Chapter 13

Real-Time and Semiquantitative RT-PCR Methods to Analyze Gene Expression Patterns During *Aspergillus*-Host Interactions

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Abstract

Aspergillus species are infamous for causing several plant and animal diseases that directly (e.g., invasive aspergillosis) or indirectly (e.g., consumption of toxic food supplies) can lead to high rates of morbidity in humans and animals worldwide. Despite progress in molecular information and manipulation of *Aspergillus* spp., including genome sequence availability and suitable transformation methodologies, efforts to control *Aspergillus* diseases are still far from satisfactory, due in part to lack of knowledge of fungal virulence attributes. In order to obtain meaningful insights on the disease mechanism(s), it is essential to detect virulence gene expression during host invasion. Here, we describe two PCR-based detection methods of *Aspergillus* gene expression in both plant and mammalian tissues. Moreover, these techniques can be employed for routine screening of large numbers of aspergilli to improve diagnosis, disease monitoring, and therapy of fungal disease.

Key words: *Aspergillus* spp., pathogen, gene expression, cDNA, real-time PCR, reverse transcriptase–PCR.

1. Introduction

Aspergillus species are opportunistic pathogens that generally require wounds or otherwise weakened hosts for colonization *(1)*. *Aspergillus* diseases affect both plants and animals *(1–3)*. For example, *A. flavus*, a common seed-infecting fungus, contaminates such hosts as corn and peanuts with high levels of the carcinogen aflatoxin *(4)*. Extreme levels of toxin production are associated with drought and other environmental stresses on the host crop, and animal diseases caused by ingesting contaminated feeds are known as aflatoxicoses *(5)*. Recent episodes of human and dog deaths

Steffen Rupp, Kai Sohn (eds.), *Host-Pathogen Interactions*, DOI: 10.1007/978-1-59745-204-5_13,

from aflatoxin poisoning have highlighted the need to detect and ameliorate the presence of this toxin and producing fungus in food and feeds worldwide *(6, 7)*. *A. flavus* is also a noted human pathogen, but the most infamous cause of aspergillosis in humans is *A. fumigatus*, a ubiquitous saprophyte. *A. fumigatus* causes a wide range of animal diseases that include fungal sinusitis, asthma, and allergic bronchopulmonary aspergillosis as well as life-threatening invasive aspergillosis (IA) associated with high mortality rates *(8, 9)*. Pathogenicity of *A. fumigatus* depends on the immune status of patients and fungal strain. There is no unique essential virulence factor for development of aspergillosis, and fungal virulence appears to be under polygenetic control *(10)*. In addition to *A. fumigatus* and *A. flavus*, several other *Aspergillus* spp. can cause aspergillosis in the immunocompromised host including the genetic model *A. nidulans*, otherwise known as a GRAS organism *(11)*.

Detection of antibodies, antigens, DNA, or mRNA represents different diagnostic strategies for fungal infections. Antibody detection in animals is limited in value due to cost of the reagents and unpredictable differences in host humoral responses *(12)*. Currently, there is no routine antibody detection method for fungal pathogens of plants. PCR methodologies for the detection of fungal nucleic acids from infected hosts may be the optimal diagnostic approach because it offers the potential of sensitivity, capabilities for multiple sampling, and target specificity *(13–17)*. Fungal mRNA detection is especially powerful as this reveals expression of genes critical for pathogenesis. Here, we detail two PCR-based methods to detect expressed fungal genes in host tissue using real-time PCR for *A. nidulans* and *A. flavus* infected peanut and semiquantitative reverse transcriptase (RT) PCR for *A. fumigatus* infected mouse.

2. Materials

2.1. Aspergillus Gene Expression in Peanut

2.1.1. Seed Infections

1. Peanut (*Arachis hypogaea*) seeds, cultivar Florunner (commercial line highly susceptible to *A. flavus* in the field).
2. Potato dextrose agar medium (PDA; Difco, Franklin Lakes, NJ): potato starchose 4 g, glucose 20 g, and 15 g agar in 1 L double distilled water (ddH_2O).
3. 20X salts solution: $NaNO_3$ 120 g, KCl 10.4 g, $MgSO_4 \cdot 7H_2O$ 10.4 g, KH_2PO_4 30.4 g, and then add ddH_2O to 1 L, autoclaved and stored at room temperature.
4. Trace elements: $ZnSO_4 \cdot 7H_2O$ 2.2 g; H_3BO_3 1.1 g, $MnCl_2 \cdot 4H_2O$ 0.5 g, $FeSO_4 \cdot 7H_2O$ 0.5 g, $CoCl_2 \cdot 5H_2O$ 0.16 g, $CuSO_4 \cdot 5H_2O$ 0.16 g, $(NH_4)6Mo_7O_24 \cdot 4H_2O$ 0.11 g, Na_4EDTA 5.0 g, and then add the solids in order to 80 mL of H_2O, dissolving each completely before adding the next.

Heat the solution to boiling, cool to 60°C, adjust the pH to 6.5 to 6.8 with KOH pellets. Cool to room temperature and adjust volume to 100 mL with ddH_2O.

5. *Aspergillus* glucose minimal medium (GMM): 20X salt solution (50 mL/L), trace elements (1 mL/L), D-glucose (10 g/L), agar (for solid media) (15 g/L) in 1 L ddH_2O, adjust pH 6.5 and then autoclave 15 min, at 15 psi (1.05 kg/cm^2), 121°C on liquid cycle.
6. *Aspergillus* species. *A. nidulans* wild-type strain RDIT9.32 and oxylipin mutants Δ*ppoB* (RDIT59.1), Δ*ppoA*;Δ*ppoC* (RDIT54.7), Δ*ppoA*;Δ*ppoB*;Δ*ppoC* (RDIT62.3) *(18)*, and *A. flavus* strain 12S (kindly provided by P. Cotty, USDA-ARS, New Orleans, LA).
7. Sterile 0.01% Tween 80 (MP Biochemical, Solon, OH, cat. no. 103170) in distilled water.

