. In brief, a total of 3 µg of genomic DNA from each sample was mixed with 40 µl of 2.5X random primer solutions. The final volume was adjusted to 88 µl with H_2O. The reaction mix was denatured at 99ºC for 5 min. Following the immediate cooling to 4ºC, 10 µl of 10X dNTP mix containing biotin labeled dCTP and 2 µl of Exo Klenow fragments (80 units) were added to the reaction and incubated at 37ºC for 2 h. Labeled

DNA fragments were purified using the supplied column and assayed by gel electrophoresis prior to being applied to the arrays. Fragments of 100-200 bp were applied to the Affymetrix Rice GeneChip® for hybridization.

Target hybridization and image acquisition

Hybridizations were conducted according to Affymetrix standard protocol for eukaryotic target hybridization. Ten µg of biotinylated fragments were mixed in 200 µl with a final concentration of 0.1 mg/ml sonicated herring sperm DNA in a hybridization buffer with 100 mM 2-N-morpholino-ethane-sulphonic acid (MES), 1 M NaCl, 20 mM EDTA and 0.01% Tween 20, denatured at 99ºC for 5 min and equilibrated at 45ºC for 5 min prior to hybridization. The hybridization mix was then transferred into the Rice GeneChip® cartridge and hybridized at 45ºC for 16 h. The hybridized arrays were washed and stained using EukGE-WS2v5_450 protocol with an Affymetrix GeneChip Fluidics Station 450. The arrays were scanned twice and the intensities averaged with an Affymetrix GeneChip Scanner 3000 using GCOS 1.4.0.036 software (Affymetrix, Santa Clara, CA). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus [31] and are accessible through GEO Series accession number GSE15071 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15071).

Data processing and analysis

Programming was done in R (http://www.R-project.org) and Bioconductor [32]. The "affy" package [33] was used to extract probe level information and examine diagnostics. In brief, the arrays were analyzed for spatial aberrations, congruence of signal

distribution between arrays and variability in percentage of present calls across arrays [22]. Perfect match probe data was scale-normalized to the average of the wild type arrays. An R script was used to calculate log ratios versus wild type at the probe level. Probes meeting log ratio criteria (e.g., less than or equal to -0.8 log ratio on $\log_2$ scale) were flagged. Probe sets with more than 50% of probes meeting the defined log ratio criteria were called potentially deleted.

Analysis began with an initial combination of threshold (-0.8) and proportion (50%) values to generate a list of candidate deletions from individual arrays. Probe sets called deleted were aligned by BLAST [34] to the publicly available Nipponbare genome sequence [18] to identify location. For validation, sequence flanking the probe set location was used to design primers specific to the region. Genomic DNA from mutants and the wild type parent IR64 were used as template for PCR to confirm probe sets called deleted or not deleted on the arrays. PCR confirmation data from 112 amplifications was used to generate Table 1. TPR is calculated as the proportion of PCR-confirmed deletions that are correctly called by the analysis. FPR1 is calculated as the proportion of confirmed non-deletions incorrectly called deleted by the method. FPR2 is calculated by counting the number of probe sets meeting defined log ratio and proportion combinations for $\log_2(WT_1/WT_2)$ and for $\log_2(WT_2/WT_1)$, where wild type replicate 1 = WT1, and wild type replicate 2 = WT2. Primers used for validation are shown in Additional file 1.

Aggregation analysis

Affymetrix rice probe sets were anchored to the Nipponbare genome using homology mapping of the probe sets to version 5 of the TIGR rice genome annotation gene models (data from http://www.ricearray.org/matrix.search.shtml). The genome positions of the TIGR gene models were used for analysis in groups of genes along a chromosomal region. Probe sets mapped to multiple gene models were not included. The genome positions of the TIGR gene models were used for analysis in groups of genes along a chromosomal region. The ratios of deleted to non-deleted gene models within a predetermined genome block (0.5, 1.0 and 2.0 Mb) were compared to the genome-wide ratios using a Fisher exact test using a sliding window analysis with the window being shifted by one half block. Blocks significantly different from the fixed ratio (at $p<0.05$) were declared as potentially contiguous deletions. Block size can be varied to determine deletion size with greater precision.

Integration and visualization of deleted gene models and genomic regions using Generic Genome Browser

The coordinates of potential deletions in gene models and contiguous genome blocks were determined relative version 5 of the TIGR rice genome annotation (http://rice.tigr.org). Probe set and deleted genome block information were coded using the General Feature Format (GFF version 2, or GFF2) and loaded directly into the genome visualization tool, Generic Genome Browser (GBROWSE) [35], which was preloaded with gene model annotation data. Each mutant with the corresponding GFF2 data was visualized against the rice genome as a separate track. Comparative

visualization of the different mutants can be done by activating their respective track in GBROWSE.

Tilling and sequence analysis

Tilling to identify SNPs was performed as described [36] using primers shown in Additional file 1. The putative gene conferring the *spl1* phenotype, a cytochrome P450 family member, was amplified from mutants E16923, D1137, D2943, and wild type IR64 using gene specific primers (see Additional file 1). The amplicons were cloned into pGEM®-T Easy (Promega, Madison, WI), and the cloned PCR products were sequenced at the CSU Proteomics and Metabolomics Facility.

Abbreviations

Diepoxybutane, DEB; Fast neutron, FN; Gamma Ray, GR; Generic Genome Browser, GBROWSE, General Feature Format (GFF); Perfect match (PM); Single nucleotide polymorphism (SNP); Targeting induced local lesions in genomes (TILLING)

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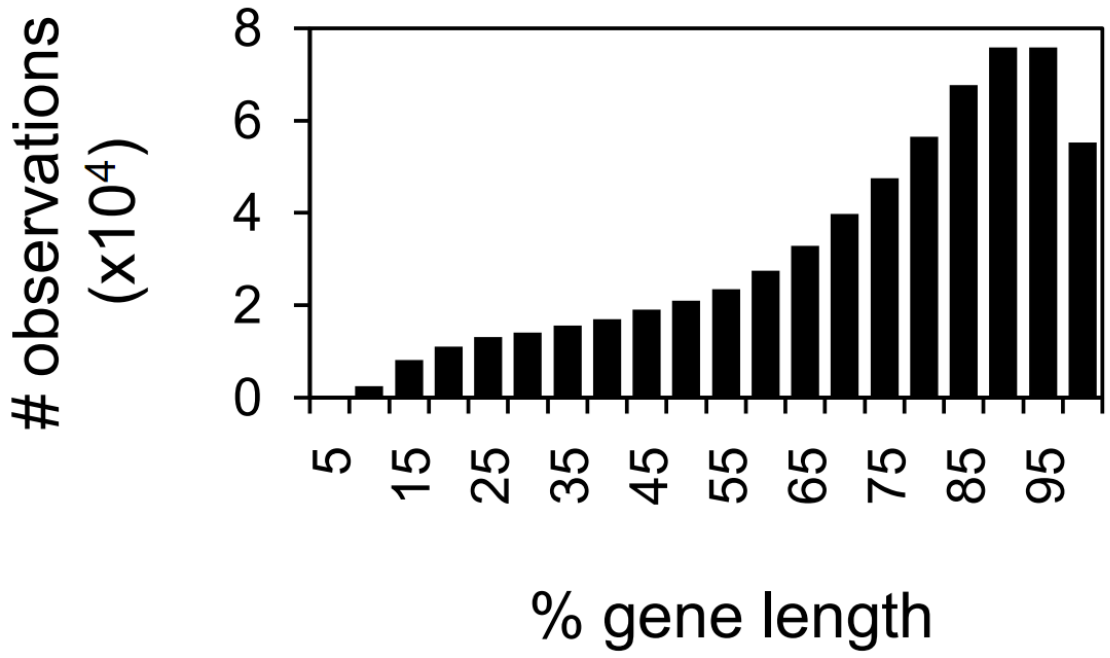
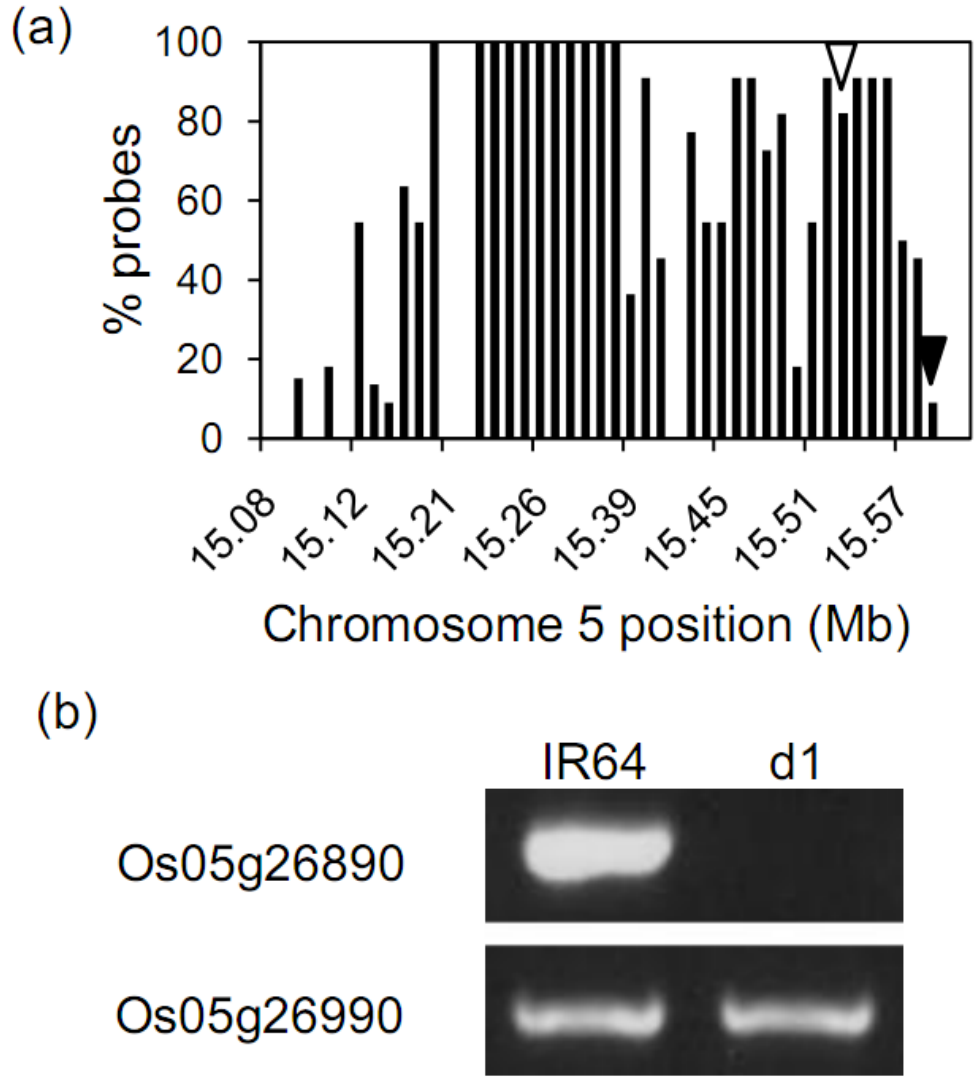
Figure 1. Distribution of probes on the Affymetrix Rice GeneChip® is biased to the 3' end of gene models.



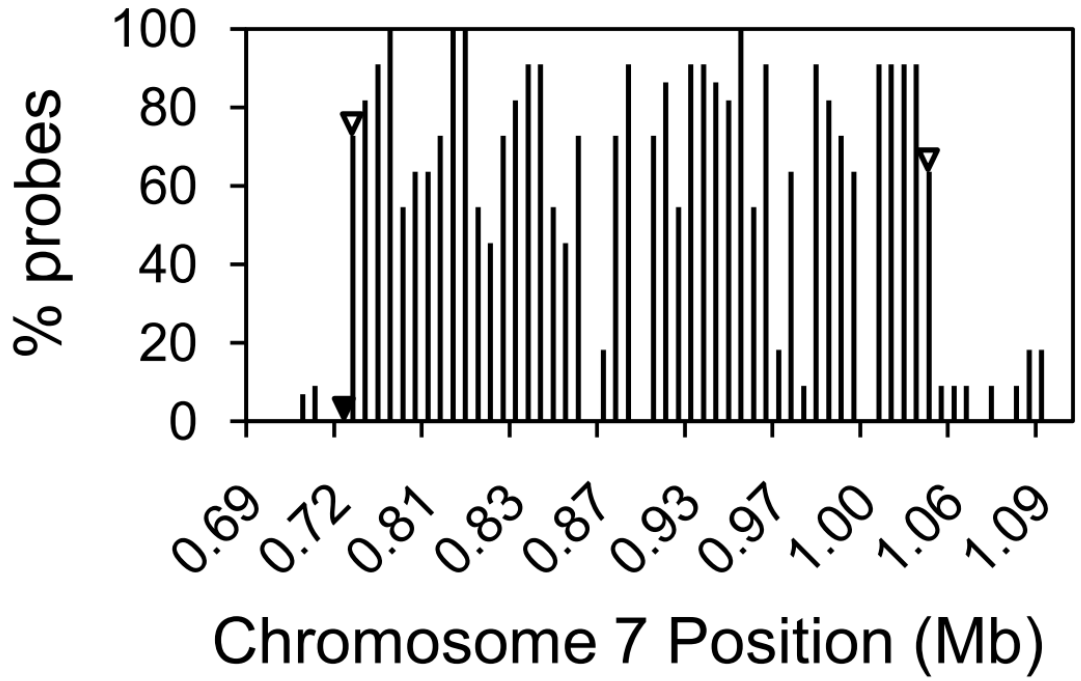
Because absolute lengths of genes vary, the genes are represented as percentage of length. The Affymetrix probes were binned into 5% intervals along the gene length. The y axis represents the number of probes on the array within a bin.

Figure 2. Mutant line d1 contains a ~500 kb deletion on chromosome 5 encompassing the *RGA1* gene.



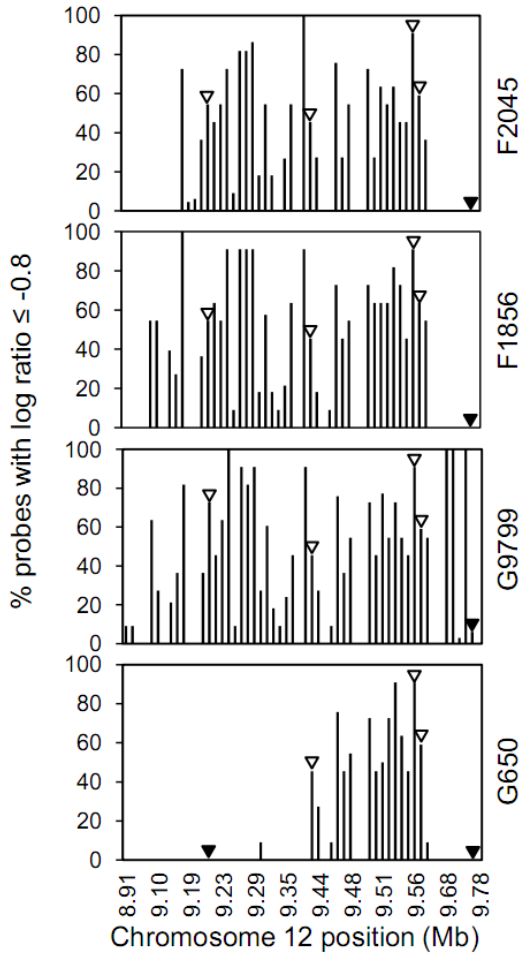
a) Gene models in the region show a high percentage of probes with $\log_2$(mutant probe intensity/wild type probe intensity) ≤ -0.8, indicating a large deletion. b) PCR confirmation of the deletion of *RGA1* (Os05g26890) relative to wild type (indicated by an open arrowhead in part a) and PCR confirmation of the right border of the deletion (Os05g26990) relative to wild type (indicated by a closed arrowhead in part a). The left border was not resolved.

Figure 3. Confirmation of a ~300 kb deletion on chromosome 7 in mutant line G282 as predicted by array hybridization using log ratio cutoff of <-0.8 for 50% or more of probes in a probe set.



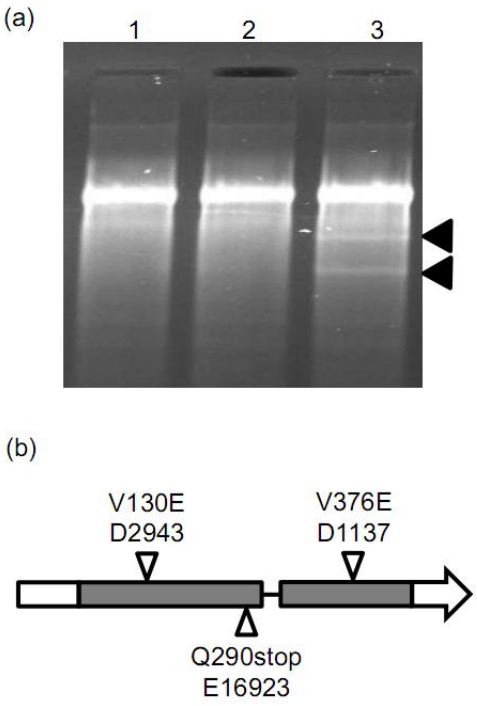
Open arrowheads indicate deletions in gene models confirmed by PCR. The closed arrowhead indicates a gene model confirmed to be present by PCR.

Figure 4. Array-based deletion discovery identifies allelic relationships among spl1 mutants.



Hybridization of genomic DNA from two confirmed allelic *spl1* mutants (G650 and F1856) and two mutants showing the distinctive *spl1* lesion mimic phenotype (G9799 and F2045) identified overlapping deletions in all four lines on chromosome 12. A log ratio cutoff of ≤-0.8 for 50% or more of probes in a probe set was used. Open arrowheads indicate deletions in gene models confirmed by PCR. Closed arrowheads indicate gene models confirmed to be present by PCR.

