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Evaluation of Double-stranded (ds) RNA from Select Isolates of *Rhizoctonia solani* by Real-Time PCR

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Abstract

The primary purpose of this research is to develop a fungal model system at the RNA level that will accurately and rapidly identify definitive signature patterns of viruses infecting the plant pathogenic fungi *Rhizoctonia solani*. Three different isolates of *R. solani* were selected for the study that represented a single phenotype that is targeted by three different viruses. The three isolates used for current analytic purposes were strains; 357, 303, and 386. All the three isolates of *R. solani* had M-size dsRNA fragments that were viral in origin and exhibited considerable sequence heterogeneity. The M-size dsRNA fragments were gel-purified and cloned into pGEM®-T Vector Systems or pJET 1.2 /blunt vector. White colonies were selected and a positive clone was sent for sequencing by Retrogen, Inc. The sequence was BLAST searched in the NCBI database. The final sequence was used to design the primer and probe through Primer 3 Input version 0.4.0 (http://frodo.wi.mit.edu/). The picked forward, reverse and probe primers sequences were sent to Biosearch Technologies (http://www.biosearchtech.com/) to synthesize both the forward, reverse primers and TaqMan Probe. The 5' end of the TaqMan probe was labeled with fluorophores dye FAM and the 3' end with quenched dye BHQ-1. Results from such an analysis using Real-Time PCR are presented in various combinations of the dsRNA from the three isolates and the cloned fragments from each. , The working concentrations of primers with the concentration of probe were tested to optimize the assay. In addition to the primes and probe concentration, the annealing temperature and number of amplification cycles were optimized to validate sensitivity of detection.

Keywords: Rhizoctonia solani, dsRNA, primers

1. Introduction

Rapid screening and detection of multiple pathogens on a single platform is still a major challenge. The Microbial Forensics in Biodefense program (administered by the Army Research Office) at Indiana University of Pennsylvania is developing a fungal model system at the RNA level to identify definitive signature patterns. The current phase of the project focuses on model organism screening, genetic content manipulation to generate genetic/molecular variants representing possible variants of bio-threat agents.

The fungal host we are using (*Rhizoctonia solani*) is a single phenotype targeted by up to thirteen different viruses. In the fungal-virus model system we have identified nucleic acid and protein signatures specific to the viruses (wild type and mutant strains) using direct nucleic acid and protein identification systems.

2. Materials and Methods

2.1 RNA Target Identification

Total RNA, Virus Purified double-stranded (ds) dsRNA was identified as the target. Extraction of dsRNA was performed with the CF-11 Column method.

2.2 Growing Cultures:

The fungal isolate was maintained on plates and grown in Malt extract broth with dextrose, the sugar found in their crop hosts. Fungi cultures were plated on Difco™ Nutrient Agar 1.5% for two weeks. Agar plugs were cut and placed in 200 mL Difco™ Malt Extract Broth. The cultures matured for 40-70 days.

2.3 Extraction Of Viral dsRNA:

The total nucleic acid was extracted using phenol-chloroform followed by elution using a CF-11 cellulose column. First, twenty grams of mycelium were frozen in liquid nitrogen and pulverized in a chloroform-isoamyl (24:1) phenol solution for 30 minutes. The slurry was centrifuged at 7000g for 10 minutes and the supernatant was removed. One gram of CF-11 cellulose and 17.6% absolute cold ethanol was added to the supernatant on ice and slowly mixed for 90 minutes. The solution was centrifuged again at 7000g for 10 minutes. The CF-11 pellet was resuspended in 200-250mL 1.18% STE-EtOH and run through a column. The nucleic acid was eluted out with 10 mL 1X STE. Eluted material was suspended in 40 µL of sodium acetate and absolute ethanol over night at -20°C. The suspension was high speed pelleted at 12,000 rpm for 35 minutes. The pellet was dried and resuspended in two washes totaling 65 µL of nuclease-free or DEPC water.

2.4 Purifying dsRNA:

The dsRNA purified using CF-11 elution method was subjected to nuclease treatments to remove contaminating DNA and ssRNA in the preparation. The RNase I enzyme used removes the fungal rRNA from the sample. Ten microliters of RNase I enzyme (1000 U/ 1 µL Ambion) and 75 µL 4x SSC, sodium chloride sodium citrate buffer, was added to the total dsRNA in 65 µL water. This solution was incubated for 30 minutes. Three washes followed: phenol, phenol-chloroform-isomyl (25:24:1), and chloroform-isomyl (24:1). The supernatant was kept overnight at -20 °C in sodium acetate and absolute ethanol. The enzyme treated dsRNA was then fractionated on a 1% TAE agarose gel [40 mm Tris, 2 mm sodium ethylenediamine tetra-acetic acid (Na_2EDTA), 20 mm anhydrous sodium acetate, and 29.6 mm glacial acetic acetic acid,pH 7.8] at 40 volts for four hours. Staining was achieved with ethidium bromide and visualized with a UV illuminator. Images were taken with the Kodak Imagine Station 4000r Pro.