2.1.2. Plant and Fungal RNA Isolation

1. RNeasy Plant Mini kit (Qiagen Ltd., cat. no. 74903).
2. Turbo DNA-free (Ambion, Inc., Austin, TX, cat. no. 1907).

2.1.3. Plant and Fungal cDNA Synthesis

1. iScript cDNA synthesis Kit (Bio-Rad Laboratories, Inc., cat. no. 170-8890).

2.1.4. Real-Time Reverse Transcriptase–PCR (Real-Time RT-PCR)

1. iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., cat. no. 170-8880).
2. RiboGreen RNA Quantitation Kit (Molecular Probes Inc., Eugene, OR, cat. no. R-11490).
3. AB-0900, Thermo-Fast 96 Semi-Skirted PCR Plate (Abgene, Rockford, IL).
4. Costar plates for RNA quantification.
5. Beacon Designer Software (Premier Biosoft International, Palo Alto, CA).
6. Primers were purchased from Integrated DNA Technologies (IDT) Inc., Coralville, IA.

2.1.5. Data Analysis

1. MyiQ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.).
2. MyiQ software package (Bio-Rad Laboratories, Inc.).

2.2. Aspergillus fumigatus Gene Expression in Mouse

2.2.1. Animals and Organism

1. ICR (Harlan Sprague Dawley, Indianapolis, IN) mice weighing 24 to 27 g.
2. Cyclophosphamide (Sigma, St. Louis, MO, cat. no. C0768).
3. Cortisone acetate (Sigma, cat. no. C3130).
4. *Aspergillus fumigatus* 293.
5. *Aspergillus* GMM.
6. Sterile 0.01% Tween 80 (MP Biochemical, cat. no. 103170) in distilled water.

2.2.2. Fungal RNA Isolation

1. Mouse lung.
2. Tissue grinder (Fisher, Hampton, NH, cat. no. 09-552-28).
3. Miracloth (Calbiochem, cat. no. 475855).
4. RNAse free DNAse (Qiagen Co., Valencia, CA, cat. no. 79254).
5. Triton X-100 (final conc. 1%, EM Science, La Jolla, CA, cat. no. TX1568-1).
6. Ultra pure water (RNAse free, Cayman, Chem. Co., Ann Arbor, MI, cat. no. 400000).
7. TriZol (Invitrogen, Carlsbad, CA, cat. no. 15596-018).
8. Phenol:$CHCl_3$:isoamyl alcohol = 24:23:1 (Ambion, cat. no. 9732), abridged as PCI.
9. Isopropanol (Fisher, cat. no. S77798).
10. 100% ethanol (AAPER Alcohol & Chemical Co., Shelbyville, KY, cat. no. DSP-KY-417).
11. Diethyl pyro-carbonate (DEPC, Sigma, cat. no. D5758).
12. Chloroform (Fisher, cat. no. C607-4).
13. Microfuge 18 (Beckman, Fullerton, CA).

2.2.3. Reverse Transcriptase–PCR (RT-PCR)

1. SuperScript III kit (Invitrogen Co., cat. no.18080-044).
2. Primers (Integrated DNA Technologies [IDT], Inc.).
3. Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA).
4. TripleMaster PCR system (Eppendorf, Westbury, NY, cat. no. 954140261).

3. Methods

The essential step of real-time and semiquantitative RT-PCR is the extraction of high-quality mRNA from infected hosts (*see* **Note 1**). Current applied methods in plant and animal tissue provide repeatable and reliable results in obtaining high-quality total RNA from fungus-infected tissue. Here, we show two alternative methods for the detection of host or fungal gene expression during host infection. Real-time PCR provides high sensitivity utilizing small samples yet is relatively expensive. An alternative, less costly method is semiquantitative RT-PCR. The success of detection is dependent on host or pathogen specific primers in both methods. If possible, it is important to design primers to allow for size differences between genomic DNA and mRNA in regions where introns are excised, thus allowing for a technical control of DNA contamination. Additionally, the primers for the first round PCR in semiquantitative RT-PCR should have high melting temperature (70°C to 76°C) to increase specificity for the target DNA amplification.

3.1. *Aspergillus nidulans* Gene Expression in Peanut

3.1.1. Seed Infections

1. *Preparation of seeds.* Mature seeds are immersed in distilled water for 5 min (*see* **Note 2**) and then the exterior brown peanut layer (testa) is removed using the fingers. The two cotyledons are separated and the embryo is carefully removed without damaging any of the cotyledon tissue. Then, cotyledons are surface sterilized by placing them in a tea ball infuser and dipping them in a beaker containing 0.05% sodium hypochlorite in sterile water for 3 min (*see* **Note 3**). The tea ball is transferred into a new beaker with sterile distilled water for 30 s (wash step), followed by a 5-s wash with 70% ethanol in a new beaker (additional sterilization step) and one more 30-s wash with sterile distilled water while shaking the tea ball. The cotyledons were drained completely and placed in a Petri dish until the time of infection. All the steps are aseptically performed in a laminar flow hood or a biosafety hood.
2. *Preparation of inoculum.* Streak a PDA Petri plate with *A. flavus* or a GMM plate with *A. nidulans* conidia from a glycerol stock culture. Incubate the plates at 29°C for *A. flavus* or at 37°C for *A. nidulans* for 3 to 5 days under dark conditions (or light if you are using *A. nidulans* strains that have the *veA* gene) until the plates are covered with conidia. Collect the spores in the hood using a spreader and 5 mL of sterile distilled water and transfer the spore suspension to a culture tube. Count the conidia using a hemocytometer to estimate the spore concentration.
3. *Seed infections.* Peanut cotyledons are inoculated with *A. flavus* or *A. nidulans* at a concentration of 10^5 conidia/mL (10 cotyledons/per 10 mL of conidia suspension). Cotyledon treatments include water control (mock inoculation) and wounding or infection with the fungi (*see* **Note 4**). For wounding experiments, cotyledons are scratched 4 times with a razor blade to a depth of ca. 2 to 3 mm. For all treatments, 20 cotyledons are immersed in 20 mL of sterile distilled water (control and wounded) or sterile water with fungal conidia in 50 mL Falcon tubes while shaking for 30 min in a rotary shaker (50 rpm). All cotyledons are incubated in the dark at 29°C for *A. flavus* or 37°C for *A. nidulans* in glass Petri dishes lined with three pieces of moist filter paper and a water reservoir (the lid of a Falcon tube containing 1 mL of sterile water) to maintain high humidity in the artificial chamber (**Fig. 13.1**). Samples for fungal or plant gene expression analysis are collected at desired time points over a time course of 5 days, by which time cotyledons are covered by the fungi. Collected samples are frozen in liquid nitrogen and kept at −80°C until the time of analysis.

