CHARACTERIZATION OF SHEATH BLIGHT PATHOGEN
*RHIZOCTONIA SOLANI AND ITS MOLECULAR
INTERACTION WITH *ORYZA SATIVA

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Abstract: Little is known about mechanisms of molecular interaction of hosts with the
necrotrophic pathogen *Rhizoctonia solani*. Detailed analysis of the pathogen population in
Arkansas, the major rice producing state in the USA, led to the identification of the most virulent
field isolate out of a total of 124 isolates. The most virulent field isolate was used to inoculate a
cultivar, Jasmine 85, that contains minor resistance genes. Messenger RNAs 16 hours after
inoculation were extracted to detect key genetic components for the interaction. Experiments
were repeated with three different molecular techniques: 1) DNA microarray, 2) Robust Long
serial analysis of gene expression (RL-SAGE), and 3) Subtractive suppression hybridization
(SSH) library. Thus far, 200 expressed genes have been characterized from an SSH library, a
profile of 22,000 rice genes from DNA microarrays, and 6720 SAGE clones of the treated and the
control, each containing 40-45 tags, were analyzed. Our current analysis suggests that a
sophisticated interaction between pathogen secreted proteins and cell wall associated host
proteins may occur during early infection and these interacting genes may be key targets for the
control of rice sheath blight disease.

Introduction: Since the molecular cloning of the first resistance (R) gene *Pto* from the
tomato conferring gene-for-gene specificity (Martin et al., 1993), significant progress has
been made in understanding the molecular mechanisms of disease resistance to
biotrophic and hemi-biotrophic pathogens. Detailed analysis of the structural and
functional characteristics of R genes has enabled better understanding of the molecular
mechanisms of R gene-mediated defense responses and also has facilitated the
development of R gene marker for marker-assisted selection (Martin et al., 2003; Jia,
2003). In contrast, the molecular mechanisms of interaction of the necrotrophic
pathogen with its host are poorly understood. In reality, a number of necrotrophic
pathogens cause significant economic damage to agricultural production worldwide.
In general, a necrotrophic pathogen kills the host quickly allowing little time for the host to effectively activate its multi-faceted defense systems. In addition, the necrotrophic pathogens have a broad host range. By changing the host, a pathogen isolate can defeat the counter-defense system acquired by the host. It has been difficult to find innate immunity among host species; and the phenotypes are often quantitative in nature and its assessment is not easy due to its interaction with the environment.

In rice, the necrotrophic pathogen *R. solani* causes sheath blight disease (Fig. 1). Significant damage has often been observed in the areas where intensified agriculture systems are practiced. Thus far, sheath blight is one of a few diseases that has increased production costs by billions of dollars worldwide.

![Figure 1. Sheath blight disease of rice (A) and *R. solani* showing the young mycelia with 45 degree and 90 degree angles (B).](image)

Disease control still heavily relies on fungicides. Increased use of genetic control methods should reduce the use of fungicides, which may benefit the environment. In the past, use of minor resistance genes alone has not effectively prevented crop losses. Sheath blight is the most damaging disease in the southern US where the majority of rice is grown. Since 2001, the molecular plant pathology group of the US Department of Agriculture - Agriculture Research Service, Dale Bumpers National Rice Research Center (DB NRRC) has led a team to study interaction of rice with *R. solani* using gene expression technology. Gene expression technology offers a unique opportunity to study molecular mechanisms of interactions of the host with the broad-host-pathogen. The technologies include sequencing unique genes from an infected host by a subtractive suppression cDNA library (Kim et al., 2000; Xiong et al., 2001), DNA microarray (Schena et al., 1995) and serial analysis of gene expression (SAGE) (Matsumura et al., 1999; Vekúlescu et al., 1995) and improved Long SAGE (RL-SAGE) (Gowda et al., 2004). Using these techniques, differentially expressed genes can be identified to understand the molecular interaction of rice with the pathogen. In practice, differentially expressed host genes can be further analyzed for their roles in resistance and subsequently used for marker-assisted selection. Differentially expressed pathogen genes can be examined as potential targets for the development of environmentally friendly control measures.

In this review, we report the progress on studies of the population biology of *R. solani* and differential host and parasite gene expression analyzed by DNA microarrays, RL-SAGE and SSH libraries.
Figure 2. Diagram of rDNA-ITS DNA primers used for PCR amplification (A). Phylogenetic comparison of rDNA-ITS region of *R. solani* isolates collected worldwide (B) (P. Singh and Y. Jia, unpublished data).
R. solani characterization
To characterize R. solani populations in rice fields, fungal isolates from sheath blight-like diseased leaves from representative areas of rice production systems in Arkansas were used. These isolates were purified and analyzed by cytology, ribosomal (rDNA)-Internal transcribed sequence spacer (ITS), anastomosis, hyphal growth rate in vitro, and pathogenicity (Y. Wamishe, Y. Jia, P. Singh and Rick Cartwright et al., unpublished data).