2.5 Agilent 2100 Bioanalyzer:

To begin the process of running the bioanalyzer, an Agilent High Sensitivity DNA Kit was used. First, the blue High Sensitivity DNA dye concentrate and red High Sensitivity DNA gel matrix were allowed to sit in a micro-centrifuge tube holder for 30 minutes, to let the dyes equilibrate to room temperature. 15µl of blue dye concentrate was added to the red gel matrix vial. The solution amounted to about 40µl, which was then pipetted into a micro-centrifuge tube. The dye solution was then vortexed for 5 seconds. The micro centrifuge tube containing the gel-dye mix was then centrifuged at 2000g ± 20% for 10 minutes at 4˚C.

Next, a new High-Sensitivity DNA chip was placed in the chip priming station. A mid-size pipet (1-20µl) was used to pipet 9.0µl of the gel-dye mix into the well marked "G" on the chip. The plunger on the syringe was positioned at 1ml, and the chip priming station was then shut. The plunger was pressed down until it was held by the clip. After 60 timed seconds, the clip was released. Five seconds later, the plunger was pulled back into the 1ml position. The chip priming station was opened and 9.0ml of gel-dye mix was pipetted into the wells marked G on the chip. Five microliters of marker green was pipetted into every sample and ladder well. No wells were left

empty. One microliter of High Sensitivity DNA ladder (yellow) was pipetted into the well marked as a ladder. One microliter of dsRNA from the 303 strain was pipetted into the wells marked 1-3 and 4-6, one microliter of dsRNA from the 386 strain was pipetted into the wells marked 7-9 and 10-11. The dsRNA from the 357 strain was administered in the same fashion, but on a separate run, using a different chip, at an earlier date. The chip was then placed horizontally into the adapter vortex for one minute at 2400rpm. Afterwards, the chip was immediately placed into the Agilent 2100 Bioanalyzer. The program was set to run for 45 minutes.

2.6 Real-Time PCR:

Before real-time PCR could be run using the plasmids of strains 357, 386, and 303, the samples were placed into a mass spectrophotometer and the concentrations were recorded. First, the software for the mass spectrophotometer was turned on, along with the machine itself. Once the machine was on and connected with the software, the UV light and visible light were turned on. A clean cuvette was placed into the cuvette holder in the open compartment of the machine. 49µl of distilled water was pipetted into the cuvette. Next, the spectrophotometer was blanked. One microliter of 357.1 was pipetted into the cuvette. Without removing the pipet, the end of the tip was used to mix the plasmid with the distilled water. The compartment was then shut and the mass spectrophotometer took a reading of the concentration of the plasmid. After the reading was finished, the cuvette was removed from the compartment and cleaned with nuclease free water and Kimtex wipes. The same procedures were followed for plasmids 357.2, 357.3, and 357.4. The concentrations for the plasmids of 386 and 303 were also measured using the same procedures.