Fig. 13.1. Infected peanut cotyledons (cultivar Florunner) with *A. flavus* (strain 12S). Seeds are placed in a glass Petri dish containing filter paper saturated with sterile distilled water and a water reservoir to keep the humidity high and are incubated in the dark. Picture was taken after 6 days incubation at 29°C.

3.1.2. Plant RNA Isolation

1. 100 to 150 mg of frozen cotyledons is macerated using a pestle and mortar in liquid nitrogen. Decant tissue powder and liquid nitrogen into an RNase-free, liquid nitrogen cooled, 2-mL microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. The 2-mL tubes are kept in liquid nitrogen until all the samples have been processed.
2. Total RNA is extracted using the RNeasy Plant Mini kit (*see* **Note 5**). Remove samples from the liquid nitrogen and immediately add 600 μL of RLT buffer (supplied in the RNeasy kit) including β-mercaptoethanol (β-ME; add 10 μL β-ME per 1 mL Buffer RLT just before use). Vortex the samples vigorously to dissolve the homogenized tissue and to disrupt the cells.
3. Transfer the lysate to a QIAshredder spin column (lilac column) of the kit placed in a 2-mL collection tube, and centrifuge for 2 min at 11,300 × *g*. Carefully transfer the supernatant of the flow-through from the collection tube to a new microcentrifuge tube. Be careful not to disturb the cell-debris pellet and avoid collecting the lipid floating on top layer that is formed.
4. Add 0.5 volume of ice-cold ethanol (96% to 100%) to the cleared lysate, and mix immediately by pipetting 5 to 6 times. Transfer 700 μL of the sample to an RNeasy spin column (pink column) placed in a 2-mL collection tube (supplied in the kit). Centrifuge for 30 s at 6700 × *g*. Discard the flow-through and add the remaining solution of each sample into the same RNeasy spin column. Centrifuge for another 30 s at 6700 × *g* and discard the flow-through. Total RNA remains in the spin column membrane.
5. Add 700 μL Buffer RW1 to the RNeasy spin column, close the lid and let the samples stand for 5 min at room temperature.

Then, centrifuge for 1 min at 6700 × *g* to wash the spin column membrane. Discard the flow-through and the collection tube.

6. Place the RNeasy spin columns (pink column) into new supplied collection tubes and add 500 μL Buffer RPE (Buffer RPE is supplied as a concentrate, and ensure that 4 volumes of 96% to 100% ethanol is added to Buffer RPE before use; that is, 44 mL of ethanol in the supplied 11 mL of Buffer RPE) to the RNeasy spin column, close the lid, and centrifuge for 30 s at 6700 × *g* to wash the spin column membrane. Discard the flow-through.
7. Add another 500 μL Buffer RPE to the RNeasy spin column and centrifuge for 2 min at 6700 × *g* to further wash the spin column membrane. Discard the flow-through.
8. Place the RNeasy spin column in a new 1.5 mL collection tube (supplied). Be careful not to carry any residual wash buffer to the final collection tube. Add 40 μL RNase-free water (supplied) directly to the spin column membrane and incubate the samples for 3 min on the benchtop. Centrifuge for 1 min at 11,300 × *g* to elute the RNA. Repeat the elution step using the first eluate to obtain a more concentrated RNA. Incubate the samples for 2 min on the benchtop and centrifuge for 1 min at 11,300 × *g*. Store the RNA (a mixture of plant and fungal RNA) at −80°C until the time you will proceed to the next steps.

3.1.3. Plant and Fungal cDNA Synthesis

1. Samples are treated with Turbo DNAfree (Ambion, Inc.) prior to reverse transcription to remove residual amounts of DNA. Use the rigorous protocol according to the manufacturer's guidelines. The absence of DNA from the RNA samples is confirmed by performing real-time PCR on 50 ng of total RNA using a control primer set (e.g., actin primers). RNA samples that yield a threshold cycle (C_t) value greater than 32 can be considered free of contaminating DNA.
2. Total RNA is quantified using the RiboGreen kit (Molecular Probes). RiboGreen is an ultrasensitive fluorescent nucleic acid stain for quantifying RNA in solution using a fluorescence microplate reader. RNA samples obtained after **step 8** of **Section 3.2** are diluted 20 times, 200 times, and 2000 times and are mixed with the RiboGreen solution following the manufacturer's protocol.
3. cDNA is generated from 1 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA) in a 20-μL reaction that includes 4 μL of 5X Buffer, 1 mL reverse transcriptase, and the remaining 15 μL consists of 1 μg of total RNA and water. The

cDNA synthesis conditions are 5 min at 25°C, 30 min at 42°C, 5 min at 85°C, hold-step at 4°C. Final cDNA synthesis products are diluted to 200 μL.