Among 140 isolates, 102 were R. solani and the rest were sclerotia-forming fungi. Consistent with the literature, heterogenic multi-nuclei were observed among isolates. The regions specific to rDNA-ITS were amplified and either cloned for sequencing and/or directly sequenced. To date, 102 isolates were sequenced and sequence comparisons of 102 isolates of rDNA-ITS sequences indicate that they are highly homogenous and are highly similar to R. solani isolates reported in the Philippines and Vietnam (Figure 2). Minor sequence variation within rDNA-ITS was observed among different isolates (Jia et al., 2004) and these sequence differences are useful for monitoring R. solani population differentiation and evolution. Anastomosis is the ability of each fungal hyphae to fuse with the same kind of hyphae (Fig. 3). Anastomosis grouping (AG) has been routinely used to determine the R. solani host range. All R. solani isolates from rice were determined to be the AG1-IA group, which is also in agreement with the literature.

Figure 3. Anastomosis fusion of two isolates RR0104 and RR0113. Hyphae from two isolates were fused together at areas where cell walls were more transparent.

The speed of hyphae growth in culture was studied to predict the virulence of the pathogen isolates. Actively growing mycelia of each isolate were randomized onto potato dextrose agar plates and the growth was measured at different time intervals after inoculation. Overall, significant differences in the speed of growth were detected among 102 isolates. Most isolates grew relatively fast; however, slow growing isolates were also identified.

Seedling assays, through a microchamber method introduced from Bangladesh by Dr. Shannon Pinson (USDA-ARS, Rice Research Unit, Beaumont, Texas) were used to determine the pathogenicity of R. solani isolates. Uniformly active mycelia were used for inoculation and the total length of the disease lesions was measured to assess disease reaction. So far, correlations of rDNA-ITS with pathogenicity among the characterized...
R. solani isolates have not been high. Using slow growing isolates, the same disease reaction as observed in the fields was also detected using seedling assays in the microchamber method. The fast growing isolates caused similar damage to the slow growing isolate in the lab indicating that the lab environment has created better infection conditions. To date, we have learned that this cosmopolitan pathogen is not diverse in the rDNA-ITS region and that its speed growth may be important in causing disease in the field.

**Determination of a critical time for preparation of mRNA**—To identify a critical time point for mRNA preparation, a time course study using visual and microscopic observation was performed after inoculation of R. solani onto detached leaves in the laboratory. The R. solani sclerotia were initially used for inoculation and significant variations were observed among different resting stages of sclerotia that cause the differences in the timing when the pathogen causes the disease. Subsequent experiments involved using mycelia grown on potato dextrose agar. It was later determined that 16 hrs post-inoculation is a critical time point for identifying differentially expressed genes.

**Detached leaf inoculation method**—To reduce the environmental effects that may influence the type of differentially expressed genes, Dr. Singh and her colleagues (P. Singh, Y. Jia, G. Eizenga and F. Lee, unpublished data) tested several methods of controlled inoculation and concluded that detached leaf inoculation was easy to perform and relatively consistent disease reactions were observed. This method requires growing rice to the V11 growth stage (Counce et al., 2000) and at least 14 cm of the leaf is needed for inoculation. The detached leaf is placed in a container with moistened filter papers. Uniform inoculum size, including synchronized activity, is essential for estimating pathogenicity (Fig. 4). In practice, inoculation of the abaxial surface of the penultimate leaf is preferred for reproducibility. After inoculation, the container is sealed with parafilm to maintain high humidity. Cool white fluorescent light is required for infection. The temperature for incubation is between 22-24 °C. Precise executions of the procedures are essential for reproducibility of the assay.

**Figure 4.** Similar amount and activity of R. solani mycelia (A) were used for inoculating the detached leaves and inoculated leaf discs were maintained in a sealed moist container (B).

**Differential Gene Expression**
This study aimed to identify differentially expressed genes post-inoculation. Jasmine 85 rice was chosen because it exhibits minor resistance to R. solani based on laboratory and greenhouse methods and as confirmed by field observations. An aggressive field isolate RR0102 was used for inoculation because it may trigger the expression of the critical
genes. The biological replication was designed to allow different scientists to perform the inoculation using the same method in different seasons from 2001-2004. Three different techniques, DNA Microarray, RL SAGE and SSH library, were used to identify common pathways.