The plasmids were now ready to be used for Real-Time PCR. First, six micro-centrifuge tubes were labeled 1-6 for plasmids 357.1, 357.2, 357.3, and 357.4 (24 tubes total). 20µl of DNA was pipetted into the tube labeled #1 for each plasmid strain. This tube served as the control, as it was not diluted. Next, 90µl of distilled water was pipetted into the tubes labeled "2-6". 10µl of DNA was added to the tubes labeled #2. The tube was then vortexed for five seconds. Next, 10µl of the contents from tube #2 was pipetted into tube #3. The tube was vortexed for five seconds. 10µl of the contents from tube #3 was pipetted into tube #4. The tube was vortexed for five seconds. 10µl of the contents from tube #4 was pipetted into tube #5. The tube was vortexed for five seconds. 10µl of the contents from tube #5 was pipetted into tube #6. The tube was vortexed for five seconds. The serial dilution of 10:90 was then complete. Next, six more micro-centrifuge tubes were labeled #1-6 for each strain of plasmid DNA (24 tubes total). 150µl of 2x iQ SYBER® supermix and 1.5µl of primer was pipetted into a micro-centrifuge tube and vortexed. Next, 12.5µl of the primer master mix (PMM) was pipetted into each of the tubes labeled #1-6 for each series in each strain. 12.5µl of DNA was pipetted into the corresponding numbered tube. So starting with 357.1, 12.5µl of DNA was added into the tube labeled #1. This was continued for every series in 357 and the series in the two other strains. The tubes were carefully mixed by lightly tapping the tubes, in order to reduce the risk of introducing bubbles. The total volume then totaled at 25µl. 12 eight-well PCR strips were placed into the thermal cycler. The series in the thermal cycler were named in the program according to which DNA sample series was pipetted in the PCR strips. The series were named in the following order: 357.1, 357.2, 357.3, 357.4, 303.1, 303.2, 303.3, 303.4, 386.1, 386.2, 386.3, 386.4. The 25µl DNA and PMM mix was then pipetted into the PCR strip in the corresponding series and well. PCR strip caps were placed on each PCR strip after every sample of the DNA and PMM mix was pipetted into the appropriate well. The lid temperature was set between 95-100˚C. The programming of the real-time PCR thermal cycler could then begin. Cycle 1 was set to 95˚C for four minutes Initial Denaturation of DNA. Cycle 2 was set at 94˚C for one minute Denaturation, then 59˚C for one minute Annealing and then 72˚C for two minutes Extension. Cycle 2 was repeated for 40 cycles. Cycle 3 was the melting curve. The real-time thermal cycler was programmed to heat the samples from 55˚C to 95˚C in increments of 0.5˚C and then collect the data after 10 seconds for each incremental step.

3. Results and Discussions

The colony morphology of the three isolates tested 303, 357, and 386 are shown Figure 1. Figure 2 shows the gel electrophoresis that depicts the vial M-size fragments that was purified using the nuclease treatment. Single size dsRNA of size 2.925 kilo base (kb) was consistently purified. All such highly purified nuclease treated dsRNA were subsequently used for reverse transcription PCR and cloning. The dsRNA was resistant to DNAse and RNAse under high salt condition. The PCR products were generated using random primers. PCR products generated depended upon the $MgCl_2$ concentration. Little or no PCR products were obtained at concentration above 3.0 mM

concentration (data not shown). The optimum concentration ranged between 2.0 and 2.5 mM (Figure 2 lanes 2 and 3). All such concentrations of $MgCl_2$ in the reaction consistently generated PCR products of sizes 2.3 and 1.0 kb (Figure 2, lanes 2 and 3, respectively). Thus, our discovery platform for the first time has developed protocol to generate PCR products from unknown dsRNA sequences belonging to the genome of pleomorphic viruses infecting the *R. solani* isolates 303, 357, and 386.

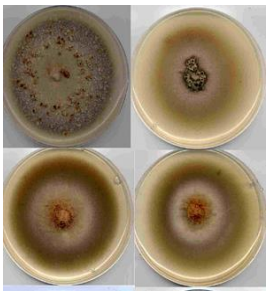

Figure 1. Colony morphology of test isolates

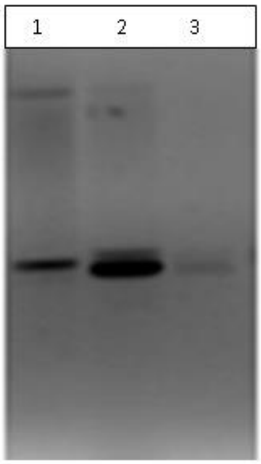

Figure 2. Ethidium bromide stained nuclease treated dsRNA from *R. solani* strains 357, 303, and 386

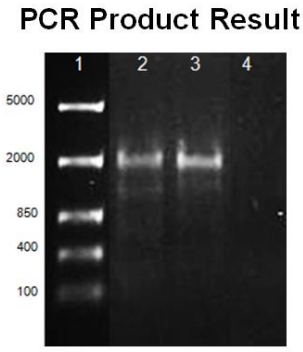

Figure 3. PCR products of cloned fragments

The PCR products thus generated were cloned into pDrive Cloning vector (Figure 4). In all 33 white colonies were selected for further analysis by fast digest for sequencing. The results from the enzyme digests of the selected 6 clones are shown in figure 5. The cloned fragment sizes were identical in lanes 2, and 3 (figure 5). The approximate

sizes of the cloned fragment were determined to be 784 bases. However, further analysis of the other clones revealed that the sizes of the cloned fragments were different {clones 4, 5, and 6 (Figure 4, lanes 4, 5, and 6). A total of 33 clones were analyzed and 13 clones that differed in sizes were further analyzed by sequencing (Retrogen, San Diego, CA, USA). The sequences were compared for similarity against the non-abundant sequence databases of EMBL, EU, using the BLAST algorithm. The primers specific to the dsRNA from strain 357 detected strains 303 and 386.