3.1.4. PCR

3.1.4.1. Real-Time PCR Primer Design

Proper primer design ensures that the selected primers are specific for the desired target sequence, bind at positions that avoid secondary structure, and minimize the occurrence of primer-dimer formation. The primers are designed based on the desired host or fungal gene using the Beacon Designer software. For instance, we have used the peanut *PnLOX2-3* cDNA sequence and the housekeeping gene "actin depolymerizing factor" (actin-DF) as a plant calibrator *(19)* and the *A. nidulans* genes α-glycosidase (AB057788), α-amylase (AF208225), and *amyR* (AF208225) and the housekeeping gene "tubulin" as a fungal calibrator. Beacon Designer software is designed to generate primer pairs suitable for real-time RT-PCR. Use housekeeping genes as reference/control genes that are expressed uniformly throughout the time course of your study. The "Avoid Template Structure" option of the software is selected to limit primer sequences to regions of little or no secondary template structure. PCR efficiency has a significant effect on the accuracy of calculated gene expression levels. Differences in efficiency can be caused by a number of factors, such as the presence of reverse-transcriptase inhibitors or primer-dimer formation. The efficiency of the primer pairs is determined on cDNA derived from peanut seed or *A. nidulans* using fivefold serial dilutions of cDNA (staring from 100 ng per reaction) in two independent experiments. Master mixes, using 2X SYBR Green reaction mix (Bio-Rad Laboratories, Inc.), from each dilution series are split into three reactions in a volume of 20 μL/well. Reactions are subjected to real-time PCR using the MyiQ Real-Time PCR Detection System and analyzed using the MyiQ software package (Bio-Rad Laboratories, Inc.) (**Fig. 13.2A**). Primer efficiencies, which are unique to each primer pair and has a major impact on the accuracy of the calculated expression ratios, are determined using the formula efficiency (E) = $10^{(-1/\text{slope})}$ with the slope determined by the MyiQ Cycler software (**Fig. 13.2B**) *(20)*. For example, in a study to quantify expression of the peanut lipoxygenases *PnLOX2-3 (19)* after *A. flavus* infection, primers RT-PnLOX2-3-F1 (5′-CCTCATCCTCCTCCTTCTTC-3′) and RT-PnLOX2-3-R1 (5′-AAGGTGTCAACGTCCAGG-3′) were used to amplify the 145-bp *PnLOX2-3* PCR fragment, and the primers RT-actin-F1 (5′-GAGGAGAAGCAGAAG-CAAGTTG-3′) and RT-actin-R1 (5′-AGACAGCATATCG-GCACTCATC-3′) were used to amplify the 106-bp actin-DF PCR fragment. The actin-DF primers had an efficiency of 2.17 and the *LOX* primers had an efficiency of 2.05. Primer efficiency close to 2.00 corresponds with 100% efficiency. For 100% efficiency,

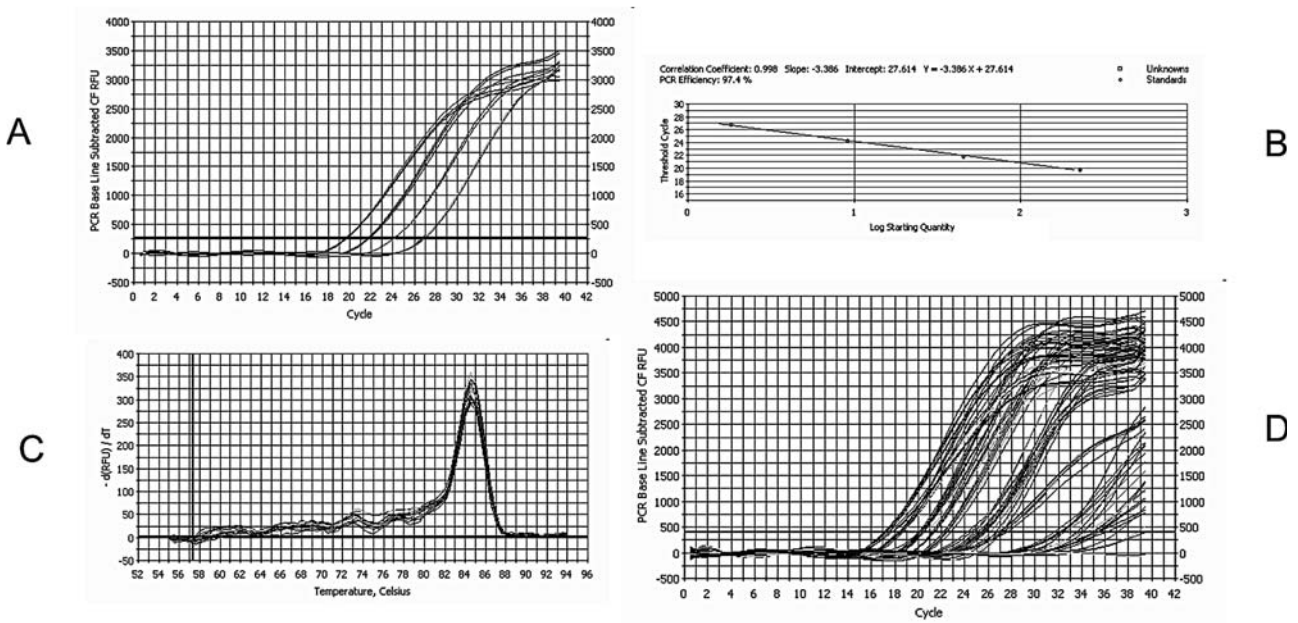

Fig. 13.2. **(A)** An iCycler software screen view showing amplification plots of a target cDNA (peanut actin) 5X dilution series to determine the efficiencies of primer sets. **(B)** Standard curve of threshold values (C_t) against the log of cDNA dilutions, generated by the iCycler software, to calculate the slope and estimate primer efficiency using the formula $(E) = 10^{(-1/\text{slope})}$. **(C)** A melt curve profile depicting the negative first derivative of the fluorescence ($-$dF/dT) versus temperature plot. The significant change in fluorescence accompanies the melting of the double-stranded PCR products. The plot of $-$dF/dT versus temperature is displaying these changes in fluorescence as distinct peaks. The software identifies the peaks and assigns melting temperatures from this plot. This analysis confirms the specificity of the chosen primers and reveals the putative presence of primer dimers. **(D)** Fluorescent data of amplification of *PnLOX2-3* and actin products derived from peanut seeds infected with *A. flavus* in real time as temperature increases or decreases. The iCycler iQ system records the total fluorescence generated by SYBR Green binding to double-stranded DNA as temperature changes and plots the fluorescence in real time as a function of temperature. The calculation of the C_t values is generated automatically by the MyiQ software.