Figure 5. Identification of a cytochrome P450 family member as a candidate for *Spl1*.



Candidate genes located in the *Spl1* region by array hybridization (Figure 4) were screened for SNPs in an EMS-generated mutant showing the *spl1* phenotype by TILLING. (a) Detection of heteroduplex by TILLING between DNA for the rice mutant E16923 and wild type parent IR64 PCR products specific for LOC_Os12g16720 (a cytochrome P450 family member). Lanes 1 and 2 are CEL1 treatments of IR64 and E16923 amplicons, respectively. Lane 3 shows the activity of CEL1 enzyme on a heteroduplex generated between IR64 and E16923 amplicons. (b) Sequencing the amplified cytochrome P450 family member from E16923 confirmed the presence of a SNP at position 290 that resulted in a stop codon. Sequence data from two DEB mutants, D1137 and D2943, showing the *spl1* phenotype revealed SNPs in LOC_Os12g16720 that caused amino acid changes.

Table 1. True and false positive rates (TPR and FPR, respectively) for different log ratio [$\log_2$(mutant PM probe intensity/wild type PM probe intensity)] and (a) proportion (probes flagged/total probes in probe set).

$\log_2$ ratio	**Proportion**	**TPR[b]**	**FPR1[c]**	**FPR2[d]**
-0.6	0.4	0.833	0.012	0.0015
-0.6	0.5	0.800	0	<0.0001
-0.6	0.6	0.767	0	0
-0.6	0.7	0.600	0	0
-0.8	0.3	0.833	0	0.001
-0.8	0.4	0.833	0	0.0002
-0.8	0.5	0.767	0	<0.0001
-0.8	0.6	0.633	0	0
-1	0.3	0.833	0	0.0002
-1	0.4	0.800	0	<0.0001
-1	0.5	0.667	0	0
-1	0.6	0.600	0	0

[a]Analysis based on PCR confirmation 30 deletions and 82 non-deletions using primers described in Additional file 1.

[b]TPR was calculated as the proportion of PCR-confirmed deletions that are correctly called by the analysis.

[c]FPR1 is the proportion of PCR-confirmed non-deletions, that are correctly called deleted by the analysis.

[d]FPR2 is the proportion of probe sets meeting defined log ratio and proportion combinations for the wild type replicates, i.e., $\log_2(WT_1/WT_2)$ and for $\log_2(WT_2/WT_1)$.

Table 2. Number of deletions predicted on each chromosome in 14 individual IR64 mutants at log ratio < -0.6 for 50% or more probes in a probe set.

Chr	**Number of deleted gene models predicted per IR64 mutant line at log ratio < -0.6 for 50% or more probes in a probe set**														
	d1	D256	D2943	G282[a]	G650	G6458	G6489	G6603	G6686	G6728	G7534	G9799	F1856	F2045	Total
1	11	0	0	64	11	0	0	5	2	0	1	0	0	0	30
2	16	0	0	38	16	1	1	4	0	1	2	0	7	1	49
3	7	0	0	37	3	4	4	9	8	4	4	0	0	4	47
4	18	0	0	24	14	3	3	5	1	3	6	0	2	4	59
5	41	0	0	34	12	3	2	6	0	2	5	0	0	2	73
6	9	0	0	20	8	2	2	5	0	2	4	0	0	2	34
7	4	0	1	62	2	1	1	7	1	1	1	0	0	1	20
8	5	0	1	20	8	0	4	4	0	1	2	0	1	0	26
9	9	1	0	21	6	2	2	4	0	2	2	0	0	2	30
10	25	0	0	16	22	4	11	4	1	4	8	1	2	4	86
11	3	0	0	27	0	3	4	8	1	3	5	0	0	3	30
12	20	1	0	26	21	1	1	2	1	1	1	28	28	27	132
Total	168	2	2	359	123	24	35	63	15	24	41	29	40	50	616

[a]Deletions predicted for G282 are not included in totals. Because the number of predictions is large, to reduce FPR, a higher stringency (for example, log ratio < -0.8 for 50% or more probes in a probe set) is recommended for this mutant.

Table 3. Predicted probe set deletions using various combinations of $\log_2$ ratio and proportion (probes flagged/total probes) or adjacent probes including TPR and FPR rates as described in Table 1 and Additional file 2.

Mutant line	**Count of probe sets predicted to be deleted for different proportion (Prop) and log ratio (LR) combinations**				**Count of probe sets predicted to be deleted for different run length (RL)[a] and log ratio (LR) combinations**		
	LR = -0.6 Prop = 0.5	LR = -0.8 Prop = 0.5	LR = -1.0 Prop =0.3	LR = -1.0 Prop = 0.4	LR = -0.8 RL = 3	LR = -1 RL =2	LR = -1 RL = 3
d1	168	45	50	39	63	66	39
D256	2	0	0	0	1	11	0
D2943	2	0	0	0	2	2	1
G282	359	89	139	69	333	560	109
G650	123	46	29	19	55	45	23
G6485	24	0	0	0	0	2	0
G6489	35	5	5	1	7	7	2
G6603	163	0	0	0	54	30	3
G6686	15	5	9	8	10	14	5
G6728	24	0	0	0	4	15	1
G7534	41	2	2	2	46	35	5
G9799	29	25	28	24	34	55	24
N1856	40	36	40	33	36	41	30
N2045	50	17	22	19	26	37	21
WT check	10	0	1	0	16	88	3
TPR	0.800	0.767	0.833	0.800	0.800	0.833	0.800
FPR1	0	0	0	0	0	0	0
FPR2	0.0002	<0.0001	<0.0001	<0.0001	0.0008	0.004	0.0002

[a]Run length is group of adjacent probes within a probe set that meet a defined log ratio cutoff.

Additional file 1. Oligonucleotide primers used in this study

TIGR v5 locus[a]	Primer[b]	Sequence 5' – 3'	T_A (ºC)[c]
LOC_Os07g02260	EP1-1F	AGGAACGGCTGGAGGTAAGTAA	57
	EP1-1R	ACACATTGTTGGACGCGTGA	
LOC_Os07g02270	HP1-1F	TCTCGATAGCTACATCCGGAAGTTG	57
	HP1-1R	ACTTAAGATTTCGTATTGCCTCACACAT	
LOC_Os07g02640	CHP1-1F	GAGCACCTTGATTGAGCATGACTG	57
	CHP1-1R	GTGCAAAGTTCCTGCGATCTCTTC	
LOC_Os07g02800	DBD1-1F	AGAAGAAGGGTGGTGATCGTAAGG	54
	DBD1-1R	GGTACTTCTGCGCAATCAGAATCG	
LOC_Os03g04490	CDKI1-1F	GGGACCTCATCTCCCAATCGTTTA	57
	CDKI1-1R	GTAGATTCCAGCCAGCATCCCTAT	
LOC_Os04g47912	EP2-1F	TAGCTCGATGTTCTCATCGTACCG	57
	EP2-1R	CAGATGATGAAGTGGTATCTCCAGC	
LOC_Os11g24170	NBLRR1-1F	GCAGAACTAAAGGAGCAGTGCAAG	57
	NBLRR1-1R	CACAGCCATGAAGATCGGTAATGC	
LOC_Os08g25799	MFTF1-1F	TGTGCCTTCAAGCTGTTTGGTC	57
	MFTF1-1R	TGCCATCTCTTTGCCAGATTGC	
LOC_Os08g26110	DLP1-1F	GGTGTTCCAAACCGGAACGAAT	57
	DLP1-1R	GCATTGGCAATTTGGCTTCGTG	
LOC_Os08g26370	HP4-1F	GAGGCAAGAGAGAGCATCAACA	57
	HP4-1R	ATCGAAGGGTATAGTACCGCCA	
LOC_Os04g21890	RPM1-1F	GCAGGAACTACGATGGAAGAGCTA	57
	RPM1-1R	CTTGGGAACCATCCCAGAACAAAC	
LOC_Os02g06160	LLPK1-1F	CAGTCAAGAAGTTTACACGTGCCG	57
	LLPK1-1R	CAGATGATGAAGACCCTACTGGCA	
LOC_Os05g26890	GPA1-1F	TTGTATGATGTAGGAGGCCAGAGG	57
	GPA1-1R	TTACAAGTTTCTGGTCTAGGGCCG	
LOC_Os05g27050	PTR2-1F	TATGCAAGTAGTGCAGGGTACGAG	57
	PTR2-1R	CTGTTCCGGATTGTGTCCTGTTTG	
LOC_Os06g09820	UK1-1F	TGTACGCAAGGCTCTCTTACTAGC	57

	UK1-1R	CTTCCATCTTCGGTGAAGGACTCA	
LOC_Os10g34230	CD5P-1F	TCAGGTCGTGGAAGTCATCTCAAG	57
	CD5P-1R	CTGTAGCCATGTAGGTGTGGACTT	
LOC_Os05g19360	HP5-1F	CCTGTCGCCTAGCTCCTT	59
	HP5-1R	AGGTCTCTCGGCAGTATTGTGTTC	
LOC_Os02g27200	MONOX1-1F	GTTTATAGGATGGCGCTTTCGGTG	59
	MONOX1-1R	CTTAGCAAAGTCCCAACGGTGAAG	
LOC_Os04g13140	VP1-1F	AGAAACTAGCCGTAGCCTTGGTAG	59
	VP1-1R	ATCATGGCGAGGCCACAGA	
LOC_Os12g16690	AK072042-2F	ATGCCGCAGCTAAATGTGGA	56
	AK072042-1R	AACCTTGTGAGTGCCAACGA	
LOC_Os12g16480	Os1022121-4F	GATTGTTAGGAACCCAATCCCTCC	56
	Os1022121-4R	CCTAGCAGAGACAACATGAACCTG	
LOC_Os12g16720	CytP450-4F	CTTGCTTCTCTGCTTAGTCACAATATT	54
	CytP450-4R	CTTCCCCACTTAACCACGGTTAG	
LOC_Os12g16720	3F	CCAACACATCTCCATTGCTG	57
	cDR	CACTCGAAGTGGTAGAG	
LOC_Os12g16720	cDF	ATCATCAAGGAGACGTT	57
	4R	AACTGGCGCTACGCTACATC	
LOC_Os12g16710	Os1005492-4F	GTATGAAGGCCCATGTTACTACCG	56
	Os1005492-4R	CATGGAGAGCTTGAGGAGGAGATA	
LOC_Os12g16520	Os1027066-4F	CTCCAGTAAATGACCACGGTTCTG	54
	Os1027066-4R	GGGCAGTATGACTTGGAGAAGATG	
LOC_Os12g16130	Os1025420-1F	CTGTCACCATCAGAGACATCTTGG	56
	Os1025420-1R	GGGATCAGGTCCACGAGAATTAAG	

Oligonucleotide primers used for validation of deletions and amplification of Spl1-gene candidates.

Additional file 2. True and false positive rates (TPR and FPR, respectively) for different log ratio [log_2(mutant PM probe intensity/wild type PM probe intensity)] and adjacent probe combinations.

Log_2 ratio	**Adjacent probes**	**TPR[b]**	**FPR1[c]**	**FPR2[d]**
-0.6	2	0.9	0.06	0.039
-0.6	3	0.867	0.012	0.003
-0.6	4	0.733	0	<0.001
-0.6	5	0.633	0	0
-0.8	2	0.833	0	0.013
-0.8	3	0.8	0	<0.0001
-0.8	4	0.5	0	<0.0001
-0.8	5	0.4	0	0
-1	2	0.833	0	0.0004
-1	3	0.8	0	0.0002
-1	4	0.433	0	0
-1	5	0.367	0	0

[a]Analysis based on PCR confirmation 30 deletions and 82 non-deletions using primers described in Table S2.

[b]TPR was calculated as the proportion of PCR-confirmed deletions that are correctly called by the analysis.

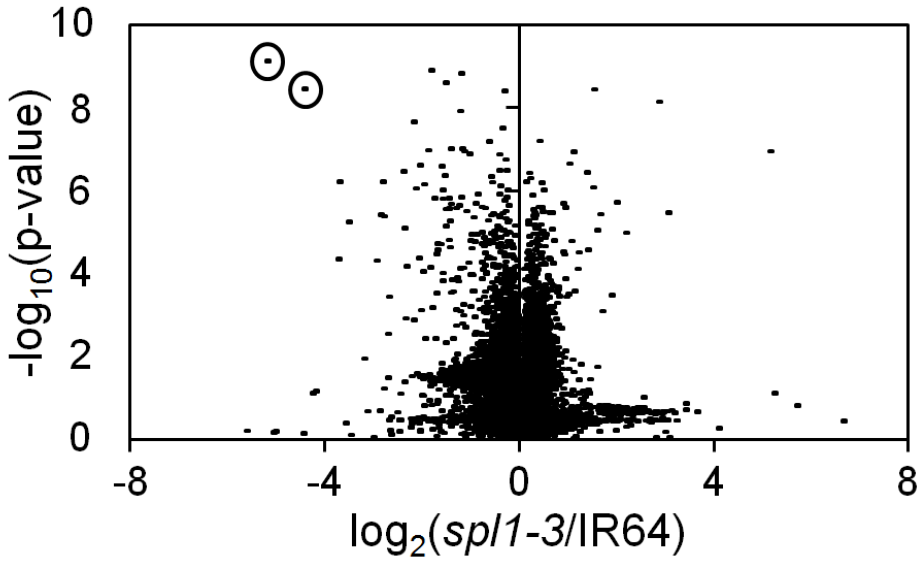
[c]FPR1 is the proportion of PCR-confirmed non-deletions, that are correctly called deleted by the analysis.

[d]FPR2 is the proportion of probe sets meeting defined log ratio and proportion combinations for the wild type replicates, i.e., $log_2(WT_1/WT_2)$ and for $log_2(WT_2/WT_1)$.

Title: True and false positive rates (TPR and FPR, respectively) for different log ratio [log_2(mutant PM probe intensity/wild type PM probe intensity)] and adjacent probe combinations.

Description: True and false positive rates for the analysis method reported by Gong, et al. [16]

Additional file 3.



Title: Volcano plot of expression data from rice *spl1* mutant G650 shows significant down-regulation of genes which are candidates for deleted genes.

Description: Data are from dual channel hybridizations comparing two rice lines, the *spl1* mutant G650 and the wild type IR64. mRNA was extracted from the youngest fully expanded leaf of six plants each. cDNA was labeled with Cy3 and Cy5 dyes and hybridized onto the Agilent Rice 22k Oligo Microarray. By plotting the $\log_2$ ratio of mutant/wild type signal intensity (x-axis) versus $1/\log_{10}$ of the p-value (y-axis) for four array hybridizations, potential deletions were identified because they exhibited large negative fold changes coupled with significant p-values. Two such deleted genes are LOC_Os12g16540 and LOC_Os12g16720 (black circles). These genes were confirmed to be deleted by PCR and by hybridization of genomic DNA to the Affymetrix Rice GeneChip (Figure 4). Indeed, other methods, such as TILLING and sequencing, indicate

that LOC_Os12g16720 is *Spl1*. However, other genes shown to be deleted by the hybridization of genomic DNA, such as LOC_Os12g16650 (Figure 4) do not have significant p-values or large negative fold changes from the expression profiles, indicating that these genes may not have been expressed in wild type plants during the time the tissue samples were taken.

CHAPTER 3

RICE 14-3-3 PROTEIN (GF14E) NEGATIVELY REGULATES CELL DEATH AND DISEASE RESISTANCE

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[†]These authors contributed equally to this work. PMM developed and evaluated the *GF14e* silenced lines, performed the *Xoo* inoculations. MB performed experiments to evaluate expression of *GF14e,* peroxidases and other defense response genes during plant development, ETI in response to *Xoo*, and after inoculation with *Rhizoctonia solani*. PMM and MB contributed equally to the conceptualization of the experiments and the preparation of the manuscript.

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SUMMARY

Plant 14-3-3 proteins regulate important cellular processes, including plant immune responses, through protein-protein interactions that affect a wide range of target proteins. In rice, some *GF14* genes, which encode 14-3-3 proteins, are induced early during interactions with *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). To determine whether *GF14e* has a direct role in resistance to disease in rice, we suppressed its expression by RNAi silencing. *GF14e* suppression was correlated with appearance of a lesion mimic (LM) phenotype in the transgenic plants at three weeks after sowing. The LM phenotype indicates an inappropriate regulation of cell death, a phenotype frequently associated with enhanced resistance to pathogens. *GF14e*-silenced rice plants displayed high levels of resistance to a virulent strain of *Xoo* compared to plants that were not silenced. Enhanced resistance was correlated with *GF14e* silencing prior to and after development of the LM phenotype, higher basal expression of a *peroxidase* gene (*POX22.3*) that was previously shown to be induced during effector-triggered immunity (ETI) in rice, and higher levels of reactive oxygen species (ROS). *GF14e* is also up-regulated during ETI between rice and *Xoo*. In addition, *GF14e*-silenced plants also exhibit enhanced resistance to the necrotrophic fungal pathogen *Rhizoctonia solani*. Together, our findings suggest that *GF14e* is a negative regulator of plant defense response gene induction, cell death, and broad spectrum resistance in rice.

INTRODUCTION

A plant's ability to resist and survive pathogen assault depends on the speed of recognition and the type of defense responses activated by the attack. Plant defense responses are classified according to the molecular mechanisms for pathogen recognition [1, 2]. One such mechanism, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), is activated by recognition of molecules common to many classes of microbes (PAMPs), and is largely responsible for conferring basal disease resistance following infection. Effector-triggered immunity (ETI), previously defined as *R* gene-mediated resistance, is activated by direct or indirect recognition of specific pathogen-produced effector molecules. ETI triggers a faster and stronger defense response and often culminates in a localized programmed cell death (PCD), referred to as the hypersensitive response (HR) [3]. The HR is correlated with a transient burst of reactive oxygen species, activation of specific defense-response genes (DR), accumulation of antimicrobial compounds, and alterations of the plant cell wall [4].