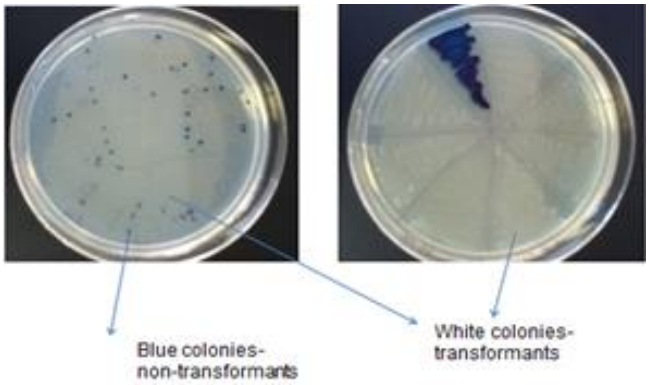

Figure 4. PCR products were cloned into pDrive (pGEM®-T) and plated onto bacterial plates containing ampicillin and X-gal. White colonies were extracted. Restriction enzyme (ECOR I) used for fast digest.

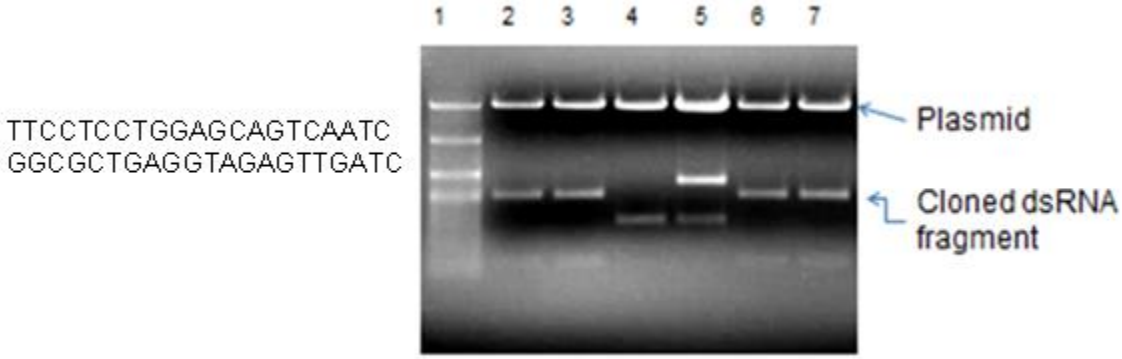

Figure 5. Analysis of cDNA clones from an isolate of *R. solani*.

In conclusion, the PCR primers designed specifically for strain 357 could identify concentration close to 10^{-1} µg – decrease of concentration of the cloned dsRNA from isolates of 303, 357, and 386 (Figure 6A) and 10^{-5} µg-decrease (Figure 6B). The primer sequences were developed for the isolate 357. Non-specific hybridization of new designed primers from 357 DNA and Vector are not detected.

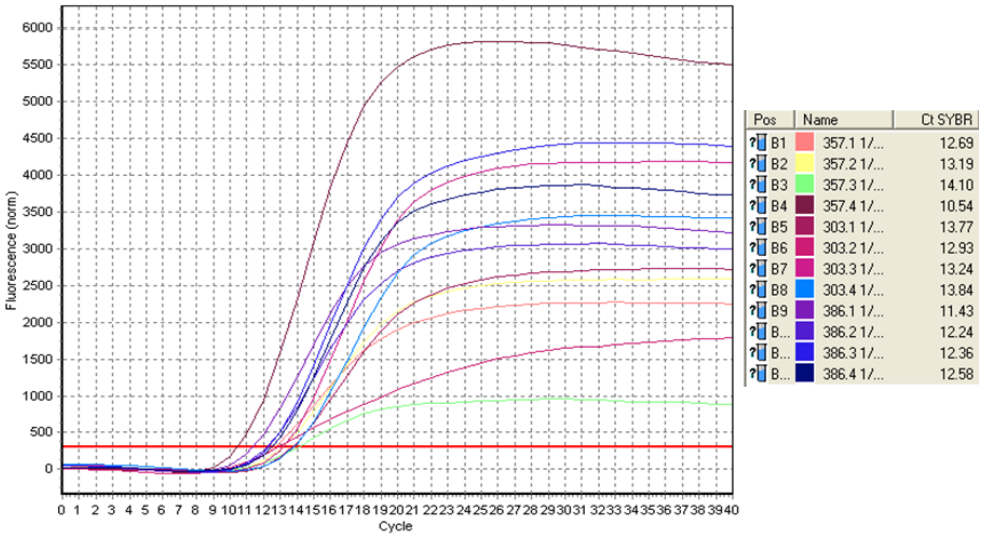

Figure 6A. Specific dsRNA primers from isolate 357 were used in a 1/10 dilution of plasmid DNA template