there will be a doubling of the amount of DNA at each cycle, for 90% the amount of DNA will increase from 1 to 1.9 at each cycle, so the factor is 1.9 for each cycle, and similarly for 80% and 70% it will be 1.8 and 1.7. Notice that a small difference in efficiency makes a large difference in the amount of final product. One must be sure, however, that the efficiency of PCR is similar for the standards as that of the "unknown" samples.

3.1.4.2. PCR Conditions

Samples are analyzed in a volume of 20 μL using iQ SYBR Green Supermix. Reactions are performed in triplicate using cDNA templates from two independent iScript reactions for each gene (e.g., *LOX* and actin) giving a total of six replicates (20 μL reactions) for each RNA sample. A master mix of SYBR Green Supermix and primers is prepared for each primer pair. Each reaction contains 50 ng of cDNA and 200 nmol of each primer. Primer stock is 5 μM and use 0.8 μL per 20 μL reaction to give a 200 nmol final concentration. Reactions are performed with the MyiQ Real-Time PCR Detection System using the default "two-step amplification plus melting curve" protocol: 95°C for 3 min followed by 40 cycles of 95°C for 1 min (denaturation) and 55°C for 45 s (annealing and elongation). The melt curve analysis is carried out after amplification and consists of 11 min at 55°C followed by 80 to 10 s steps with a 0.5°C increase in temperature at each step (**Fig. 13.2C**). The melt curve analysis is essential to demonstrate the lack of variation and the specificity of PCR products as well as the absence of primer dimers. Amplified products can also be analyzed by agarose gel electrophoresis and sequencing analysis for further confirmation. The calculation of the threshold values (C_t) is generated automatically by the MyiQ software (**Fig. 13.2D**).

3.1.5. Data Analysis

The amount of target RNA (*LOX* or degradative enzyme RNA in this case) is determined at each time point by first normalizing the target RNA to the internal standard RNA (actin for peanut or tubulin for *A. nidulans*) using the $2^{-\Delta C_t}$ formula or $2^{(C_t \text{ internal standard}) - (C_t \text{ target})}$ *(20–22)*. To determine the relative expression ratio of the *LOX* or the fungal gene at each treatment, the normalized *LOX* or fungal gene value is divided by the calibrator value. For instance, the calibrator for gene expression in cotyledons is the average of the normalized values of control (mock-inoculated) samples. The average, standard error, and coefficient of variation (CV) are determined for each set of six replicate reactions. The entire analysis is replicated in three independent experiments for each time point. An example of the results produced is shown in **Fig. 13.3**. **Figure 13.3A** shows real-time PCR analysis of the transcriptional kinetics of *PnLOX2-3* that occurs during *A. flavus* colonization in comparison with mock-inoculation *(19)*. The calibrator for this experiment was the average of normalized transcript from

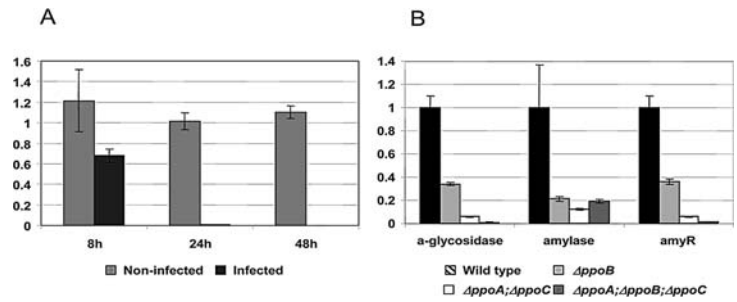

Fig. 13.3. **(A)** Quantification of plant host genes. Suppression of peanut *PnLOX2-3* after *A. flavus* infection as revealed by real-time PCR analysis after 8 h, 24 h, and 48 h. **(B)** Quantification of *Aspergillus* genes. The expression of three *A. nidulans* genes (α-glycosidase, α-amylase, *amyR*) in wild type and the oxylipin mutants Δ*ppoB*, Δ*ppoA;*Δ*ppoC,* Δ*ppoA;*Δ*ppoB;*Δ*ppoC*. The transcript level is represented as the relative quantification of the expression level of each target gene of interest (*PnLOX2-3* in part **A** or α-glycosidase, α-amylase, *amyR* in part **B**) and was determined by first normalizing the target RNA to the internal standard RNA (actin for peanut or tubulin for *A. nidulans*), and then the normalized target RNA value was divided by the calibrator consisting of the average of the normalized values of control (mock or wild-type inoculated samples) at each time point. The average and the standard errors were calculated for each set of six replicate reactions.

mock-inoculated cotyledons. The expression of *PnLOX2-3* is downregulated at 8 h, 24 h, and 48 h postinoculation. Another example (**Fig. 13.3B**) shows the transcriptional kinetics of fungal α-glycosidase, α-amylase, and the transcription factor *amyR* in different *A. nidulans* oxylipin mutants (Δ*ppo*) during the peanut colonization and infection *(18)*. The calibrator for this experiment was the average of normalized transcript from *A. nidulans* wild-type for each gene. The expression of α-glycosidase, amylase, and *amyR* is downregulated in all strains examined during the fungal-host interaction.