One approach to understanding the molecular basis of the HR is to identify mutants with visible phenotypes that resemble pathogen-induced HR lesions. A large number of mutants exhibiting abnormal regulation in cell death pathways and phenotypes mimicking the HR have been identified in plants such as maize, *Arabidopsis*, barley, and rice [5-11]. These mutants form spontaneous HR-like lesions in the absence of pathogen infection and are called lesion mimics (LM). The constitutive activation of cell death and defense pathways in some LM mutants suggests that these mutations might involve genes

regulating the HR in wild-type plants [12]. Genes controlling lesion mimics in several plants species have been cloned [8, 12-19].

14-3-3 proteins are conserved phosphopeptide binding proteins present in many isoforms in plants and animals [20]. These proteins are associated with a wide range of metabolic processes, organellar functions, nuclear localization, transcription control, and response to abiotic and biotic stresses [21-26]. Kinases and phosphatases are regulated by these proteins, suggesting that 14-3-3 proteins are integral components in eukaryotic signal transduction [27-29]. The 14-3-3 family consists of acidic 30 kDa proteins that form homo- and hetero-dimers and are located in the cytosol, nucleus, and nuclear matrix [30, 31]. 14-3-3 proteins interact with phosphorylated proteins (clients), influencing the client's activity, stability or subcellular localization [25, 32, 33]. To perform these important roles, the 14-3-3 proteins interact physically with their clients by recognition of consensus sequences [34, 35].

Several lines of evidence associate 14-3-3 protein function with plant resistance against pests and pathogens. Early reports show that *14-3-3* genes are differentially regulated during disease defense responses [36-39] and after mechanical wounding, insect chewing, and treatment with elicitors of wound-responsive gene expression [26, 40]. Furthermore, 14-3-3 proteins bind and regulate various enzymes involved in plant defense responses. The *Arabidopsis* ascorbate peroxidase (APX3), an enzyme involved in oxidative stresses, directly interacts with a 14-3-3 (GF14-λ) [41]. An ankyrin-repeat containing protein

(AKR2), which is an interacting partner of APX3 and GF14-λ, negatively regulates transcription factors that mediate defense responses in *Arabidopsis* [10]. GF14-λ also interacts with and positively regulates the RPW8.2 *R* gene which confers resistance to the fungal pathogen *Golovonomyces* spp. in *Arabidopsis* [42]. Most recently, a tomato 14-3-3 protein (TFT7) was shown to directly interact with the mitogen-activated protein (MAP) kinase kinase kinase (MAPKKKα), and this interaction is required for programmed cell death induced by Pto- and MAPKKKα-mediated signaling in tomato [43].

Rice 14-3-3 proteins are implicated in stress responses as well as plant development [31, 44]. Some of the eight predicted *14-3-3* genes in rice [31] are suggested to play a role during stress responses because of their interactions with target proteins involved in several plant stresses, including defense responses [45]. For instance, GF14b and GF14f interact with the benzothiadiazole-induced MAP kinase 1 (BIMPK) that is induced by the rice blast fungus and is involved in systemic acquired resistance (SAR) signaling [45]. In addition, members of the *GF14* family, including *GF14e*, are up-regulated after inoculation with avirulent and virulent strains of bacterial and fungal pathogens and are also differentially regulated by abiotic stresses and after treatment with defense-related phytohormones [31]. In addition, genetic markers corresponding to the pathogen responsive *GF14e* gene co-localize with QTLs against rice blast and sheath blight on rice chromosome 2 [46, 47].

In this study, we assessed the role of *GF14e* in rice disease resistance by suppressing its expression using an RNAi silencing approach. *GF14e*-silenced transgenic plants showed spontaneous HR-like lesions and enhanced resistance to a virulent strain of *Xoo*. The enhanced resistance correlates with high expression of a rice peroxidase gene and higher accumulation of reactive oxygen species. Silencing *GF14e* also enhanced resistance to the necrotrophic sheath blight pathogen *Rhizoctonia solani* (*Rs*). We propose that this 14-3-3 protein isoform acts as a negative regulator of cell death and broad spectrum resistance in plants. To our knowledge this is the first report of a 14-3-3 in plants that acts as a negative modulator of cell death and broad spectrum resistance against biotrophic and necrotrophic pathogens.

RESULTS

RNAi silencing of *GF14e* induces a lesion mimic (LM) phenotype

Using the Michigan State University Rice Genome Annotation Project Database, eight *14-3-3* gene members were predicted on rice chromosomes 1, 2, 3, 4, 8, and 11. These genes were described previously and named *GF14a* through *GF14h* [31]. The cDNAs corresponding to these eight genes share 55-85 % nucleotide similarity (Figure 1a). *GF14* members that were pathogen responsive (*e*, *b*, *c*, and *f*, Chen *et al.*, 2006) were most closely related in a phylogenetic dendrogram (Figure 1a).

Silencing of *GF14e* expression was achieved by transformation of rice variety Kitaake with an RNAi vector containing a 350 bp region from the first exon of *GF14e*.

Expression of *GF14e* was reduced in the 20 screened T_0 transgenic rice lines that contained the 350 bp trigger sequence (Figure 1b). However, no independent transgenic line that contained the trigger sequence exhibited complete silencing of *GF14e*.

At 3-4 weeks (wk) after transfer to soil, T_0 transgenic plants suppressed for *GF14e* displayed spontaneous HR-like lesions (LM phenotype). The small (<5 mm), discrete, brown necrotic lesions characteristic of the LM phenotype occurred in the T_0 through T_3 generations (e.g., Figure 1c). Silencing of three other types of defense response genes, such as genes encoding germin-like proteins (*OsGLPs*) [48] (Figure 1c), chitinases, and oxalate oxidases, did not exhibit a lesion mimic phenotype (data not shown).

Lesion mimic phenotype correlates with the *GF14e* suppression

Presence of the LM phenotype in T_0 transgenic plants correlated with reduced expression of *GF14e*, as estimated by RT-PCR (Figure 2a). High nucleotide identity (85%) was observed between the 350 bp region used for silencing and other *14-3-3* family members, particularly the closely related members *GF14b* and *GF14c* (Figure 1a, S1). Thus, we explored the possibility that the LM phenotype might result from co-silencing of *GF14a, b, c, d*, and *g* in addition to *GF14e* by using RT-PCR and quantitative real time PCR (qPCR). The gene expression of other *GF14* members was assessed in the same 20 T_0 transgenic rice plants (Figure 1) tested for *GF14e* silencing by RT-PCR analysis. Neither the expression of the closely related *GF14c* nor the more distantly related *GF14a* were co-silenced in the *GF14e*-silenced T_0 plants (Figure S2). The expression of *GF14b, c, d*

and *g* was assessed by quantitative real time PCR (qPCR) in T_3 segregating transgenic plants. Transgenic plants exhibiting the LM phenotype showed silencing of *GF14e* (5-9, 17, and 24) and expression of the hygromycin phosphotransferase (*hpt*) transgene (Figure 2b, S3). Transgenic plants with wild type levels of *GF14e* expression (5-15, 21, and 30) did not show *hpt* transgene expression or the LM phenotype (Figure 2b, S2). *GF14b*, *GF14d*, and *GF14g* were not co-silenced by the *GF14e* RNAi construct. Two out of three *GF14e*-silenced plants with the LM phenotype showed reduced expression of *GF14c* (5-9 and 17). However, silencing *GF14c* does not contribute to the LM phenotype (Figure 2b) because plant 5-24 exhibited the LM phenotype and showed suppression of *GF14e* but not *GF14c*.

_GF14e_-silenced plants show enhanced resistance to bacterial and fungal rice diseases

Since occurrence of LM on plants is commonly associated with enhanced resistance to pathogens [8-10, 12], we asked if *GF14e*-silenced LM plants exhibited enhanced pathogen resistance. Wild type (WT) plants and 67 T_1 transgenic plants segregating for the LM phenotype were inoculated with a virulent strain of the bacterial blight pathogen, *Xoo* strain PXO99A, at 7 wk after sowing (WAS). WT plants and segregants that did not show the lesion mimic phenotype (NLM) developed typical bacterial blight disease lesions, including water-soaking followed by appearance of gray-brown lesions (data not shown). Lesions occurring on *GF14e*-silenced plants (LM plants) were restricted, with mean lesion lengths much shorter than the NLM ($P < 0.0001$) (Figure 3a). The high level of resistance observed in T_1 LM plants correlated with lower bacterial numbers ($P <$

0.0001) (Figure 3b). Silencing of *GF14e* also correlated with enhanced resistance to sheath blight disease ($P < 0.05$) caused by the necrotrophic fungal pathogen *Rhizoctonia solani* (*Rs*, Figure S4). These results suggest that *GF14e* is a negative regulator for broad spectrum disease resistance in rice.

Enhanced *Xoo* resistance precedes onset of the LM phenotype and correlates with up-regulation of a peroxidase gene

The strong resistance against *Xoo* observed in the *GF14e*-silenced plants with LM could have been a consequence of the extensive cell death resulting from LM development, rather than a result of *GF14e* suppression. The LM phenotype did not appear until approximately 3 WAS, allowing us to test bacterial blight resistance in a segregating T_3 population before and after appearance of the LM phenotype. Plants were inoculated with the virulent *Xoo* strain PXO99A at 2 (no visible LM lesions), 5 (few LM lesions) (data not shown), and 7 WAS (leaves completely covered by LM lesions). Enhanced resistance to *Xoo* was observed in *GF14e*-silenced plants at every stage analyzed, regardless of the presence of visible LM lesions at the time of inoculation (Figure 4). Enhanced resistance was independent of the visible presence of cell death because 2-wk-old *GF14e*-silenced plants showed enhanced resistance prior to cell death development when compared to the non-silenced segregants (NLM) ($P = 0.05$) (Figure 4a). However, as plants aged and developed more LM lesions, resistance increased ($P < 0.005$ for plants inoculated at 7 WAS) (Figure 4b).

Yin *et al*. (2000) demonstrated that expression of defense response genes was activated in some rice LM mutants as the lesions developed. Therefore, we assessed expression of two defense response genes, a *peroxidase* (*POX22.3*) and *ethylene insensitive 2* (*OsEIN2*), in *GF14e*-silenced plants. Genes encoding for peroxidases are associated with ETI against *Xoo* in rice [49, 50] and EIN2 is a positive component in ethylene signaling in rice which is known to be associated with resistance against necrotrophic pathogens such as *Rs* (Jun *et al*., 2004; Glazebrook, 2005).The transcripts of these two genes were examined in the uninoculated tissue of the T_3 *GF14e*-silenced segregating progeny used in the experiment above at 2 and 5 WAS. Occurrence of the LM phenotype in *GF14e*-silenced plants correlated with *hpt* transgene expression. NLM (control) plants did not show *hpt* transgene expression or the LM phenotype (Figure S3).

Compared with the non-silenced segregants (NLM), the *GF14e*-silenced plants (LM) showed high levels of expression of *POX22.3* at 2 WAS ($P < 0.005$) (Figure 5a). However, at 5 WAS, expression of *POX22.3* was reduced in the LM plants compared with the 2 WAS LM plants ($P < 0.05$). At this time, the expression of this gene was slightly higher in the *GF14e*-silenced plants with LM when compared to NLM segregants but this enhanced level was not statistically significant in the T_3 generation (Figure 5a). Expression of *OsEIN2* gene was not different among the transgenic plants at any stage analyzed (Figure 5b). These results suggest that increased expression of *POX22.3* in the *GF14e*-silenced plants occurred regardless of the presence of the LM phenotype, and the induction of the gene correlated with the resistance observed in silenced plants.

The LM phenotype and enhanced level of expression of *POX22.3* in *GF14e*-silenced plants suggested that ROS (hydrogen peroxide, H_2O_2) might be accumulating due to increased peroxidase activity in the *GF14e*-silenced plants. To detect ROS, we used 3, 3' –diaminobenzidine (DAB), a histochemical reagent that acts a substrate for peroxidases when combined with H_2O_2 [51] to examine leaves of T_2 *GF14e*-silenced plants at 2 and 5 WAS. Brown staining due to H_2O_2 accumulation was observed in the leaves of *GF14e*-silenced plants at both 2 and 5 WAS (LM, Figure 5c). No visible staining was observed in leaves from non-lesion mimic segregants (NLM, Figure 5c) or wild type Kitaake plants (data not shown). The sites of H_20_2 accumulation in the 5 WAS LM plants correlates with the LM lesions on the leaf. The enhanced ROS in the T_2 plants suggests higher levels of POX activity and correlates with the higher expression of *POX22.3* in the LM silenced plants with respect to the NLM plants (Figure S5). These results suggest that increased peroxidase activity leading to ROS accumulation may contribute to enhanced pathogen resistance in *GF14e*-silenced plants.

GF14e is up regulated during ETI against bacterial blight

Previously, Chen *et al*. (2006) [31] (Chen *et al.*, 2006) showed that *GF14e* was up-regulated during compatible and incompatible interactions between rice and the bacterial blight (*Xoo*) and rice blast (*Mo*) pathogens. We analyzed *GF14e* expression during an incompatible interaction (ETI) by inoculating transgenic Kitaake rice expressing the resistance gene *Rxo1* with *Xoo* carrying the recognized effector gene (*avrRxo1*). *Rxo1* is a non-host resistance gene identified from maize [52] and confers ETI when it recognizes the pathogen effector *AvrRxo1* [53]. Cell death, or a hypersensitive response, resulting

from the *Rxo1-avrRxo1* interaction was visible at 48 h post inoculation (hpi) in the inoculated plants (data not shown). We measured *GF14e* expression at several time points before the visible HR was detected. *GF14e* expression increased as early as 4 hours post inoculation (hpi) of *Rxo1* plants and remained elevated up to 24 hpi (Figure 6).

Because *GF14e* is upregulated during ETI and is a negative regulator for POX, we tested the expression of *PO-C1,* a gene encoding a cationic pathogen-induced peroxidase that is specifically and rapidly upregulated during ETI against *Xoo* in rice [50, 54]. Interestingly, although up-regulated initially, an ETI-responsive peroxidase, *PO-C1*, was down-regulated by 8 hpi, correlating with the increased *GF14e* expression (Figure 6). These results argue that in addition to its role during basal resistance in rice, *GF14e's* role in ETI might be through the negative modulation of peroxidase gene expression that is required for ETI against *Xoo*.

DISCUSSION

14-3-3 proteins are regulators (adapters, chaperones, activators or repressors) in many biological processes in plants [32]. There is increasing indirect and direct evidence to support a role for 14-3-3 proteins during disease resistance in many plant species (for review, see [55], including the co-localization of 14-3-3 gene markers with disease resistance QTL [46, 56, 57]. *GF14* members that were pathogen and defense-related hormone responsive (*b*, *c*, *e*, and *f*, Chen et al., 2006) were the most closely related based on phylogenetic analyses (Figure 1a). In this study we provide direct evidence that

GF14e plays a role in rice resistance against diseases caused by *Xoo*, causal agent of bacterial blight, and *Rs*, the necrotrophic fungal sheath blight pathogen. Understanding basal resistance against *Rs* is essential because no single gene resistance for sheath blight is currently available for breeding purposes [47, 58-61]. In addition to the function of *GF14e* in broad spectrum disease resistance, our results suggest a role during ETI in rice during an incompatible interaction with the bacterial pathogen *Xoo*. We propose that *GF14e* is likely not only a negative regulator of broad spectrum resistance, but it is also a negative regulator of rice cell death pathways because *GF14e*-silenced plants show spontaneous HR-like lesions (LM phenotype) (Figure 1b). In contrast to our findings, the 14-3-3 members that have so far been shown to affect ETI, PTI and cell death are positive regulators because silencing of these members results in loss of resistance and cell death development (Yan *et al*., 2009; Oh *et al*., 2010a).

The nucleotide region chosen as an RNAi trigger (Figure S1) shows high sequence identity among the *GF14* gene members leading to the possibility that the resistance and LM phenotypes observed in the rice *GF14e*-silenced plants were due to i) co-silencing of other *GF14* gene members, ii) altered expression of other *GF14* gene members resulting from suppression of *GF14e*, or, iii) a combination of both. However, the specific silencing of *GF14e*, the occurrence of a LM phenotype, and enhanced resistance in the transgenic plants were all correlated, suggesting a role for *GF14e* (Figure 2, 3, and 4). Furthermore, of the four *GF14* gene members tested for co-silencing (*GF14b*, *c*, *d*, and *g*), only *GF14c* was down regulated in one of the *GF14e*-silenced plants (Figure 2b). We conclude that co-silencing of *GF14c* is not responsible for the cell death and resistance

phenotypes observed because transgenic plant, 5-24, which exhibited LM lesions and enhanced resistance to *Xoo*, was silenced for *GF14e*, but was not silenced for *GF14c* (Figure 2b, 4b). Consistent with our findings, heterozygous *GF14c* knockout (KO) rice plants did not show LM [44], but instead showed early flowering [44]. Although we observed early flowering, stunted growth, and poor seed set in some of the *GF14e*-silenced LM plants relative to NLM (Figure S6), we did not test whether reduced *GF14c* expression in these plants correlated with these developmental related phenotypes.

