Analysis of the Fusarium virguliforme Transcriptomes Induced during Infection of Soybean Roots Suggests that Enzymes with Hydrolytic Activities Could Play a Key Role in Root Necrosis

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Introduction

Sudden death syndrome (SDS) caused by the fungal pathogen, *Fusarium virguliforme,* is a major threat to soybean production in North America. There are two major components of this disease: (i) root necrosis and (ii) foliar SDS. Root symptoms consist of root necrosis with vascular discoloration that extends upto several nodes and internodes into the stem. Foliar SDS symptom is characterized by interveinal chlorosis and necrosis in leaves which finally curl and fall off, and in severe cases by flower, pod abscission and immature seed formation. A major toxin involved in initiating foliar SDS has been identified. Nothing is known about how root necrosis develops. In order to unravel the mechanisms used by the pathogen to cause root necrosis, the transcriptome of the pathogen in infected soybean root tissues of a susceptible cultivar (Essex) was investigated. The transcriptomes of the germinating conidia and mycelia were also examined. Of the 14,845 predicted *F. virguliforme* genes, we observed that 12,017 (81%) were expressed in germinating conidial spores and 12,208 (82%) in mycelia and 10,626 (72%) in infected soybean roots. Of the 10,626 genes induced in infected roots, 224 were transcribed only following infection. Expression of several infection-induced genes encoding enzymes with oxidation-reduction properties suggests that degradation of antimicrobial compounds such as the phytoalexin, glyceollin could be important in establishing the biotrophic phase. Enzymes with hydrolytic and catalytic activities could play an important role in the transitioning of the pathogen from biotrophic to necrotrophic phase. Expression of a large number of genes encoding enzymes with catalytic and hydrolytic activities during late infection stage suggests cell wall degradation by some of these enzymes could be involved in root necrosis and establishing the necrotrophic phase in this pathogen.

Methods

1. Plant materials and inoculation

Soybean [*Glycine max* (L.) Merrill] cultivar, 'Essex' highly susceptible to *F. virguliforme*, was sown in vermiculite under dark conditions at 22°C. The 7-day old seedlings were uprooted and used for root infection. *F. virguliforme* Mont-1 isolate was maintained on Bilay agar plates and sub-cultured on 1/3 PDA agar plates. On 1/3 PDA plates, *F. virguliforme* Mont-1 isolate produces characteristic blue mass containing conidial spores in two weeks of growth. The roots of the Essex seedlings were infected with *F. virguliforme* Mont-1 conidial spores (10^7 spores/mL) and incubated under dark conditions until harvesting of infected root tissues. In the control samples, roots were treated with sterile water.

2. RNA extraction from germinating conidial spores, mycelia and root samples

F. virguliforme infected root samples were collected at 3, 5, 10 and 24 days post inoculation (dpi) (based on visual symptoms) from three independent experiments for sequencing transcripts. For Real time-PCR and semi-quantitative RT-PCR (qRT-PCR) analyses, root samples were harvested at 1, 3, 5 and 10 dpi either following *F. virguliforme* infection or treatment with water. In each experiment, roots of five plants were pooled for each treatment and frozen in liquid nitrogen and stored at -80°C until further use. RNA samples of infected roots from three experiments, collected 3 and 5 dpi, were bulked and named as "early infection" whereas the pooled RNA sample isolated from the infected roots, collected 10 and 24 dpi, were termed as "late infection" time point for deep sequencing. Germinating conidial spores were grown for 12 h in liquid modified Septoria medium (MSM). Mycelia were harvested from spores grown in MSM liquid media for two weeks. Total RNAs were isolated from the germinating conidial spores, mycelia and infected root tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, U.S.A.).

3. cDNA library preparation for sequencing transcripts

Total RNAs, 10 µg each from early and late time points of *F. virguliforme* infected soybean root tissues, germinating conidial spores, and mycelia were used to purify poly $(A)^+$ RNAs using oligo (dT) attached to the magnetic beads (Promega, Madison, WI). Poly $(A)^+$ RNAs were fragmented into short sequences in the presence of divalent cations at 94°C for 5 min. RNA samples were reverse transcribed using a cDNA synthesis kit from Illumina (Illumina, Inc. San Diego, CA, U.S.A.).