3.2. Aspergillus fumigatus Gene Expression in Mouse

3.2.1. Animals and Organism

1. Six-week-old, outbreed Swiss ICR mice (Harlan Sprague Dawley) weighing 24 to 27 g are immunosuppressed by intraperitoneal injection of cyclophosphamide (150 mg/kg) on days −4 and −1, and 3 before and after infection, and with a single dose of cortisone acetate (200 mg/kg) on the morning of infection. A clinical isolate, *A. fumigatus* 293, is used for mouse infection.
2. Streak-inoculated fresh conidia from 3-day-old GMM Petri dish cultures grown at 37°C are collected by scraping conidia off the agar top using 0.01% Tween 80 in distilled water. Typically two Petri dishes suffice. Conidia are counted using the hemocytometer and resuspended in 0.85% NaCl to a concentration of 10^8 conidia/mL (*see* **Notes 6** and **7**). The mice are anesthetized via halothane inhalation in a bell jar

on day 0. Sedated mice are inoculated by nasal instillation of 50 μL of 10^8 spores/mL (10 mice) or nasal instillation of 50 μL of 0.85% NaCl (10 mice, mock control). All mice are monitored 3 times daily for morbidity. For RNA examination, mouse lung is collected at 3 days after the infection and homogenized for the subsequent experiment described below (we note here that lung biopsy is an important component to human diagnosis, so it would be feasible to perform RT-PCR on human tissue also).

3.2.2. Isolate Pathogen RNA

1. At day 3, all mice are sacrificed in a CO_2 chamber, and all lungs from each group are combined per treatment and homogenized using a tissue grinder (Fisher, cat. no. 09-552-28) with 10 mL of phosphate-buffered saline. Homogenates are passed through 1 ply of Miracloth (Calbiochem, San Diego, CA) to remove large tissue debris (*see* **Note 8**).
2. The flow-through from the 10 lungs is rapidly frozen with liquid nitrogen. The flow-through (ca. 10 mL) is then treated with DNAse (4 units/mL) (RNase Free DNase, Qiagen) and Triton X-100 (final conc. 1%) for 20 min at 37°C. The treated flow-throughs are spun down in 50 mL Oak Ridge tubes at 3000 × *g* and the supernatant discarded.
3. Pellets are resuspended in 4 mL of 1% Triton X-100 and then homogenized for 1 min. The homogenate is spun down in 50 mL Oak Ridge tubes at 3000 × *g* and again the supernatant is discarded.
4. Repeat **step 3**.
5. Pellets are washed twice with sterile distilled water and spun down at 3000 × *g* to discard supernatant.
6. Each pellet (ca. 100 μL) is lyophilized overnight and then mixed with 1 mL TRIzol (Invitrogen Co.). The TRIzol homogenate is incubated for 5 min at 15°C to 30°C to permit the complete dissociation of nucleoprotein complexes.
7. Next, 200 μL chloroform is added to the homogenate, shaken vigorously, and centrifuged at 9600 × *g* for 15 min at 4°C. The supernatant, containing total RNA, is extracted with equal volumes of phenol:chloroform:isoamylalcohol = 24:23:1 (PCI) to get high-quality RNA (*see* **Note 9**).
8. The extracted supernatant is collected and mixed with 500 μL isopropanol (2-propanol) for precipitation of total RNA.
9. After 10 min incubation at room temperature, the mixture is centrifuged at 9600 × *g* for 10 min at 4°C.
10. After removing supernatant, the RNA pellet is washed with 1 mL 75% ethanol, vortexed, and spun at 3800 × *g* for 5 min at 4°C.

11. RNA pellet is air-dried for 10 min (*see* **Note 10**).
12. The dried pellet is dissolved in 40 μL diethyl pyrocarbonate (DEPC, 1 mL/L) treated water by incubating at 60°C for 10 min.
13. A 250-fold dilution is used to measure the concentration of RNA in the sample by spectrophotometer.

3.2.3. Reverse Transcriptase–PCR (RT-PCR)

1. The RNA sample dissolved in DEPC-treated water is used for cDNA synthesis using SuperScript III kit (Invitrogen; *see* **Note 11**).
2. The following 20 μL reaction volume can be used for 10 pg to 5 μg of total RNA.
3. To synthesize the first-strand cDNA, add the following to a nuclease-free microcentrifuge tube:
   (a) 1 μL of oligo(dT) 20 (50 μM) or 50 to 250 ng of random primers or 2 pmol of gene-specific primer.
   (b) 10 pg to 5 μg total RNA.
   (c) 1 μL 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH).
   (d) Sterile, distilled water up to 13 μL. This mixture is heated to 65°C for 5 min and incubated on ice for at least 1 min. The contents of the tube are collected by brief centrifugation and the following are added:
   (e) 4 μL 5X First-Strand Buffer.
   (f) 1 μL 0.1 M DTT.
   (g) 1 μL RNaseOUT Recombinant RNase Inhibitor (Optional: cat. no. 10777-019, 40 units/μL; *see* **Note 12**).
   (h) 1 μL of SuperScript III RT (200 units/μL; *see* **Note 13**).
4. The contents of the tube are mixed by pipetting gently up and down (*see* **Note 14**).
5. The reaction is inactivated by heating at 70°C for 15 min. The cDNA (the first-strand reaction) can now be used as a template for amplification in PCR (*see* **Note 15**).
6. The following primers are used to amplify an *A. fumigatus* specific gene, *gliI* (a biosynthetic gene in the gliotoxin gene cluster) from the cDNA pool. **Figure 13.4A** shows the relative positions of the primary and secondary primers used in **step 6** and described below.
   (a) Primary amplification. 5′TCGTTGCTGGAGAATCTGCTGTATGA3′ and 5′ACAAAGGGAATCTGGTGGAAGGCGA3′ will amplify a 0.6- to 0.7-kb PCR product using TripleMaster PCR system (Eppendorf) (**Fig. 13.4B**, lanes 2 and 3). (The 0.6- to 0.7-kb is not seen in this gel, this is a common occurrence, hence the requirement of the secondary amplification step. There is a nonspecific