Eukaryotic cells contain multiple 14-3-3 isoforms, these isoforms can interact each other to form homo- and hetero-dimers [25, 62]. Suppression of *GF14e* might affect the expression of other *GF14* family members and affect this protein oligomerization and as a result, protein functions. In our study, we observed that some *GF14e*-silenced plants showed reduced *GF14c* expression and higher expression of *GF14d* (Figure 2b). Co-silencing might not be the explanation for reduced expression of *GF14c;* it is possible that reduced expression of *GF14e* affects the expression of this other family member. Similarly, the higher expression of *GF14d* in *GF14e*-silenced plants could be a result of a compensatory effect of the largely depleted pool of *GF14e*, with *GF14d* partially fulfilling the function of *GF14e*.

Lesion mimic mutants in plants frequently result from disruption in cell death pathways leading to expression of defense response genes and the spontaneous appearance of HR-like lesions, both of which may contribute to enhanced resistance to pathogens [9, 63].

This was the case for *GF14e*-silenced plants, in which suppression of this specific family member resulted in induction of HR-like lesions and enhanced resistance against two distinct rice pathogens (Figure 3, S4). Cell death in plants may be a consequence of mis-regulation of genes in the cell death pathways and/or disruption of cellular homeostasis by a wide range of metabolic perturbations [9]. Spontaneous cell death phenotypes and enhanced resistance to pathogens have been attributed to overproduction of toxic components, such as reactive oxygen species (ROS) and phenolic compounds [64, 65]. At 7 WAS, when the LM was most severe, the *GF14e*-silenced plants exhibited a high level of resistance ($P < 0.005$) (Figure 4b). Furthermore, we observed production of ROS in developing lesions in the silenced lines (Figure 5c). It is possible that when the LM phenotype is fully developed, toxic compounds associated with cell death increase to levels inhibitory to pathogen penetration and/or multiplication. However, because *GF14e*-silenced plants were more resistant to a virulent pathogen before visible development of necrotic tissue in the LM (2 WAS), we conclude that the presence of the necrotic tissue *per se* was not the cause of enhanced resistance (Figure 4a). Instead, we propose that *GF14e* functions in the regulation of disease resistance or susceptibility. The role of *GF14e* in rice cell death pathways and resistance is consistent with the finding that antisense silencing of a client of GF14λ in *Arabidopsis*, AKR2, resulted in plants with LM and enhanced defense responses leading to pathogen resistance [10]. Interestingly, overexpression of GF14λ in *Arabidopsis* caused HR-like cell death and enhanced resistance against the powdery mildew pathogen, but did not have an effect against bacterial pathogens. In this case GF14λ is a positive regulator of cell death and resistance (Yang *et al*., 2009). In tomato, a 14-3-3 protein also acts as positive regulator

of cell death and resistance (Oh *et al*., 2010a). It will be interesting to determine how these positive and negative regulators of plant cell death and resistance relate to each other during plant immunity.

In a previously described rice LM mutant, *spl11*, lesion development was correlated with enhanced resistance to multiple isolates of the hemibiotrophic pathogen *M. oryzae* and the biotrophic *Xoo* and activation of several defense response genes including the rice *peroxidase POX22.3* [9, 12]. Plant peroxidases have been implicated in resistance to both biotrophic and necrotrophic pathogens [49, 50, 54, 66-71]. Interestingly, in the *spl11* mutant, *POX22.3* gene expression was elevated only in fully expanded leaves with few lesions, but not in leaves with many lesions [12]. Similarly, we observed higher expression of *POX22.3* in the *GF14e*-silenced plants before appearance of LM lesions (2 WAS) relative to non-silenced plants. As with *spl11*, *GF14e*-silenced plants at 5WAS that exhibited full development of HR-like lesions (LM phenotype) did not show significant *POX22.3* up regulation (Figure 5a). Importantly, *GF14e*-silenced plants were resistant at all stages independent of the LM development. One possible explanation is that constitutive activation of *POX22.3* in the *GF14e*-silenced plants at early stages induces several POX-related defense responses including oxidation of phenolic compounds, ROS production, cross-linking of cell wall materials, and cell wall lignification that could limit pathogen penetration and/or arrest pathogen growth [49]. Consistent with this, we observed accumulation of H_20_2 in silenced lines by using DAB staining (Figure 5c).

The enhanced resistance observed in *GF14e*-silenced plants against the necrotroph *Rs* was somewhat unexpected because necrotic responses in plants usually enhance susceptibility to necrotrophic pathogens [72, 73]. Necrotrophs such as *Botrytis cinerea* promote virulence in their hosts by inducing HR-like cell death and production of ROS, and by inhibiting plant scavenging proteins such catalases [74, 75]. Recently, plants overexpressing a rice peroxidase showed enhanced resistance against three different necrotrophic pathogens [70, 76] indicating a role for peroxidases during broad spectrum resistance against necrotrophs. In the *GF14e*-silenced plants, GF14e-dependent activation of scavenging genes encoding for peroxidases (Figure 5) may interfere with the virulence mechanism of necrotrophic pathogens like *Rs*.

Resistance against necrotrophs is generally mediated through JA/ET signaling pathways [72, 73]. In rice, EIN2 was shown to be required for ethylene (ET) signaling [77], however, we did not observe changes in the expression of *OsEIN2* gene in the *GF14e*-silenced plants at any stage analyzed (Figure 5b). This result suggests that *OsEIN2* does not contribute to the enhanced resistance against *Rs* observed in the *GF14e*- silenced plants (Figure S4). On the other hand, supporting a role for *GF14e* during resistance against necrotrophs in rice, *GF14e* has been shown to be induced by exogenous application of methyl jasmonate (MeJA) and ethephon (ETH, a precursor for ethylene) [31]. It will be interesting to assess the involvement of these hormones during resistance against *Rs* in the *GF14e*- silenced plants.

Finally, *GF14e* is rapidly up regulated during ETI-mediated resistance against a bacterial pathogen in wild type Kitaake plants (Figure 6). If GF14e is a negative regulator of cell death and plant resistance, then type III effectors from *Xoo* might modulate the expression of *GF14e* to suppress plant immune responses, thereby hijacking the immune responses. In support of this hypothesis, several 14-3-3 protein isoforms recently were shown to interact in yeast two hybrid assays with bacterial effector proteins [78, 79]. Moreover, although the biological significance has not been elucidated, a bacterial effector from *X. campestris pv. vesicatoria* that suppresses PTI was shown to interact with several 14-3-3 protein isoforms in tomato [78]. Current research is addressing if *Xoo* effectors that suppress plant immune responses target GF14e, and to understand how *GF14e* modulates PTI and ETI in rice.

Materials and methods

Phylogenetic analysis of *GF14* family members

The *Hordeum vulgare* mRNA sequence corresponding to a *14-3-3* gene (accession no: Y14200.1) 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 *14-3-3* gene members from significant rice BAC hits. All sequences corresponding to different members in rice 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). cDNAs corresponding to eight GF14 members were used to conduct phylogenetic and bootstrapping analyses using MEGA version 4 [80].

Construction of RNAi vector and plant transformation

A 350 bp region in the first exon of *GF14e* was amplified from rice cultivar IR64 genomic DNA using the primers AP004003F1: 5'-CACCGTGAGGAGAATGTGTACATGGCT-3' and AP004003R1: 5'-CTTGGACTCTGGAGCAGTGGATGAA-3'. The PCR product was cloned into pENTR/D-TOPO vector (Invitrogen). The entry clone (pENTR/*GF14e*) was introduced into the pANDA vector via an LR clonase reaction using Gateway LR Clonase Enzyme Mix (Invitrogen) [81], and the clone was transformed into *Agrobacterium tumefaciens* strain EHA105. pANDA-*GF14e* was introduced into rice cv. Kitaake by *Agrobacterium* -mediated transformation as described [82].

DNA and RNA analysis

Rice genomic DNA was isolated from leaf tissue using a modified CTAB procedure [83], and 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 using the manufacturer's protocol. RNA concentrations were estimated by UV absorbance. Total RNA was treated with one unit of DNase (Promega) per ug total RNA, and cDNA was

synthesized using the Superscript III reverse transcriptase kit (Invitrogen). cDNA amplifications were performed using HotStar Taq DNA Polymerase (Qiagen). For amplification of the *GF14* transcripts, gene specific primers were designed in the 5' and 3'untranslated regions (UTR) (Table S1). *EF-1alpha* and *hygromycin* resistance genes were amplified as internal controls for each cDNA sample using previously published primers [48].

For quantitative real time PCR (qPCR), each primer pair was tested using the Bio-Rad iCycler and BioRad iQ SYBR Green Supermix (Bio-Rad), and efficiency was determined using a serial dilution of cDNA template from Kitaake wild type leaf tissue. Each reaction was repeated in triplicate. Primers with efficiency between 90 and 100% were used in subsequent experiments. SYBR green melt curves were examined to determine primer specificity. For amplification, 40 repeats of a two-step cycling protocol was performed: 95 °C for 15 sec, 60 – 62 °C 30 sec. RNA extracted from 2, 5 and 7 wk old plants was used to generate cDNA, and qPCR was performed on each plant for each gene specific primer (Table S1). Relative gene expression was determined using the ΔΔ Ct method with expression of *EF1alpha* in wild type tissue as an internal control (ddCT) [84].

Plant growth and pathogen inoculations

T_o transgenic plants that had been transferred to soil, and T_1 through T_3 segregating progenies germinated from seed, were grown with a photoperiod of 16 h light/8 h dark in

a growth chamber with photon flux of 135 μmol$m^{-2}s^{-1}$ and day/night temperatures of 28 /26 ºC. Suspensions (5 x 10^8 CFU/ml) of virulent *Xoo* (strain PXO99A) [85] were used to inoculate the second youngest and third youngest fully expanded leaves from the main tiller (M) and lateral tiller (L) of 7 wk-old T_1 segregating progeny (67 plants) using the leaf clipping method [86]. Disease was assessed at 2 wk after inoculation by measuring lesion lengths. Bacterial counts were done in 20 T_1 segregating plants including 10 T_1 LM that were resistant to *Xoo* PXO99A and 10 T_1 NLM segregant lines (no transgene) that were susceptible to *Xoo* PXO99A. The second youngest inoculated leaf in the lateral tiller (L2) was collected at 2 wk after inoculation and macerated in 5 ml sterile water using extraction bags and a hand model homogenizer (Bioreba). A 1:10 dilution series (7 dilutions) was made and 20 ul of each dilution was spotted three times onto nutrient agar plates containing 20 ug/ml of cephalexin and 50 ug/ml cycloheximide. Plates were incubated at 28^{o}C and bacterial colonies were counted at 48 h after incubation.

For experiments examining the effect of lesion mimic phenotype on susceptibility, 2 and 5 wk old plants were inoculated with *Xoo* by clip inoculation [86], and lesion lengths were measured at 7 dai. At the time of inoculation, leaves from the same plant, but which were not inoculated, were harvested for expression analyses using qPCR. Relative changes in gene expression were determined by comparing to uninoculated wild type Kitaake plants of the same age. Linear regression and p-values were calculated using the lm function in the statistical package [87]. To assess role of *GF14e* in ETI, cultures of *Xoo* PXO99A harboring a plasmid with the *avrRxo1* effector gene were grown on nutrient agar at 28 ^{o}C, and suspended in sterile distilled water to OD_{600} of 0.8. A

transgenic Kitaake rice line containing the maize resistance gene *Rxo1* was syringe infiltrated with the culture suspension as previously described [53]. Leaves were harvested at 0, 4, 8, 12 and 24 h post-inoculation and RNA extracted for qPCR analyses.

A detached leaf assay [88] was used to assess disease severity caused by *Rs* on 2-wk-old leaves from T_3 transgenic rice. In short, 1 cm plugs from *Rs* cultures grown on potato dextrose agar (PDA) at 28 ^{o}C were placed mycelia-side-up on leaves, and the inoculated leaves were incubated at 28 ^{o}C in continuous light for 48 h. Agar plugs were removed and the leaves were scanned using a CanoScan EX scanner (Canon, USA). Lesion area was measured using the program ImageJ 1.42q (http://rsb.info.nih.gov/ij). The percentage of disease area was calculated by dividing the disease area over the total leaf area inoculated.

Diaminobenzidine (DAB) staining

Leaves from T_2 *GF14e*-silenced plants were sampled at 2 and 5 WAS and immediately immersed in a 1 mg/ml solution of 3,3'-diaminobenzidine (DAB, Sigma, USA) at pH 3.8 [51]. Immersed leaves were incubated overnight in the dark at room temperature in a shaker. Leaves were then decolored in 75% ethanol and scanned using a CanoScan EX scanner (Canon, USA).

Segregation of the LM phenotype

Kitaake wild type plants and 97 T_1 segregants of a T_o *GF14e*-silenced LM plant were grown in a growth chamber. The presence or absence of the LM phenotype was recorded in all the individuals of the progeny and the LM phenotype was correlated with the presence of the transgene by PCR analysis using primers corresponding to the *hpt* gene [48]. Segregation ratios of the lesion mimic phenotype in the T_1 progeny were tested by a Chi Square (X^2) test.

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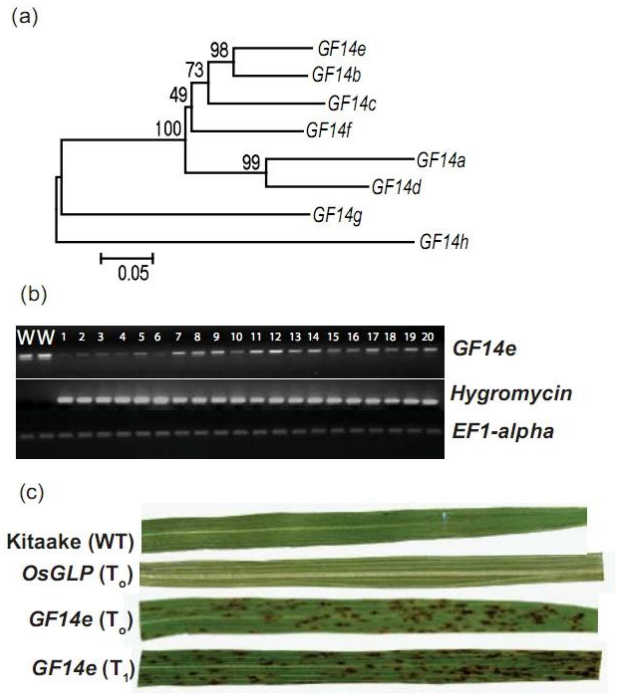
Figure 1. Silencing of *GF14e* in rice.



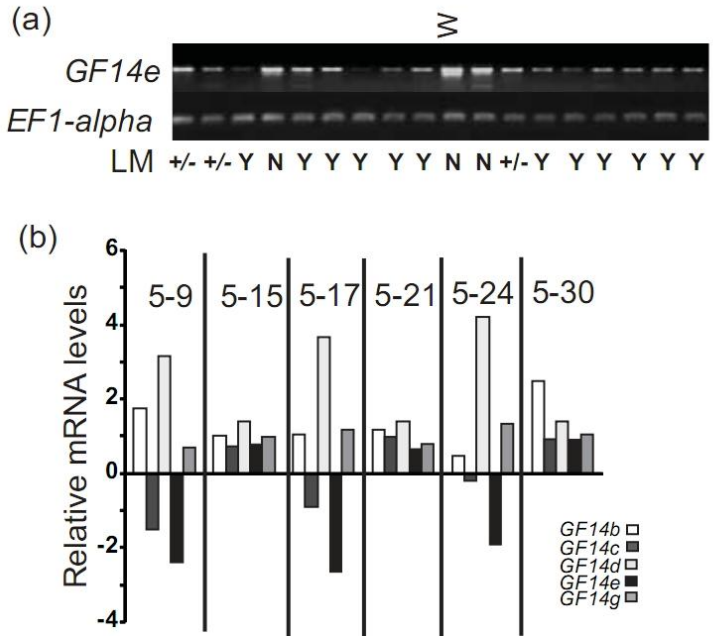
(a) Neighbor-joining cladogram of cDNA of rice *GF14* gene members. Bootstrap values are shown above well-supported nodes. The tree is drawn to scale (0.05), with branch lengths representing the number of base substitutions per site.

(b) Transcript analysis of *GF14e* in uninoculated T_0 lines containing the silencing construct using reverse transcriptase PCR (RT-PCR). Twenty independent transgenic rice lines expressing the RNAi construct were screened for *GF14e* silencing, along with wild type (WT) control lines, by RT-PCR using gene-specific primers. *EF1-alpha* was amplified as an internal control. The hygromycin resistance transgene was successfully amplified from these transgenic plants.

(c) Appearance of the lesion mimic (LM) phenotype on leaves from *GF14e*-silenced T_0 and T_1 transgenic plants. The lesion mimic phenotype (LM) is observed as dark brown

spots on the leaves of *GF14e*-silenced plants. The LM phenotype was not observed on wild type Kitaake plants (WT) or on T_0 plants silenced for a germin-like protein (*OsGLP*).

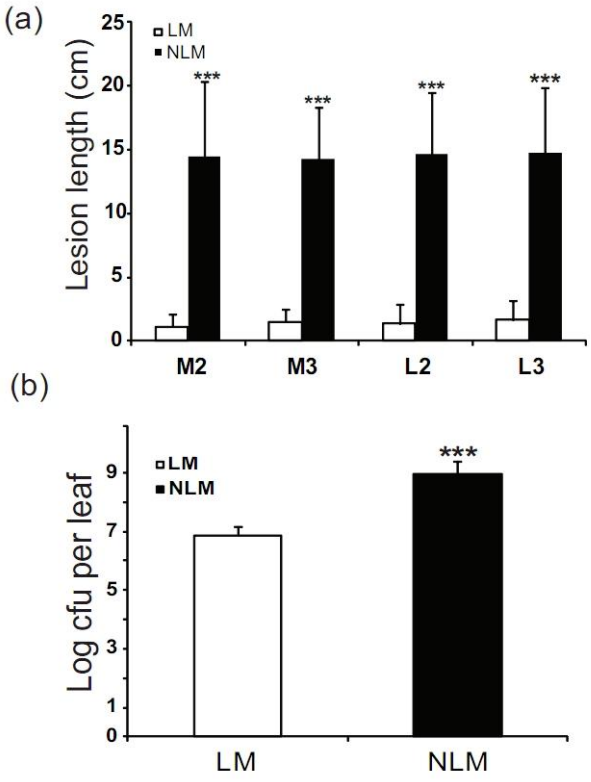
Figure 2. Correlation of the lesion mimic (LM) phenotype with *GF14e* silencing.