Differential host gene expression revealed by DNA microarray The first snapshot of gene expression of the host reacting to the pathogen was revealed by DNA microarray. Agilent arrays with oligo-nucleotides specific to 22,000 rice genes were obtained and messenger RNAs prepared from Jasmine 85 treated with the same virulent isolate were labeled with fluorescence dye Cy3 and Cy5. As shown in Figure 5, induced and suppressed genes were visible by different colors of each gene (Figure 5).

Figure 5. A scatter plot showing 22,000 rice genes hybridized with mRNA from Jasmine 85 (A); Hybridization spots were enlarged (B); Log10 ratio of control versus treated gene expression (C).

Differential gene expression revealed by RL SAGE In this work, Jia and Wang et al (unpublished data) created two RL SAGE libraries from control and sheath blight inoculated (treated) Jasmine rice cultivar. About 3456 and 3264 reads were obtained
after sequencing from control and treated libraries respectively. From these sequences, 40-42 bp ditags were extracted to obtain individual RL-SAGE tags. About 90,230 and 78,218 total tags were obtained from control and treated libraries respectively, of which 20,233 and 24,049 were unique. The SAGE tags were matched to several rice databases like TIGR-EST, TIGR-Annotated genes (SEQ), rice whole genomic sequence, and KOME full-length cDNAs. In the sheath blight control library, about 45.02% of total RL-SAGE tags were matched to TIGR ESTs, 35% of tags were matched to the sense strand and 9% to the antisense strand, showing their similar expression in the cell. More detailed bioinformatics analysis and RT-PCR confirmation are being performed.

**Differential gene expression revealed by SSH library** To confirm results generated using DNA microarray and RL-SAGE, 200 genes were sequenced from a subtracted cDNA library of Jasmine 85 post-inoculation. In this work, Dr. Singh and her colleagues constructed a SSH cDNA library where the background genes were eliminated by hybridizing the treated samples with the control. Thus far, a total of 200 genes were sequenced. Searching of the current GenBank database, few genes were predicted to be involved in photosynthesis indicating the library subtraction was complete since the most abundant plants genes are involved in photosynthesis. The putative functions of a large portion of genes were not determined (Figure 6). Confirmation of expression is being sought by northern blots and RT-PCR.

**Figure 6.** Diagram showing different categories of expressed genes (the percentage was calculated based on a total of 200 expressed genes).

**Progress on recombinant inbred lines of Lemont with Jasmine 85**
Recombinant inbred lines are useful tools for studying quantitative traits since homozygous advanced generation lines descend from an F2 generation. To map these up-regulated genes from Jasmine 85, an F2 population of 200 progeny of the cross of Lemont with Jasmine 85 generously provided by Dr. Anna McClung (USDA-ARS, Beaumont, TX) was used to develop recombinant inbred lines (RIL) using single seed descent. A single seed from each recombinant line was used for advancing to the next generation. F5 seedlings are presently being produced and grown in the greenhouse. F9 RILs are likely to be advanced for mapping in 2007. Eventually candidate genes
identified from this project will be mapped onto rice chromosomes for verification and their function will be validated by methods of reverse genetics.

In conclusion:
Characteristics of \textit{R. solani} in rice fields provide some useful information for integrated cultural practices. Using different platforms and biological replications, we have identified some clues of molecular interactions between rice and \textit{R. solani}. The trends of host and parasite gene expression learned from this project are important for designing molecular strategies to control sheath blight disease. Several candidate genes encoding proteases, protein kinases, and transcription factors were found to play critical roles in response to \textit{R. solani} infection. We hypothesize that upon inoculation, \textit{R. solani} may produce extracellular enzymes to degrade the host cell wall and cell membranes for invasive growth, and at the same time, \textit{R. solani} may activate sophisticated plant defense responses through membrane associated protein kinases. These above mentioned candidate genes are being further studied.

Future perspectives:
The genome size of rice is 640 mega bases which is the smallest among monocots. Draft genome sequences have been available for three years (Goff et al., 2002; Yu et al., 2002). In addition, high-density maps (McCouch et al., 1997, 1988, 2002), systems highly amenable to genetic transformation (Christou et al., 1997; Hiei et al., 1994), and useful genetic stocks (Leung et al., 2001; Jia et al., 2004) are available. These features make rice an excellent model for studying the molecular mechanisms of host-parasite interactions.

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