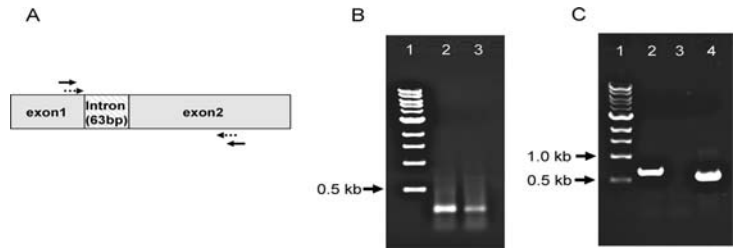

Fig. 13.4. **(A)** Diagram of *glil* gene (1428 bp) and location of the primary (solid arrows) and secondary (nested) primers (dashed arrows). **(B)** Primary RT-PCR amplification of *glil* from mouse lung infected with *A. fumigatus* or mock-inoculated. Lane 1, 1 kb size marker; lane 2, 10 μL out of 50 μL of RT-PCR tube of total RNA from mock-inoculated lungs; lane 3, 10 μL out of 50 μL of RT-PCR tube of total RNA from infected lungs. The lower band in both lanes is nonspecific amplification whereas the *glil* fragment is not seen in this gel. **(C)** Secondary PCR amplification with nested primers of *glil* from 2 μL out of 50 μL the primary RT-PCR amplifications above. Lane 1, 1 kb size marker; lane 2, PCR from genomic *A. fumigatus* DNA; lane 3, 10 μL out of 50 μL of PCR from RT-PCR product of mock-inoculated lungs; lane 4, 10 μL out of 50 μL of PCR from RT-PCR product of infected lungs. The fragment in lane 4 is smaller than the fragment in lane 2 as an intron was excised in lane 4. (Reprinted with permission from American Society for Microbiology from Bok, J. W., Chung, D., Balajee, S. A., Marr, K. A., Andes, D., Nielsen, K. F., Frisvad, J. C., Kirby, K. A., and Keller, N. P. 2006. GliZ, a transcriptional regulator of gliotoxin biosynthesis, contributes to *Aspergillus fumigatus* virulence. *Infect. Immun.* **74**, 6761–6768.)

band that is not amplified by the secondary primers, **Fig. 13.4C**). Contained in the PCR tube are

(i) 5 μL 10X PCR Buffer
(ii) 1 μL 10 mM dNTP Mix
(iii) 0.2 μL Sense primer (20 pmol)
(iv) 0.2 μL Antisense primer (20 pmol)
(v) 0.2 μL DNA polymerase (5 units/μL)
(vi) 2 μL cDNA (from first-strand reaction)
(vii) Autoclaved, distilled water to 50 μL

Heat the reaction to 95°C for 2 min to denature. Perform 35 cycles of PCR (95°C for 1 min (denaturation), 68°C for 30 s (annealing), and 68°C for 1 min (elongation); primer melting temperature is 76°C based on GC content and expected PCR product is 0.6 to 0.7 kb.

(b) Secondary Amplification. 2 μL of the above PCR reaction is used as a template with nested primers, (5′TGTTGATCGAGACGCCGTTCTG3′ and 5′CAGAGCGGCTCGATTCTGGTG3′, **Fig. 13.4C**, lanes 3 and 4; *see* **Note 16**). The size difference between lane 2 (a genomic DNA control) and lane 4 (cDNA) in **Fig. 13.4C** is due to an intron (63 bp) in *gliI* gene. This result indicates that cDNA was synthesized and detected successfully by this method from infected mouse lung tissue.

4. Conclusions

Aspergillus species are opportunistic pathogens attacking plants, animals, and humans. Pathogenesis is a complex mixture of pathogen, host, and environmental attributes that perhaps can be best revealed during the actual course of pathogen ingress. Here we present two methods that have allowed analysis of both fungal and host gene expression *in situ*. Such studies are key components in elucidating mechanisms of pathogenesis in aspergillosis infections.

5. Notes

1. Intact and high-quality of total RNA isolated from seeds is difficult and the operator needs to be especially vigilant with pipetting technique as plant components can interfere with the quality of extracted RNA.
2. Immersion of peanut seeds in distilled water for 5 min facilitates the removal of testa and prevents the formation of wounds on the cotyledons.
3. All solutions are prepared in water that has a resistivity of 18.3 MΩ-cm and total organic content of less than 5 ppb.
4. Wounding experiments are needed to differentiate the plant host genes that specifically respond to fungal infections and not to wound damage.
5. To avoid RNA degradation problems during the extraction procedure, do not process more than six samples at the same time.
6. We suggest using 0.01% Tween-80 in water to prepare conidial suspensions as conidia are hydrophobic. Immediately make a conidial resuspension in 0.85% NaCl for injection in mouse.
7. Fresh conidial suspensions are recommended for both plant and animal inoculations due to changes in spore physiology over time.
8. Removal of lung tissue by filtration can clog the miracloth. Alternatively, you can use forceps to remove large sections of tissue. The lung can be cut into smaller pieces prior to homogenization, which aids in extraction.
9. After extraction of total RNA from lung tissue with TRIzol, additional extraction with PCI is an important step to increase RNA quality.
10. Do not overdry your RNA pellet. The overdried RNA pellet will be difficult to resuspend in solution.
11. SuperScript III Reverse Transcriptase is a version of M-MLV RT that has been engineered to reduce RNase H activity

and provide increased thermal stability. The enzyme can synthesize cDNA at a temperature range of 45°C to 60°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases. Because SuperScript III RT is not significantly inhibited by ribosomal and transfer RNA, it can be used to synthesize cDNA from total RNA. For difficult or high-GC-content templates, use a 60°C cDNA synthesis temperature.