(a) Assessment of the silencing of the *GF14e* gene and lesion mimic development. Expression of *GF14e* in T_0 transgenic lines and an untransformed Kitaake plant (W) was assessed by using RT-PCR. The occurrence of a lesion mimic phenotype was scored as Y (necrotic lesions over the entire leaf), +/- (few necrotic lesions on the leaf), and N (no lesions). *EF1-alpha* was used as an internal control for RT-PCR.

(b) Analysis for co-silencing of rice *GF14* gene members using quantitative real time PCR (qPCR). Primers specific to rice *GF14b*, *c*, *d*, *e* and *g* were used in qPCR reactions with cDNA from an inoculated leaf of a T_3 segregating population of transgenic plants. Plants 5-9, 5-17 and 5-24 showed the LM phenotype and plants 5-15, 5-21, and 5-30 do not show LM. The resulting CT values were normalized to uninoculated Kitaake wild type plants and reference gene *EF1-alpha*.

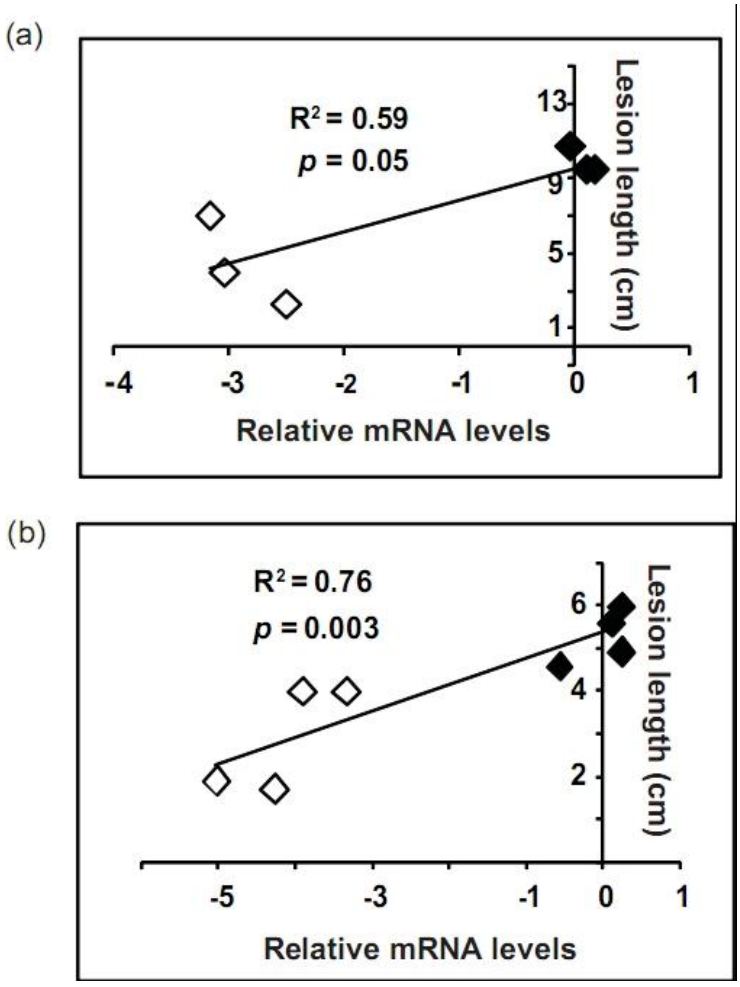
Figure 3. *GF14e*-silenced rice plants show enhanced resistance to the bacterial blight pathogen.



(a) Average lesion length after inoculation of T_1 segregants with *Xoo* PXO99A. Lesion lengths (cm) were measured at 14 dai on four different leaves, including 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. Asterisks indicate differences (***= $P < 0.0001$, Student's t test) in lesion lengths between LM (open bar) and NLM (filled bar) plants.

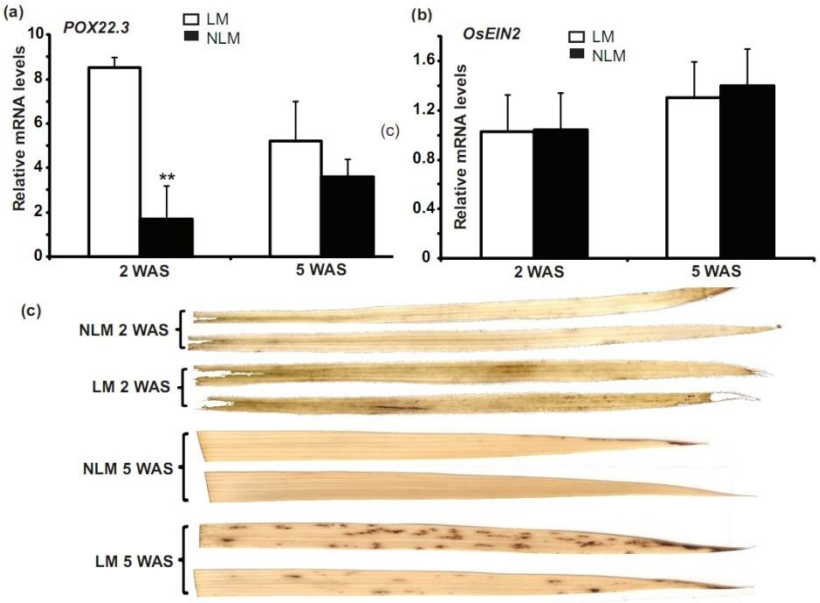
(b) Bacterial numbers in the L2 leaves from 10 T_1 LM and 10 T_1 NLM plants at 14 dpi with *Xoo* PXO99A. Asterisks indicate differences (***= $P < 0.0001$, Student's t test) in bacterial numbers between LM (open bar) and NLM (filled bar) plants.

Figure 4. *GF14e* silencing and bacterial blight resistance precede appearance of the lesion mimic phenotype.



(a , b) 2-wk-old plants (a) and 7-wk-old plants (b) were inoculated with *Xoo* PXO99A and bacterial lesion length in cm (y-axis) were measure at 7 dai. *GF14e* expression (x-axis) was assessed relative to uninoculated wild type plants of the same age. Plants showing lesion mimic phenotype (LM) are indicated as open diamonds and plants with no lesion mimics (NLM) are indicated as closed diamonds. The experiment was repeated twice with similar results. Correlation coefficient and p-value were calculated by linear regression.

Figure 5. Up-regulation of a defense response peroxidase gene in *GF14e*-silenced plants.

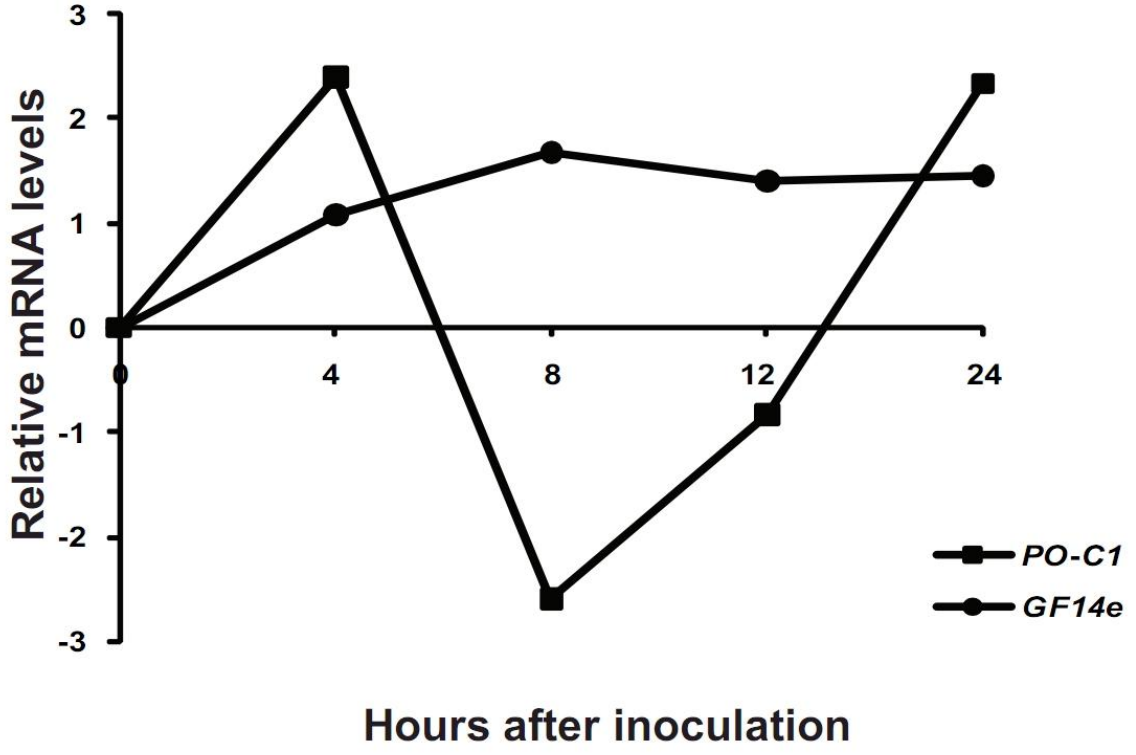


(a) Peroxidase gene (*POX22.3*) is up-regulated in the *GF14e*-silenced plants. Relative *POX22.3* mRNA levels in uninoculated tissue of 2 and 5 wk-old (2 WAS and 5 WAS, respectively) LM and NLM plants inoculated with *Xoo* (PXO99A) were measured by qPCR at 14 dpi. Asterisks indicate differences (**= $P < 0.005$, Student's t test) in expression levels of *POX22.3* between LM (open bar) and NLM (filled bar) plants.

(b) The rice ETHYLENE INSENSITIVE 2 gene (*OsEIN2*) expression is not altered in the *GF14e*-silenced plants. Relative *OsEIN2* mRNA levels were measured by qPCR as in (a). Expression values were compared to uninoculated wild type Kitaake and normalized to reference gene *EF1-alpha*. Asterisks indicate differences (**= $P < 0.005$, Student's t test) in expression levels of *OsEIN2* between LM (open bar) and NLM (filled bar) plants.

(c) Reactive oxygen species accumulate in the developing lesions on leaves from *GF14e*-silenced lines. Brown staining due to H_2O_2 accumulation and peroxidase activity was detected by DAB, and was observed in the leaves of 2 WAS and 5 WAS T_2 *GF14e*-silencing plants (LM), but not in the non-lesion mimic (NLM) segregants. All LM plants developed lesions by 3 WAS.

Figure 6. *GF14e* is upregulated and peroxidase *PO-C1* is up- then down-regulated during ETI resulting from the interaction of the *Rxo1* resistance gene and the *avrRxo1* effector gene.



Rice plants carrying the resistance gene *Rxo1* were inoculated with *Xoo* harboring a plasmid with the cognate effector *avrRxo1*. The HR resulting from ETI is clearly visible at 24 hpi. Expression of *GF14e* and *PO-C1* were assessed by qPCR. Relative *GF14e* or *PO-C1* mRNA levels were compared at 4, 8 12 and 24 hpi to the uninoculated control (0 h) and normalized to reference gene *EF1-alpha.*

Table S1. Oligonucleotides primers used in this study.

MSU v6.1 Locus[a]	Primer[b]	Sequence 5'→ 3'
LOC_Os04g38870	GF14b-qF*	GGCTGTGATTGTTGTCGGGAAA
	GF14b-qR*	GAAGTGCCACCAAGCAGTTCAA
LOC_Os08g33370	GF14c-qF*	TAGATGCCTCATGCTGCTGTCA
	GF14c-qR*	AGACGGACCACTAGCACCAAAT
LOC_Os11g34450	GF14d-qF*	TCAGGACATTGCTCTCGCAGAT
	GF14d-qR*	ACCAAGGCTGTCCAGTTCTGAT
LOC_Os02g36974	GF14e-qF*	AGGATATTGCCCTGGCAGAGTT
	GF14e-qR*	TGCAAGATTGCAAGCACGGT
LOC_Os01g11110	GF14g-qF*	AGTTGAGCTGCTGAGTAACCCT
	GF14g-qR*	AAGAGCGATCAGCGAGTGCTTA
LOC_Os07g06190	OsEIN2-qF*	GCCCACCATCTCTTACCTTCAG
	OsEIN2-qR*	GCCCAAACCAGATAACCTCGA
LOC_Os07g48020	POX22.3-qF*	ACGACATAAACGGGCCACAC
	POX22.3-qR*	TAGGTGCTAATGCCATGGCTG
LOC_Os07g48050	POC1-qF*	AGAGCCATGTCCATCATCAAGAGC
	POC1-qR*	CTCGTTTCCCGACAACAGAACAGA
LOC_Os03g08050	EF1 alpha-qF*	AGCCTCGTTCAAATGGTGGT
	EF1 alpha-qR*	TAGTGCACATTGCGAGCAGA
LOC_Os02g36974	AP004003 F1+	GGATTCCTTTATCTGGCACTATTGAAG
	AP004003 R1+	GTGAATCAAGAA CCATCGTGCACAC
LOC_Os08g33370	AP003881-F2+	TTCTGGAGAGACCCCATGGCTTTC

LOC_Os08g33370	AP003881-F2[+]	TTCTGGAGAGACCCCATGGCTTTC
	AP003881-R2[+]	GACAGCAGCATGAGGCATCTAAATC
LOC_Os08g37490	AP004643-F2[+]	CTCAACTCAACTCCACCCGAATC
	AP004643-R2[+]	GTAAGCAAAGACCCG ACGAAAGG
LOC_Os03g13170	Ubiquitin-F[+]	CCAGGACAAGATGATCTGCC
	Ubiquitin-F[+]	AAGAAGCTGAAGCATCCAGC

[a]MSU Rice Genome Annotation: http://rice.plantbiology.msu.edu/

[b]F = forward primer, R =reverse primer

* Primers used for qPCR analyses

+ Primers used for RT-PCR

Figure S1. Multiple sequence alignment showing the sequence similarity among the *GF14* gene family member in rice for the 350 bp region of *GF14e* that used as a target for RNAi (indicated as PM6).

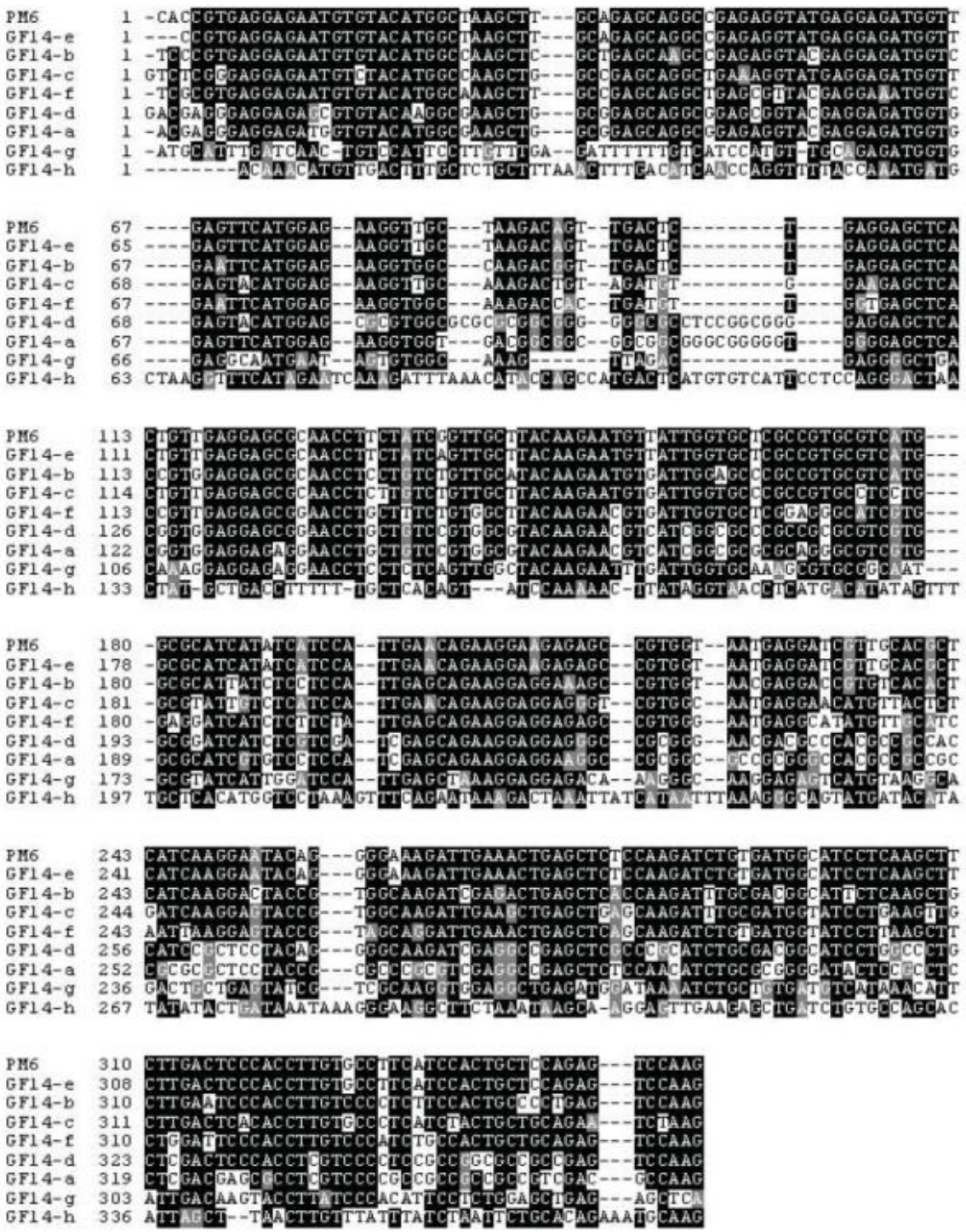


Figure S2. *GF14c* and *b* gene members are not co-silenced in the *GF14e*-silenced plants.