4. Sequencing of the *F. virguliforme* transcripts isolated from infected soybean roots, germinating conidial spores and mycelia

cDNAs of an individual RNA sample were sequenced in a single lane of the Illumina NGS platform GAII (Illumina, Inc. San Diego, CA, U.S.A.) at the DNA Facility, Iowa State University. The raw sequence reads were generated using Solexa GA pipeline 1.6. Adaptor sequences were trimmed off from the raw 75 bp sequence reads and reads were aligned to the *F. virguliforme* genome sequence (http://fvgbrowse.agron.iastate.edu/) using Bowtie program. The draft *F. virguliforme* genome sequence has been shown to contain 14,845 predicted genes. The generated SAM (Sequence Alignment/Map) output for each condition was used to extract mapped reads for corresponding genes using unix script command. The reads per kb per million reads (RPKM) for each gene was calculated according to the formula $R=10^9C/NL$. CIMminer was used to generate color-coded Clustered Image Maps (CIMs) ("heat maps") representing "high-dimensional" data sets such as gene expression profiles (http://discover.nci.nih.gov/cimminer/). A heat map was generated in one matrix CIM for the normalized values for each gene after RPKM analysis. TargetP1.1 (http://www.cbs.dtu.dk/services/TargetP/) was used to identify the candidate secreted proteins.

5. Identification of the candidate *F. virguliforme* virulence genes

We looked for infection-induced *F. virguliforme* genes that showed high identity to functionally characterized virulence genes by running NCBI BlastX program against nr database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Genes showing high identity to known virulence genes were tabulated.

6. Semi-quantitative RT-PCR and quantitative real-time PCR (qRT-PCR) analyses

Semi-quantitative PCR of several induce *F. virguliforme* genes was conducted to validate the expression profiles deciphered from deep sequencing of single RNA samples pooled from three biological experiments. Genes were selected randomly from individual functional categories. Total RNA samples were isolated from the *F. virguliforme*-infected or water treated etiolated root samples of soybean cv. Essex at 1, 3, 5 and 10 days post inoculation or water treatment by using TRIzol (Invitrogen, Carlsbad, CA, U.S.A.). qRT-PCR for a few selected genes were conducted on an iCycler sequence detection system (Bio-Rad; Hercules, CA, U.S.A) using SYBR Green fast qPCR master mix (Bio-RAD; Hercules, CA, U.S.A).

Results

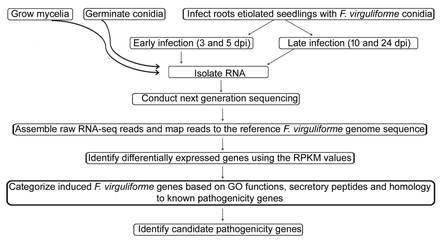


Fig 1. Flow diagram of the steps followed in conducting the transcriptomic analysis.

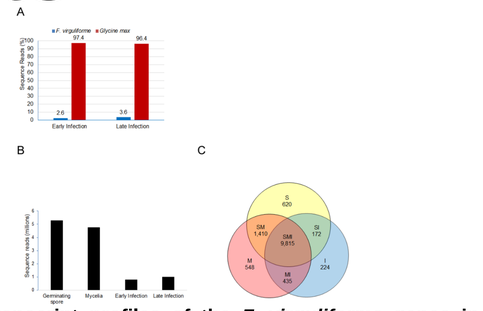


Fig 2. Transcript profiles of the *F. virguliforme* genes in various tissues

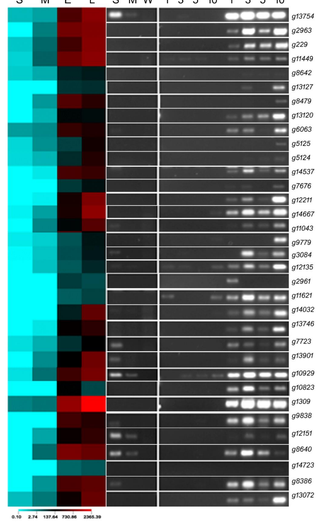


Fig 3. Semi-quantitative RT-PCR of selected genes from various functional categories of genes.