12. When using less than 50 ng of starting RNA, the addition of RNaseOUT is essential.
13. If generating cDNA longer than 5 kb at temperatures above 50°C using a gene-specific primer, the amount of SuperScript III RT may be raised to 400 U (2 μL) to increase yield.
14. The reaction temperature should be increased to 55°C for gene-specific primers. Reaction temperature may also be increased to 55°C for difficult templates or templates with high secondary structure.
15. Amplification of some PCR targets (those >1 kb) may require the removal of RNA complementary to the cDNA. To remove RNA complementary to the cDNA, add 1 μL (2 units) of *E. coli* RNase H and incubate at 37°C for 20 min.
16. The second round of PCR with nested primers is recommended to extract low levels of fungal transcripts in host tissues. Ideally, it is good to design primers that encompass an intronic region in the amplified nucleic acid as this allows for an internal control to show that RNA, not DNA, is amplified.

Acknowledgments

This work was supported by NIH 1 R01 Al065728-01 and NSF IOB-0544428 (subcontract S060039) and NSF MCB-0236393 to NPK. We thank David K. Willis (University of Wisconsin-Madison) for introducing us to the real-time RT-PCR methodology and for many fruitful discussions. We thank Marisa K. Trapp for assistance in formatting this article.

References

1. Raper, K. B., and Fennell, D. I. (1965) *The Genus Aspergillus* pp. ix, 686 p., Williams & Wilkins, Baltimore.
2. Campbell, C. K. (1994) Forms of aspergillosis, in (Powell, K. A., Renwick, A., and Peberdy, J. F., Eds.), pp. 313–320, *The Genus Aspergillus*, Plenum, New York.
3. St Leger, R. J., Screen, S. E., and Shams-Pirzadeh, B. (2000) Lack of host specialization in *Aspergillus flavus*. *Appl. Environ. Microbiol.* **66**, 320–324.
4. Payne, G. A. (1998) Process of contamination by aflatoxin-producing fungi and their impact on Crops, in (Sinha, K. K., and Bhatnager, D., Eds.), pp. 279–306,

Mycotoxins in Agriculture and Food Safety, Marcel Dekker, New York.

5. Robens, J. F., and Richard, J. L. (1992) Aflatoxins in animal and human health. *Rev Environ Contam. Toxicol.* **127**, 69–94.
6. Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., Nyamongo, J., Backer, L., Dahiye, A. M., Misore, A., DeCock, K., and Rubin, C. (2005) Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environ. Health Perspect.* **113**, 1763–1767.
7. Leung, M. C., Diaz-Llano, G., and Smith, T. K. (2006) Mycotoxins in pet food: a review on worldwide prevalence and preventative strategies. *J. Agric. Food. Chem.* **54**, 9623–9635.
8. Denning, D. W., and Stevens, D. A. (1990) Antifungal and surgical treatment of invasive aspergillosis: review of 2,121 published cases. *Rev. Infect. Dis.* **12**, 1147–1201.
9. Rementeria, A., Lopez-Molina, N., Ludwig, A., Vivanco, A. B., Bikandi, J., Ponton, J., and Garaizar, J. (2005) Genes and molecules involved in *Aspergillus fumigatus* virulence. *Rev. Iberoam. Micol.* **22**, 1–23.
10. Latge, J. P. (2001) The pathobiology of *Aspergillus fumigatus. Trends Microbiol.* **9**, 382–389.
11. Dotis, J., and Roilides, E. (2004) Osteomyelitis due to Aspergillus spp. in patients with chronic granulomatous disease: comparison of *Aspergillus nidulans* and *Aspergillus fumigatus. Int. J. Infect. Dis.* **8**, 103–110.
12. Young, R. C., and Bennett, J. E. (1971) Invasive aspergillosis. Absence of detectable antibody response. *Am. Rev. Respir. Dis.* **104**, 710–716.
13. Bretagne, S. (2003) Molecular diagnostics in clinical parasitology and mycology: limits of the current polymerase chain reaction (PCR) assays and interest of the real-time PCR assays. *Clin. Microbiol. Infect.* **9**, 505–511.
14. Scherm, B., Palomba, M., Serra, D., Marcello, A., and Migheli, Q. (2005) Detection of transcripts of the aflatoxin genes *aflD, aflO*, and *aflP* by reverse transcription-polymerase chain reaction allows differentiation of aflatoxin-producing and non-producing isolates of *Aspergillus flavus* and *Aspergillus parasiticus. Int. J. Food. Microbiol.* **98**, 201–210.
15. Chen, R. S., Tsay, J. G., Huang, Y. F., and Chiou, R. Y. (2002) Polymerase chain reaction-mediated characterization of molds belonging to the *Aspergillus flavus* group and detection of *Aspergillus parasiticus* in peanut kernels by a multiplex polymerase chain reaction. *J. Food. Prot.* **65**, 840–844.
16. Hummel, M., Spiess, B., Kentouche, K., Niggemann, S., Bohm, C., Reuter, S., Kiehl, M., Morz, H., Hehlmann, R., and Buchheidt, D. (2006) Detection of *Aspergillus* DNA in cerebrospinal fluid from patients with cerebral aspergillosis by a nested PCR assay. *J. Clin. Microbiol.* **44**, 3989–3993.
17. Munoz, P., Guinea, J., and Bouza, E. (2006) Update on invasive aspergillosis: clinical and diagnostic aspects. *Clin. Microbiol. Infect.* **12**, 24–39.
18. Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R., and Keller, N. P. (2005) Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans. Microbiology.* **151**, 1809–1821.
19. Tsitsigiannis, D. I., Kunze, S., Willis, D. K., Feussner, I., and Keller, N. P. (2005) *Aspergillus* infection inhibits the expression of peanut 13S-HPODE-forming seed lipoxygenases. *Mol. Plant. Microbe. Interact.* **18**, 1081–1089.
20. Rotenberg, D., Thompson, T. S., German, T. L., and Willis, D. K. (2006) Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *J. Virol. Methods.* **138**, 49–59.
21. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
22. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. *Methods.* **25**, 402–408.