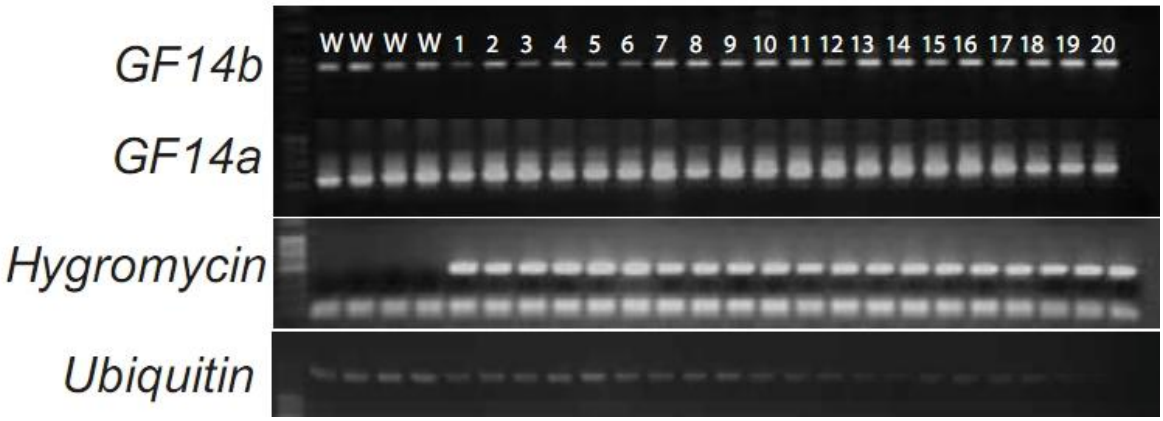


Figure S3. Expression of the hygromycin resistance (*hpt*) transgene in the T_3 generation of *GF14e*- silenced transgenic plants.

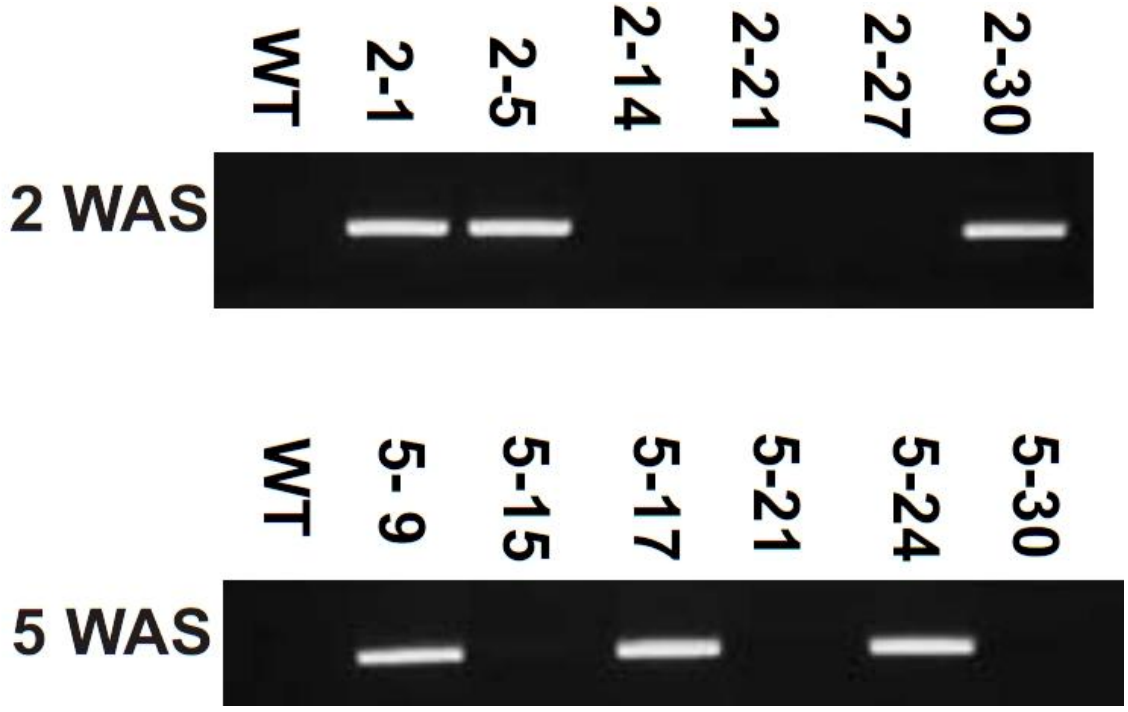


Figure S4. Lesion mimic plants show enhanced resistance to *Rhizoctonia solani (Rs)*.

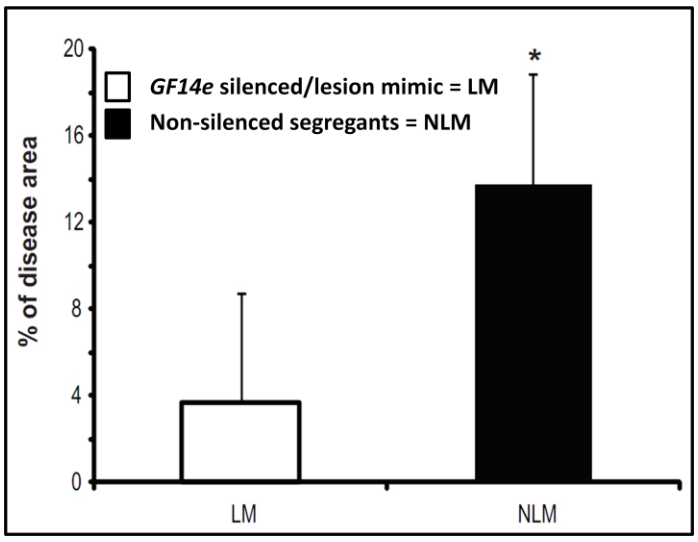
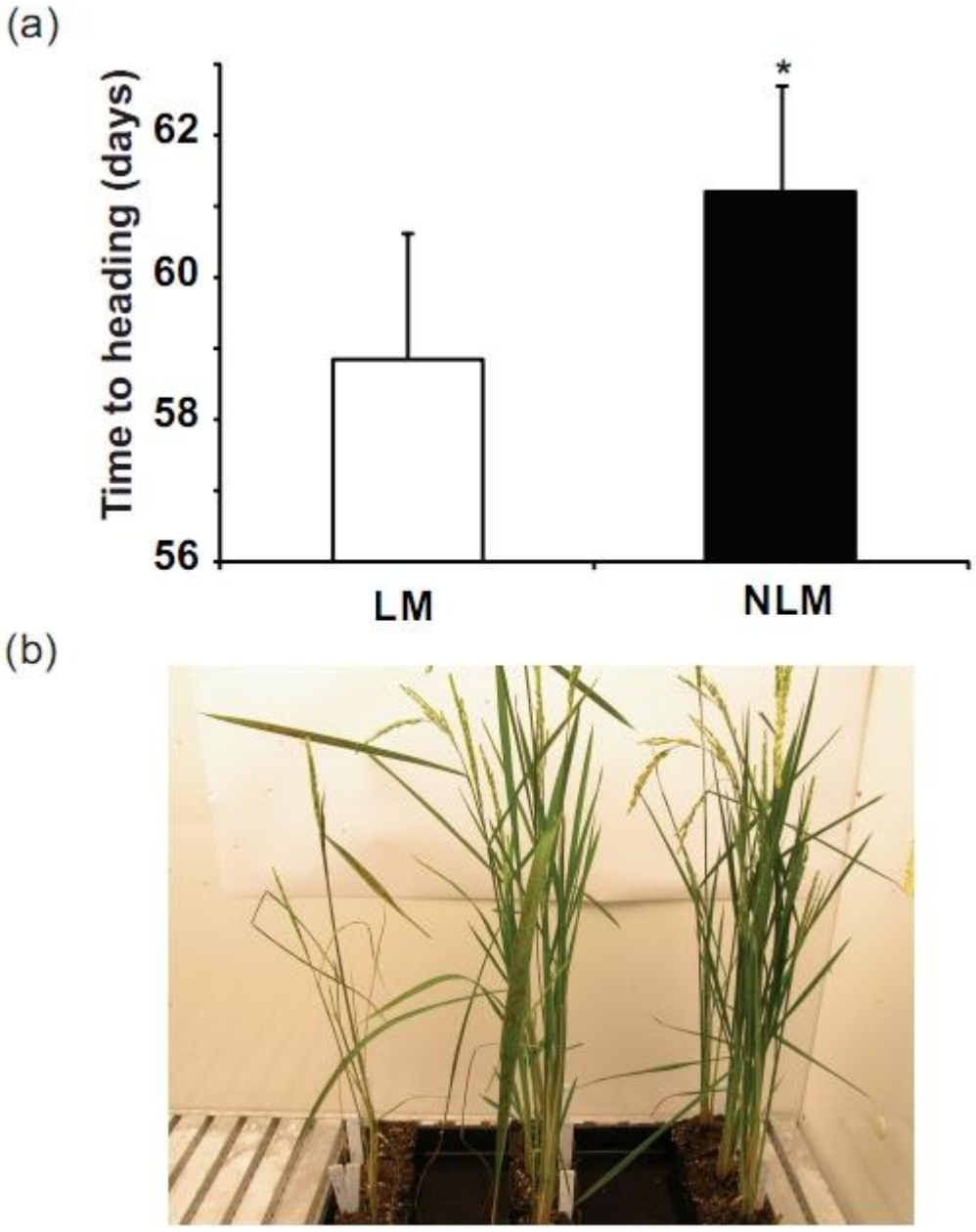


Figure S5. Developmental related phenotypes of the *GF14e*-silenced plants.



CHAPTER 4

GF14E NEGATIVELY REGULATES PTI AND ETI THROUGH WRKY TRANSCRIPTION FACTORS

Introduction

14-3-3 proteins are ubiquitous in all eukaryotes. This family has been implicated in many plant cellular processes, including regulation of nitrogen metabolism [1], energy production [2] and transcriptional control in response to hormones [3]. The 14-3-3 proteins also are predicted to contribute to plant disease and defense responses. Recent studies have shown that an interaction between a 14-3-3 protein and a bacterial effector protein (AvrRxv) is necessary for effector triggered immunity (ETI) in tomato and *Xanthomonas campestris* pv. *vesicatoria* interactions [4]. The details of how this class of proteins function in defense responses is only beginning to emerge. For example, in tomato, the 14-3-3 protein Tft7 positively regulates defense signaling through a MPKKK [5], and in Arabidopsis, *GRF6* positively regulates basal and resistance gene-mediated resistance to a fungal pathogen [6].

In rice, there are eight 14-3-3 genes, named *GF14a-h* [7]. Only the function for *GF14c* has been described [8, 9]. *GF14c* regulates multiple metabolic processes, including flowering and phytohormone signaling [8, 9]. Three rice *GF14* family members, *GF14b,*

GF14c and *GF14e*, were predicted to play a role in defense responses based on transcriptional activation during defense responses with pathogens [7]. In a previous paper, we examined the role of *GF14e* in rice defense responses by using a transgenic RNAi approach (Chapter 3). With reduced *GF14e* expression, we observed a lesion mimic (LM) phenotype and enhanced resistance to two distinct rice pathogens, the biotrophic bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* and the necrotrophic fungal pathogen *Rhizoctonia solani*. In contrast to the observations of positive regulation in tomato [5] and Arabidopsis [6], our work indicated that GF14e negatively regulates plant resistance to multiple pathogens in rice.

Generally, plant disease resistance or immunity is described as PAMP triggered immunity (PTI) triggered by pathogen associated molecular patterns (PAMPs) and ETI triggered by effectors (for review, see [10]). The two are distinguished by the speed of immune response activation, duration, and effectiveness; ETI responses are faster, longer lasting and more effective than those in [10]. Although the accumulation of information now suggests that this distinction is artificial, and that there is a continuum between PTI and ETI [10]), there is utility in asking if the resistance regulated by 14-3-3 proteins such as GF14e is more similar to ETI or to PTI. There are, for example, defense response genes and regulatory genes whose upregulation is distinct between the two. For example, of four peroxidase genes characterized in rice, one, *Pox8.1*, is upregulated during PTI- and ETI based defense responses in rice [11], two (*PO-C1* and *Pox22.3*) are ETI specific [11, 12]. The fourth, *Pox5.1*, is transiently induced during wounding, and is not associated with PTI and ETI responses [11].

Our previous work hinted that *GF14e* negatively regulated ETI by inhibiting the activation of defense response enzymes and the accumulation of reactive oxygen species (ROS) (Chapter 3). Enhanced resistance in the *GF14e* silenced lines was correlated both with higher expression of an ETI-associated peroxidase gene (*POX22.3*) and higher levels of ROS (Chapter 3). ROS are a hallmark of defense responses, generating an inhospitable environment for pathogens, as well as acting as signaling molecules [13]. Additionally, ROS such as H_20_2, serve as substrates for peroxidases; peroxidases use the H_2O_2 to oxidize a wide variety of organic molecules by removing a hydrogen atom [14]. Defense responsive peroxidases, specifically, use H_2O_2 to enhance plant cell wall structures, such as lignin, that can impede pathogen ingress [14]. The enhanced expression of the ETI-related peroxidase and accumulation of ROS supported a role for GF14e as a negative regulator of ETI, but did not indicate if GF14e also regulates PTI, nor did it provide clues as to how GF14e functions to inhibit resistance.

Understanding the biological functions of 14-3-3 proteins requires first identifying the client interacting protein, and, second determining how the 14-3-3's affect that function. 14-3-3 proteins bind phosphorylated client proteins at the consensus sequence R/K-X(1,2)-pS/pT-X-P [15]. Relevant to this study, one activity exhibited by a rice 14-3-3, *GF14c*, is the binding of a transcription factor (TF), *OsBZR1* or a putative transcriptional modulator, *OsHd3a*, and sequestering them away from the nucleus to limit their ability to activate transcription [8, 9]. Upon upstream signaling by brassinosteroid or for flowering

initiation, OsBZR1 and OsHd3a are dephosphorylated, allowed to dissociate from the 14-3-3 complex and translocate to the nucleus, where transcriptional activation of their specific target genes occurs [8, 9]. These studies provide insight into 14-3-3 protein function in modulating transcriptional responses in rice. That *GF14c* in both of these studies acts as a negative regulator of a downstream response provides a starting point for the mechanism of defense regulation observed by *GF14e* (Chapter 3). However, to develop a model for *GF14e* function in defense signaling, a GF14e client is needed. Based on our previous work with *GF14e* silenced plants (Chapter 3) and the function of GF14c, we hypothesized that GF14e binds and negatively regulates a positive regulator of plant defense responses, potentially a transcription factor or transcriptional modulator.

WRKY transcription factors (TFs) have been implicated in modulation of defense response gene expression. They bind W-box cis elements (T/C-TGAC-T/C) in gene promoters, regulating the gene's transcription [16]. This family of TFs is one of the largest transcription factor families in plants, and they are involved in the regulation of gene expression for a wide variety of plant functions including germination, cold stress tolerance and disease resistance [16-18]. WRKY TFs suppress transcription of genes in some pathways, while activating transcription of genes in other pathways. WRKY TFs have been shown to interact with Arabidopsis 14-3-3 proteins by using tandem affinity purification experiments [19]. In rice, several WRKY TFs are induced during defense responses, and the promoters of several defense response genes are enriched in WRKY boxes [20]. However, there is no information on whether rice WRKY TFs are regulated

by or are clients of 14-3-3 proteins, and how any interaction would affect defense responses.

Our goal is to understand how GF14e negatively regulates disease resistance in rice. In this study, we show that (1) PTI and ETI-related defense response genes, including peroxidases and WRKY TFs, are transcriptionally activated in *GF14e* silenced lines, (2) signaling for GF14e-regulated defenses is through the SA pathway, and (3) some defense WRKY TFs have potential 14-3-3 interaction domains. Our results support the model that GF14e negatively regulates WRKY TFs that are positive regulators of rice defense responses

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Results

***GF14e* silenced lines show differential regulation of defense response genes**

To determine if enhanced resistance in *GF14e* silenced lines is associated with PTI and/or ETI-based, expression of four peroxidases was analyzed by using qPCR. *Pox8.1* is upregulated during PTI- and ETI based defense responses in rice [11], *PO-C1* and *Pox22.3* are ETI specific [11, 12], and *Pox5.1* a wound-inducible peroxidase [11]. Three peroxidase genes, *PO-C1*, *Pox22.3* and *Pox8.1*, showed enhanced expression that correlated with *GF14e* silencing at 2 and 5 weeks after sowing (WAS) (Figure 1). In contrast, *Pox5.1* did not show enhanced expression in these silenced lines. These results suggest that *GF14e* negatively regulates both PTI and ETI, and is not involved in regulation of wound responses.

Upregulation of peroxidases during defense responses can be associated with signaling through salicylic acid (SA) [14] mediated pathways. Since SA mediated signaling has been shown to be antagonistic to jasmonate (JA) and ethylene (ET) signaling [21], we tested the expression of genes associated with these pathways. *OsEIN2* and *OsERG3* are associated with ET pathways, while *JAmyb* genes are typically associated with JA pathways. No differential regulation of these genes was correlated with *GF14e* silencing (Figure 2 and data not shown). Taken together with the findings that PTI- and ETI-associated peroxidases are upregulated and a wound-inducible peroxidase (*Pox5.1*) is not induced (FIGURE 1), these results suggest that *GF14e* negatively regulates SA-mediated signaling, but does not regulate JA or ET pathways.