In this study, a one-step real-time RT-PCR was introduced and designed in order to reduce the cost and time requirements for reverse transcription. After identifying the inserted sequence of interest, forward primer and reverse primer were designed and optimization of the assay was performed on total dsRNAs from the isolate 357. During the optimization, various parameters were determined in order to obtain an optimum detectable signal (Ct value) from the intensity of the fluorescence (FAM), the efficiency of the assay and the number of cycles. A standard curve from ten-fold dilutions of total dsRNA of 357 from 10^{-1} to 10^{-6} µg was constructed for each assay and the efficiency of the reaction was calculated. With optimal parameters, it was able to gain an efficiency of 104 ±10.44% (data not shown). Generally, PCR efficiencies should be between 90 % and 110 %. When the efficiency is too low (< 90 %) or too high (> 110 %), the sensitivity of assay will be affected and the accuracy of the results may be questionable. Low efficiency can be caused by poor primer or target binding from primer dimmer formation between oligonucleotides in the reaction mix, or from un-optimized oligonucleotide and reagent concentrations in a reaction. However, high PCR efficiency generally results from having a nonlinear reaction, which could mean that the reaction has too much target[4].

In order to confirm the absence of contamination, two negative controls have been introduced in the real time RT-PCR assay. No template control was primarily tested for the absence of contaminants among the PCR buffers and reagents. On the other hand, no reverse transcriptase control was to confirm that there were no DNA contaminants in the testing samples (data not shown). In subsequent experiments, the specificity and the reproducibility of the assays were analyzed. Since only total dsRNAs template from RS357 isolate was tested positive in the assay, the determined analytical specificity was 100% (Figure 6A). Negative control samples were showing negative results, which demonstrated that the contamination had been avoided. In addition, the assay was highly reproducible due to the multiple repeats of the same dilution that resulted in nearly identical standard curves and the statistics indicated only small variations in the intercept and slope, respectively. Additional data obtained from the real time RT-PCR assays would be the limitation of detection for the presence of dsRNAs template from cloned isolates from 357 and 386 (Figure 6B). The concentration that could be detected by the real time TaqMan RT-PCR assay was in the range of 10^{-1} to 10^{-3} µg.

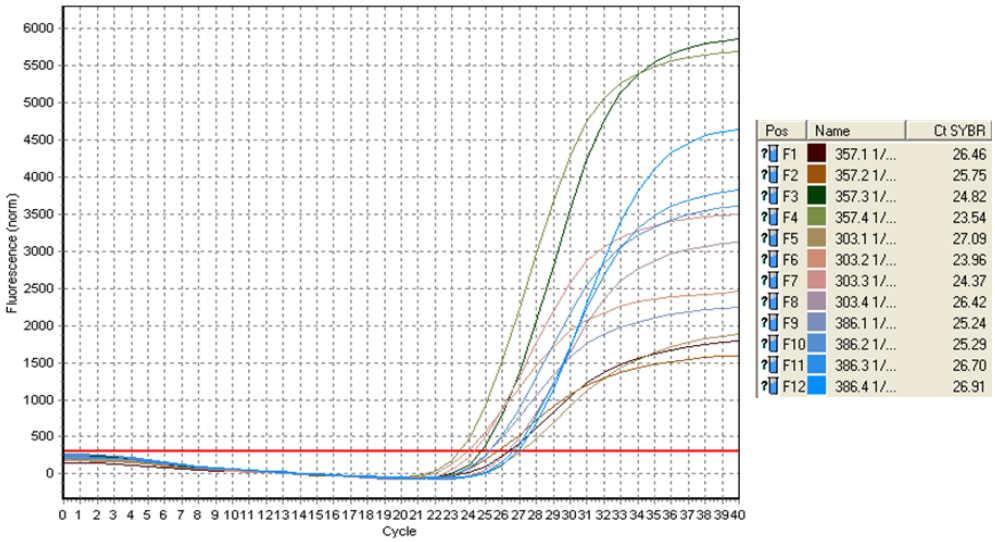

Figure 6B. Specific dsRNA primers from isolate 357 were used in a 1/100,000 dilution of plasmid DNA template

4. Acknowledgements

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5. References Cited

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