Upregulated peroxidases have multiple W-boxes

WRKY TFs are known to regulate various defense response genes in rice [22]. To identify possible transcriptional regulators of the peroxidase genes, we examined the 1-kb upstream sequences for the presence of W-box promoter elements. With the exception of *Pox5.1*, the wound-inducible peroxidase, all of the 1 kb upstream regions contained at least one W-box (Table 1). *PO-C1*, the most highly upregulated peroxidase in *GF14e* silenced lines, had four W-boxes, the most of all peroxidases analyzed (FIGURE 1, TABLE 1). The occurrence of multiple W-boxes in the peroxidase promoter regions suggests these genes might be regulated by WRKY TFs.

WRKY TFs are differentially regulated in *GF14e* silenced lines

Previous work suggests that a number of WRKY TFs are positive regulators of PTI and/or ETI [16, 20](A. Seck, unpubl. data). *OSWRKY53* is associated with PTI and ETI [23, 24](A. Seck, unpubl data), while *OSWRKY68* is associated with ETI only [20](A. Seck, unpubl. data). To date, *OSWRKY24* has only been associated with PTI [20]. The expression of *OsWRKY24*, *53* and *68* was examined in *GF14e* silenced lines. *OsWRKY24* consistently showed upregulation associated with *GF14e* silencing (Figure 3). At 2 and 5 WAS *OsWRKY53*, the putative PTI and ETI WRKY, showed upregulation associated with LM phenotype and suppression of *GF14e* expression, while a putative ETI response WRKY TF, *OsWRKY68* showed consistent upregulation associated with silencing and the lesion mimic phenotype at 5 WAS, but not at 2 WAS (FIGURE 3). These results suggest that *GF14e* negatively regulates PTI- and ETI- associated WRKY TFs.

WRKY TFs contain 14-3-3 interaction domain sequences

Previous studies have shown that plant 14-3-3 proteins can interact with WRKY TFs [19]. To determine if the defense response WRKY TFs tested above could potentially interact with 14-3-3 proteins, sequences from previously identified 14-3-3-interacting proteins from rice, barley and Arabidopsis [4, 8, 25-27] were aligned to generate a consensus sequence (Figure 4) [28]and this sequence was used to predict 14-3-3 interacting proteins from the entire rice proteome (MSU annotation, V6). Five WRKY TFs were identified from among the more than 1,000 predicted interactors (TABLE 2). *OsWRKY24*, *53* and *68*, the three defense response WRKY TFs shown to be upregulated

in the *GF14e*-silenced plants, were not among the five WRKY TFs identified in the original screen for 14-3-3 interactors. The screen had used a stringent search for the consensus sequence and may have missed diverged interaction domains. A relaxed screen specifically of *OsWRKY24, 53* and *68* for sequences that fit the rationale of 14-3-3 interactions was performed, but relationships were weak. Although not conclusive, these results suggest that *OsWRKY24, 53* and *68* are not likely 14-3-3 interactors. However, *OsWRKY13* was identified in the initial screen as having the consensus 14-3-3 interaction domain. Thus, *OsWRKY13,* which has been implicated in modulating defense responses in rice [20], is a candidate for a *GF14e* interactor.

Discussion

GF14e, a 14-3-3 gene family member, was predicted to encode a negative regulator of rice defense responses (Chapter 3). In this study, we demonstrate that GF14e regulates both PTI and ETI-mediated responses, and provide insights into how GF14e functions to regulate plant defense. Based on examination of expression of different peroxidase and WRKY TF genes, including those associated with PTI, ETI, and wound-responses, as well as genes associated with various signaling pathways (SA, JA, and ET), we propose that GF14e negatively regulates both PTI and ETI-mediated responses that signal through the SA pathway. The upregulation of WRKY TFs in *GF14e* silenced lines suggest that GF14e negatively regulates defense responsive WRKY TFs. A few WRKY TFs contain sequences consistent with14-3-3 interaction domains; whether or not GF14e directly interacts with one or several WRKY TFs remains to be examined. However, given the

data presented here, we conclude that *GF14e* acts at the level of or upstream of WRKY activation during plant defense responses.

Microbial perception, be it during a PTI or ETI response, orchestrates reprogramming of a plants' transcription to favor defense (for review see [29, 30]). Intriguingly, gene profiles elicited during PTI and ETI are highly similar, but vary in timing and intensity [31, 32]. Transcripts differentially regulated during both types of immunity include genes involved in signal transduction and downstream responses such as genes encoding transcription factors and active oxygen generating /cell wall modifying genes [31]. Thus, the increased expression of WRKY transcription factors and peroxidase genes related to both PTI and ETI in the *GF14e*-silenced rice was not unexpected. Furthermore, these results are consistent with the previously observed accumulation of ROS and the upregulation and increased activity of several peroxidases during PTI and ETI [11, 12, 33]. *GF14e*-silenced rice plants also were shown to accumulate ROS, particularly in the developing lesion mimics (Chapter 3). These data taken together suggest that *GF14e* negatively regulates both PTI and ETI.

Genes upregulated as a consequence of *GF14e* silencing were largely associated with SA-mediated signaling. Expression of genes associated with JA, including a wound-inducible peroxidase, or ET signaling were not affected by *GF14e*-silencing. This result indicates that *GF14e* negatively regulates SA-mediated defense responses, though the accumulation of SA in the silenced lines was not tested. Studies using WRKY TF

overexpressing plants showed significant downregulation of JA and ET-related signaling genes, and upregulation of SA-related genes [20].

WRKY TFs are key regulators, both positive and negative, of both PTI and ETI (for review, see [16]). For example, in barley, HvWRKY1 and HvWRKY2, which bind to and repress basal defense genes [34]. The WRKY DNA binding domain, or W-box, named after the almost invariant WRKY amino acid sequence at the N-terminus, is about 60 aa long, and is the defining feature of WRKY TFs. The peroxidases which were upregulated in the *GF14e*-silenced plants contained between two and four W-boxes (Table 1). In barley, the transcription factor HvWRKY38 requires two W-boxes for efficient binding, and interestingly, those W-boxes are in opposite orientation. In some cases, multiple boxes have a synergistic effect on transcription. In line with this, expression of *PO-C1*, which had four W-boxes in different orientations (Table 1), was consistently higher than expression of the other two induced peroxidases (Fig. 1). Enrichment of WRKY binding domains in promoter regions of peroxidase genes up-regulated in the *GF14e* silenced lines suggested WRKY TF control their expression, and that GF14e negatively regulates these WRKY TFs.

Almost all WRKY TFs bind to the same core sequence, so knowing that W-boxes are present doesn't reveal which specific WRKY TFs is involved. Adjacent sequences have been shown to be required for binding site preference in Arabidopsis, and in fact, comparisons of the sequence context surrounding the W-Boxes specific for particular

WRKY TFs has been used in limited examples to predict specificity [35]. However, no such information is available for the rice WRKY TFs and W-Boxes. Thus, for this analysis of WRKY TF interaction with 14-3-3 proteins, from the >100 WRKY TFs in rice, we focused on a few whose role in ETI and/or PTI had been predicted [20, 24, 36]. In Arabidopsis, 14-3-3 proteins were shown to interact with WRKY TFs using tandem affinity purification experiments [19]. A search for the presence of a concensus sequence for a14-3-3 interaction domain among all rice transcripts identified five WRKY TFs, of which two have been implicated in defense responses. These findings are consistent with (but do not prove) an interaction of GF14e with one or more WRKY TFs.

Model for GF14e function in defense responses

Overall, these results support the model that GF14e negatively regulates WRKY TFs that are positive regulators of rice defense responses. We propose that *GF14e* negatively regulates cell death and resistance in rice in a manner similar to previously described functions of 14-3-3s in other plant systems where the 14-3-3 protein sequesters a transcription modulator in the cytoplasm [8, 9]. Following an upstream signal, the client transcription factor is dephosphorylated and allowed to translocate to the nucleus, where it activates transcription of target genes. This model proposes that GF14e physically interacts with a WRKY TF and retains it in the cytoplasm prior to upstream defense signaling that leads to the dephosphorylation of one or several WRKY TFs, dissociation of the TF from the 14-3-3 protein and its subsequent translocation to the nucleus, where it activates transcription of defense response genes (Figure 5). Interestingly, there are no reports of insertion mutants in *GF14e* coding sequence. This, coupled with the

observation that plant transformations with artificial microRNA constructs designed to silence *GF14e* and the observation that no silenced line showed complete silencing of the target suggest the possibility that *GF14e* knockouts may be lethal.

Materials and methods

Plant and microbe growth conditions

Plants were grown in greenhouse conditions in the Colorado State University greenhouses as described [37]. Rice lines (T_3) silenced for *GF14e* expression were previously generated by RNAi (Manosalva, Bruce and Leach, accepted pending revision). At 2 and 5 weeks after sowing, the youngest fully expanded leaf was removedand immediately frozen in liquid nitrogen for total RNA extraction.

Total RNA extraction and genomic DNA digestion

One leaf from each plant was ground to a fine powder under liquid nitrogen and immediately mixed with TRIzol reagent for extraction of RNA according to the manufacturer's instructions (Catalogue #15596-018, Invitrogen, Carlsbad, CA, USA). The extracted RNA was resuspended in HPLC water, and treated with an RNase-free DNase (RQ1, Catalogue #M6101, Promega, Madison, WI, USA) to remove DNA contamination. Decontaminated RNA was again extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and quantified using a NanoDrop spectrophotometer (ND1000, NanoDrop Technologies, Wilmington, DE, USA). RNA quality was determined by gel electrophoresis.

cDNA generation and qPCR

For generation of cDNA, anchored oligo dT primers were used to amplify 2 ug of total RNA in a reverse transcriptase reaction (SuperScript III, Catalogue #18080-051, Invitrogen, Carlsbad, CA, USA). Twenty-five ul reactions were prepared in triplicate for each primer pair using Biorad's SYBR green supermix (Catalogue #170-8880, Biorad, Hercules, CA, USA). Primer combinations used in this study were designed using the IDT PrimerQuest program (http://www.idtdna.com) and are listed in Table 3. All primer pairs were tested for efficiency and specificity in a dilution series followed by SYBR green melt curve analysis. All primer pairs used in this study had an efficiency > 90%.

Bioinformatics

14-3-3 interaction sequences were aligned from published manuscripts [4, 8, 25-27] and used to generate a sequence logo [28]. The sequence logo consensus was used to search for proteins containing this sequence in the MSU version 6.1 rice peptide sequences using ScanProsite [38]. Promoter sequences from the MSU version 6.1 1 kb upstream database were examined for the presence of a W-box ([T/C]TGAC[T/C]) on either strand using a custom PERL script (R. Davidson, Unpublished).

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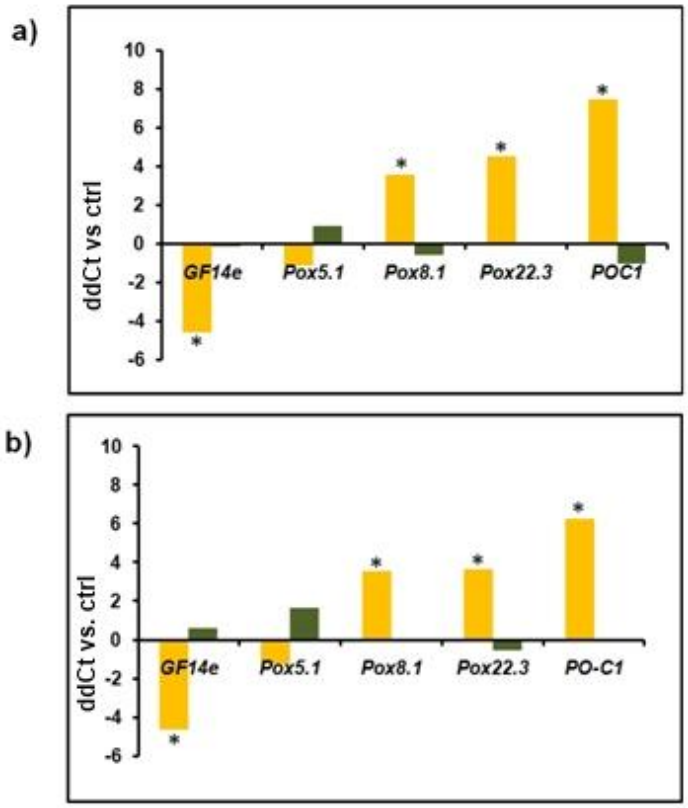
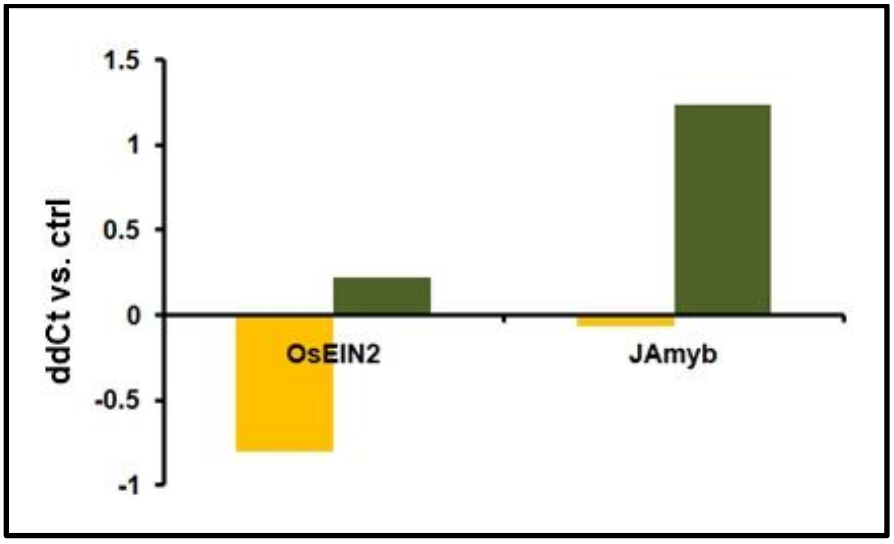
Figure 1. *GF14e* silenced lines show significant upregulation of defense response peroxidases



Gene specific primers were used in qPCR with cDNA generated from a segregating T2 population of *GF14e* silenced plants of two different ages. Relative expression of *GF14e*, *Pox5.1*, *Pox8.1*, *Pox22.3*, and *PO-C1* was examined in *GF14e* silenced LMs (yellow) and segregants (green) at a) 2 WAS and b) 5 WAS. Each bar represents measurement from two independent plants from each phenotypic class. All data are normalized to wild type Kitaake using *EF1alpha* as an internal control. Bars marked with an asterisk are significant at $p < 0.05$ in a Student's t-test.

Figure 2. Ethylene and jasmonate signaling pathways are not affected in *GF14e* silenced plants



Gene specific primers were used on cDNA from a segregating T2 population of *GF14e* silenced plants. Relative expression of *OsEIN2* and *JAmyb* was examined in *GF14e* silenced LMs (yellow) and segregants (green) at 5 WAS. Student's t-test indicated no significant difference between the two phenotypic classes.

Table 1. Peroxidases upregulated in GF14e silenced lines have multiple W-boxes in 1 kb upstream sequence

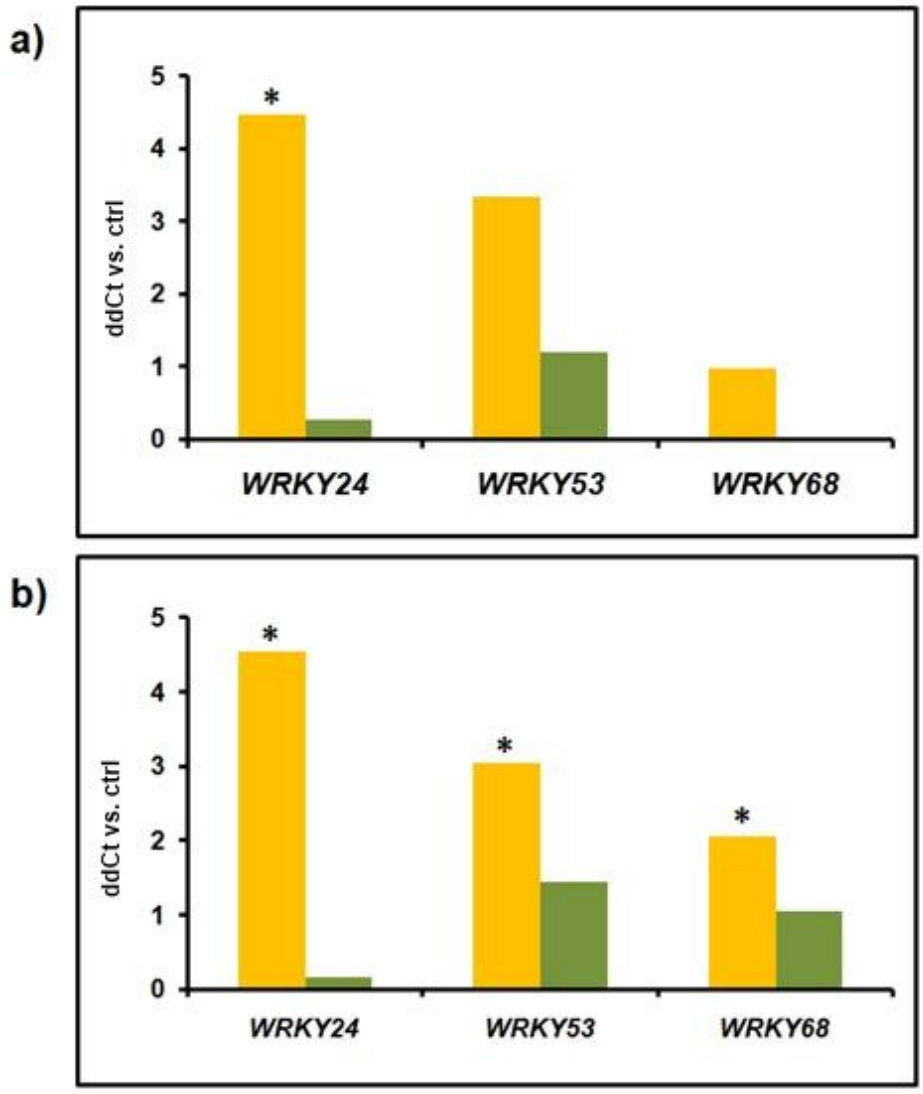
MSU v6.1 locus[a]	Gene[b]	W-box count (S/A)[c]
LOC_Os07g48040	*Pox5.1*	0/0
LOC_Os07g48010	*Pox8.1*	2/1
LOC_Os07g48020	*Pox22.3*	2/0
LOC_Os07g48050	*PO-C1*	1/3

[a] Published peroxidase sequences [11,12] were used in a BLAST search against the MSU version 6.1 annotation of the public rice cv. Nipponbare genome sequence [39] to identify corresponding genes.

[b] Gene names as published in research identifying these genes [11,12].

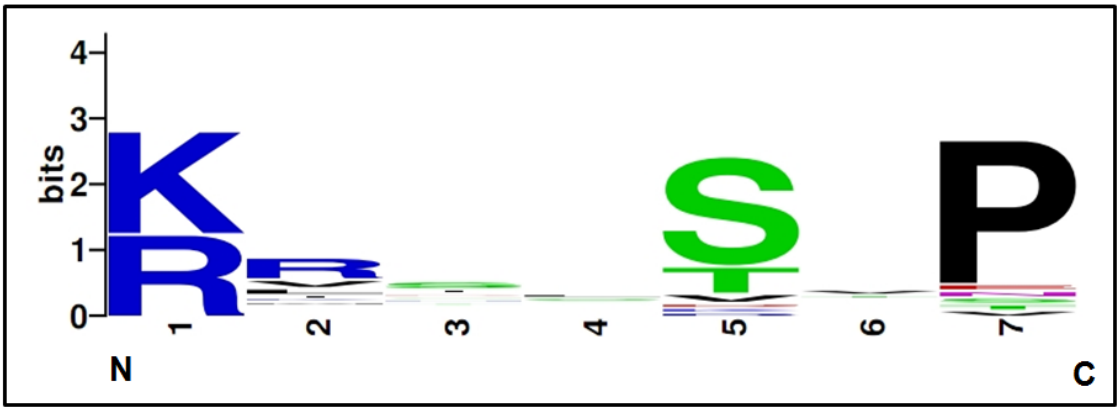
[c] W-box elements in the 1kb upstream sequence from the MSU version 6.1 annotation of the rice genome were identified on both strands using a custom PERL script (R. Davidson, unpublished). S – sense strand A – antisense strand

Figure 3. Rice WRKY transcription factors are significantly upregulated in *GF14e* silenced rice plants



Gene specific primers were used on cDNA from the same plants as Figure 1. Relative expression of *OsWRKY24*, *OsWRKY53* and *OsWRKY68* was examined in *GF14e* silenced LMs (yellow) and segregants (green) at a) 2 WAS and b) 5 WAS. Bars marked with an asterisk are significant at $p < 0.05$ in a Student's t-test between each phenotypic class.

Figure 4. Alignment of multiple putative 14-3-3 interaction domain sequences to generate a consensus sequence



Putative 14-3-3 interaction domains identified in multiple studies of confirmed clients [4,8, 34-36] were aligned using ClustalW [40]. The resulting alignment was used to generate a consensus sequence logo [28] [RK]-[RV]-X(2)-[ST]-X-P, which was then used in a scan of the rice proteome for putative 14-3-3 clients in rice [38].

Table 2. Rice WRKY transcription factors contain putative 14-3-3 interaction domains

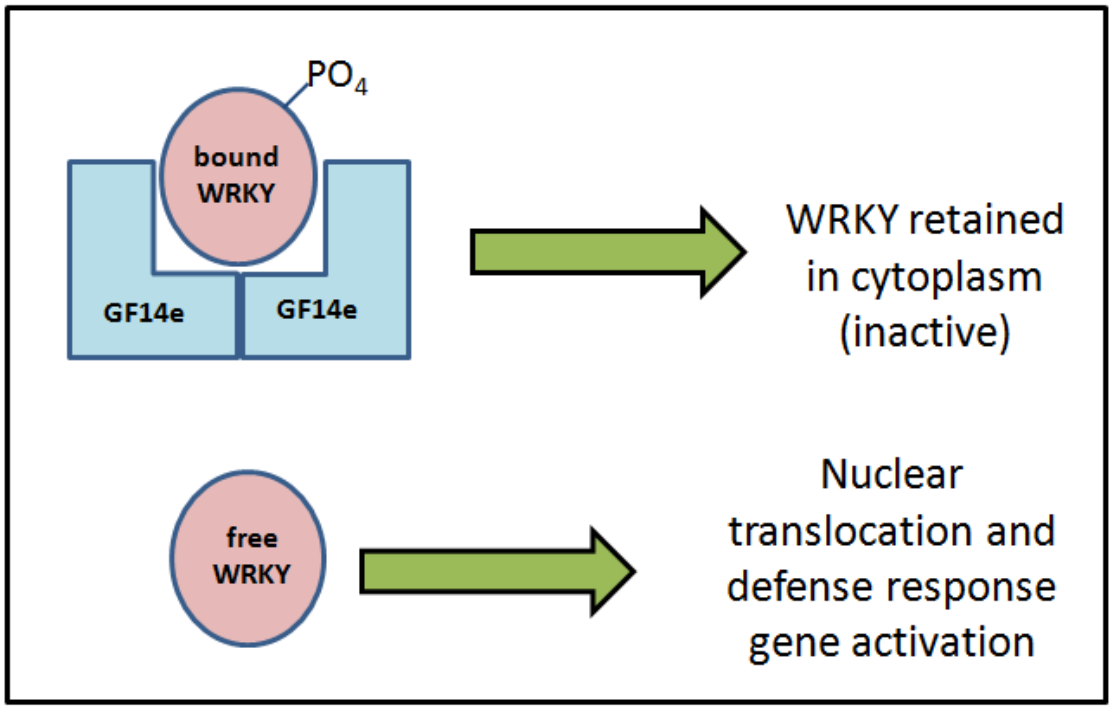
MSU v6.1 locus[a]	Gene[b]	Interaction domain (position)[c]
LOC_Os03g58420	*OsWRKY6*	KRRSSPP (65-71)
LOC_Os01g54600	*OsWRKY13*	KRVVSVP (74-80)
LOC_Os01g53040	*OsWRKY14*	KRVVTVP (69-75)
LOC_Os08g38990	*OsWRKY30*	RVGYSTP (111-117)
LOC_Os06g30860	*OsWRKY31*	RVAASGP (168-174)

[a] Locus identifier from the MSU version 6.1 annotation of the public rice cultivar Nipponbare genome [39].

[b] Gene name associated with the MSU version 6.1 annotation [39]

[c] Putative 14-3-3 interaction domain sequence and amino acid position within the primary amino acid sequence as identified using the query sequence [RK]-[RV]-X(2)-[ST]-X-P generated from an alignment of multiple 14-3-3 interaction domains [28].

Figure 5. GF14e negatively regulates defense responsive WRKY transcription factors



Cytoplasmic GF14e binds a phosphorylated WRKY TF and sequesters it in the nucleus. Upon unidentified upstream defense signaling, the TF is dephosphorylated and dissociates from the GF14e complex, allowing it to translocate to the nucleus where it activates defense response gene expression.

Table 3. Oligonucleotide primers used for qPCR in this study

MSU v6.1 Locus[a]	Primer[b]	Sequence 5'→ 3'
LOC_Os02g36974	GF14e-qF	AGGATATTGCCCTGGCAGAGTT
	GF14e-qR	TGCAAGATTGCAAGCACGGT
LOC_Os07g06190	OsEIN2-qF	GCCCACCATCTCTTACCTTCAG
	OsEIN2-qR	GCCCAAACCAGATAACCTCGA
LOC_Os07g48020	POX22.3-qF	ACGACATAAACGGGCCACAC
	POX22.3-qR	TAGGTGCTAATGCCATGGCTG
LOC_Os07g48050	POC1-qF	AGAGCCATGTCCATCATCAAGAGC
	POC1-qR	CTCGTTTCCCGACAACAGAACAGA
LOC_Os03g08050	EF1 alpha-qF	AGCCTCGTTCAAATGGTGGT
	EF1 alpha-qR	TAGTGCACATTGCGAGCAGA
LOC_Os07g48040	Pox5.1-qR	AGCCTTGCTCCTTCTACAGATAGC
	Pox5.1-qR	TTGGTCTCGTTGTATAGCCTCGTC
LOC_Os07g48010	Pox8.1-qF	CTTCGACCTCGAGAACCTCATCAA
	Pox8.1-qR	TTGGTCTCGTTGTAGATCCTTCCC
LOC_Os01g61080	OsWRKY24-qF[c]	TTCTTCCAGAACTCGCTCTACTGA

	OsWRKY24-qR[c]	ACAAGTACAAAACCCCCCTAAACTAC
LOC_Os05g27730	OsWRKY53-qF	CTTCAAGGAGCAAGAGCAGCAAGT
	OsWRKY53-qR	GCCGTTGTAGGTGCACTTGTAGTA
LOC_Os04g51560	OsWRKY68-qF[c]	CGGCAATCAGCTCGAAGATC
	OsWRKY68-qR[c]	GTAGTAGCCCCGTGGGTAAGG
LOC_Os11g45740	JAmyb-qF	TGTGTATCTGGTGTAACAATGG
	JAmyb-qR	TCATCATCACCATGCACATG

[a]MSU Rice Genome Annotation: http://rice.plantbiology.msu.edu/

[b]F = forward primer, R =reverse primer

[c] Primer design from Qiu, et al. [20]

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

Plant disease and defense are governed by a complex exchange of signals and responses. This dissertation work describes a technological advance to facilitate the study of the signal transduction process (as well as other plant processes) and the characterization of the role for one gene family member involved in regulation of plant defense signaling.

The study in Chapter 2 demonstrates that deleted rice genes and genomic regions can be localized by hybridization of genomic DNA to oligonucleotide arrays. The technique is broadly applicable to discovery of deletions in other organisms, provided microarrays with sufficient coverage are available. With the development of next generation sequencing technologies, the microarray technique could be considered to be obsolete for rice, though it can still be used to inform future research using the mutant collection developed by the International Rice Research Institute [1]. For example, our data suggests that approximately 5,000 mutant hybridizations has a 92% probability of finding a mutation in every gene in the rice genome [2]. In addition, the genome browser developed as part of that study is amenable to accepting data from diverse deletion discovery methods.

We used the technique to discover a deleted gene that was responsible for a lesion mimic phenotype in rice. Following our publication [2], Fujiwara et al. used a map-based cloning approach to clone the gene involved in the *spl1* or the Sekiguchi lesion

phenotype; their work confirmed our report that the *spl1* phenotype was caused by a mutation in a chromosome 12 cytochrome P450 [3]. They also demonstrate that this gene catalyzes the enzymatic conversion of tryptamine to serotonin. *spl1* lesion mimics show enhanced resistance to pathogens [4], indicating a possible role for tryptamine and serotonin in defense responses.

Our research begins to describe a function for a member of the 14-3-3 gene family (*GF14e*) in rice defense responses. In Chapter 3, transgenic *GF14e* silenced plants were developed, and silencing of *GF14e* was correlated with the expression of a lesion mimic phenotype and with enhanced resistance to both fungal and bacterial pathogens. This data, coupled with the observed enhanced expression of known defense response genes, indicates that *GF14e* is a negative regulator of defense responses in rice. In contrast to our findings, in Arabidopsis a 14-3-3 protein is predicted to be a positive regulator of defense responses. Purwestri et al [5] and Bai et al [6] showed that rice 14-3-3 protein GF14c interacts with client proteins mediating distinct responses (specifically, brassinosteroid signaling and control of flowering). Given this information, it is logical to predict that 14-3-3 proteins in rice can have disparate effects on multiple pathways. In the Purwestri study [5], only GF14c was identified as interacting with the client Hd3a, contrasting to Bai's report [6] that all eight of the rice 14-3-3 proteins interact with OsBZR1. Activity of 14-3-3 proteins is dependent on the phosphorylation state of the client, that is, post-translational modification. Since their activity is dependent on client modification, it is possible for 14-3-3 proteins to have a wide variety of effects on numerous clients, with the particular changes in client activity being based in alteration of kinase/phosphatase activity. The work in this thesis demonstrates that one function for

GF14e is the modulation of defense responses. However, as other studies have identified multiple functions for 14-3-3 proteins in rice, it is reasonable to consider that it may function in other responses.

Chapter 4 begins to unravel a molecular function for the GF14e protein. In defense responses, after activation of early signaling events by pathogen attack, elicitor signals are often amplified through the generation of secondary signal molecules such as salicyclic acid (SA), ethylene, and jasmonate. Genes associated with the SA pathway, but not the ethylene and jasmonate pathways, were upregulated in *GF14e*-silenced plants, suggesting that the defense response regulated by *GF14e* signals through the SA-mediated pathway. This result was particularly interesting given the observation that *GF14e*-silenced lines show enhanced resistance to a necrotrophic fungal pathogen (*Rhizoctonia solani*). Resistance to this class of pathogen is often associated with jasmonate signaling [7]. However, recent studies have described an association between constitutive expression of a rice peroxidase previously associated with resistance to a bacterial pathogen [8] and resistance to a necrotrophic fungal pathogen in carrot [9]. This suggests a function for *GF14e* in broad spectrum resistance.

GF14e-silenced plants were upregulated for defense response peroxidases that contain W-boxes in their promoters; a wound-response peroxidase lacking W-boxes in its promoter did not show upregulation in these plants. Additionally, these plants show enhanced expression of WRKY transcription factors predicted to be involved in defense responses in prior studies [10, 11]. The enhanced expression of these genes in (2 WAS) was not significant, but the trend, which was towards upregulation. Examination of a larger number of silenced lines would be required to determine significance.

Since almost all WRKY TFs bind to the same core sequence, the presence of W-Boxes in gene promoters may predict that WRKY TFs are involved in regulation, but it does not reveal which specific WRKY TFs are involved. Given that rice has genes for over 100 WRKY TFs, narrowing the candidates for regulation by *GF14e* is not trivial. For this work, I used two criteria to narrow the WRKY TF candidates: (1) the presence of a putative 14-3-3 interaction domain, and (2) a suggested role for the WRKY TF in plant defense or disease responses. Of the WRKY TF predicted to have a 14-3-3 interaction domain, only two, *OsWRKY13* and *OSWRKY14*, had been implicated in defense responses [11]. Attempts were made to determine expression of these two WRKY TFs in the *GF14e*-silenced lines, but the primers did not work. As these are particularly interesting WRKY TFs, work is in progress to develop new primers and determine their expression in the silenced lines.

Taken together, the information in Chapters 3 and 4 suggest that GF14e may function as a negative regulator of defense responses at the level of WRKY transcription factors. The hypothesis proposed in Chapter 4 suggests that GF14e sequesters WRKY transcription factors in the cytoplasm, preventing nuclear localization. To address this hypothesis, I suggest the following approaches. First, yeast two hybrid and/or bimolecular fluorescence complementation experiments could be performed with GF14e and the WRKY TFs predicted to interact with 14-3-3 proteins (*OsWRKY13* and *OSWRKY14*) to determine if the protein products interact. This approach, using yeast or a heterologous plant expression system, may not be well-suited to the post-translational changes required for interaction with 14-3-3 proteins. To address this possibility, the use of phosphomimetic mutants of the putative client proteins may be required [12].

Additionally, WRKY proteins, as transcription factors, have been shown as autoactivators in Y2H experiments [13]. By truncating the coding sequence, autoactivation in these putative clients may be avoided [13].

Second, to determine if nuclear translocation can be prevented in the presence of GF14e, I propose that 2) Expression analysis of WRKY transcription factors predicted to interact with 14-3-3 proteins and 3) Transient co-expression of epitope tagged *GF14e* be co-expressed with the predicted 14-3-3 interacting WRKY TFs tagged with GFP in *Nicotiana benthamiana*. If GF14e can prevent nuclear location of the WRKY TF, then the GFP would not be detected in the nucleus (relative to a WRKY TF that does not interact with GF14e or predicted 14-3-3 interacting GFP tagged WRKY TFs expressed alone). To address the possibility that *GF14e* may act upstream of WRKY TFs or that GF14e may be involved in other processes, I propose that tandem affinity purification (TAP) experiments be performed with TAP-tagged GF14e and an extraction of rice proteins. Rice proteins that are identified in this experiment could provide evidence for GF14e's participation modulating defense responses upstream of WRKY TF activation.

In summary, this work provides tools for understanding the regulation of plant defense signaling in plants. Chapter 2 provides a valuable tool to identify induced genomic deletions not only in rice, but provides a framework for the description and dissemination of information in plant species with little genomic information. For example, a recent publication described the use of a similar technique and its application in citrus [14]. Chapters 2 and 3 begin to unravel an important gene family's contribution to plant

defense responses. As research continues, the role of the 14-3-3 family's involvement in multiple plant processes, in particular, their regulation of defenses, in broad spectrum and R-gene mediate responses will continue to evolve.

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