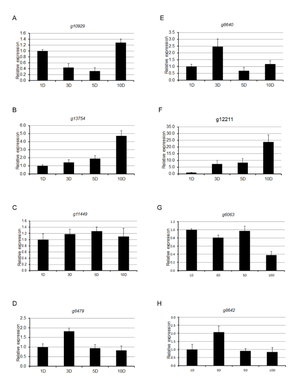


Fig 4. Quantitative RT-PCR of a few selected genes, evaluated in RT-PCR analyses.

Sample	Number of genes	% of genes expressed	Number of unique genes
Germinating conidial spores	12,017	81	620
Mycelia	12,208	82	548
Infection	10,626	72	224
(a) Early infection	9,545	64	18
(a) Late infection	10,344	70	79

Table 1. *F. virguliforme* genes expressed in germinating conidial spores, mycelia and infected soybean root tissues.

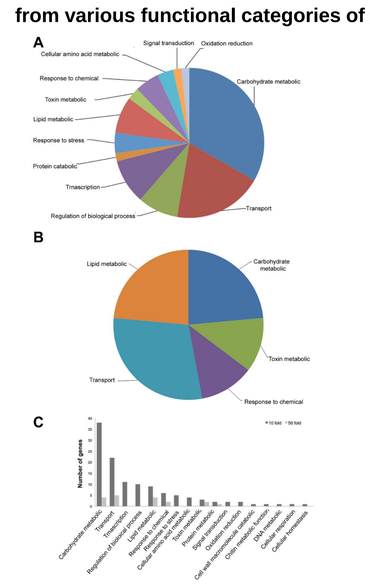


Fig 5. Categories of *F. virguliforme* genes based on BLAST2GO analyses for biological processes.

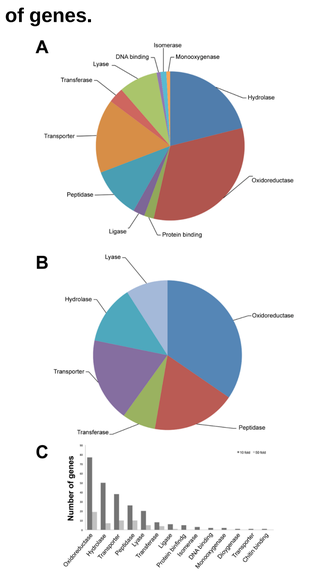
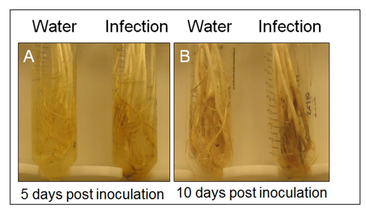


Fig 6. Categories of *F. virguliforme* genes based on BLAST2GO analyses for molecular functions.



Visual syptoms of root infection by *F. virguliforme*

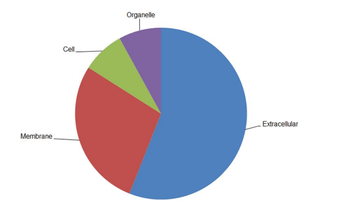


Fig 7. Categories of putative secretory *F. virguliforme* genes based on BLAST2GO analyses for cellular locations. Categories of putative secretory *F. virguliforme* genes based on cellular locations.

Conclusions

1. Our deep sequencing approach identified 10,626 (72%) of the 14,845 predicted *F. virguliforme* genes that have shown to have at least three sequence-reads in the infected soybean root tissues.
2. We have also identified 27 candidate virulence genes based on their homologies to functionally characterized virulence genes
3. Expression of several infection-induced genes encoding enzymes with oxidation-reduction properties for degradation of antimicrobial compounds such as the phytoalexin, glyceollin could be an important virulence mechanism in this pathogen to establish during early biotrophic phase
4. Genes pertaining to early induced genes, toxin genes (FvTox1), root necrosis, hydrolases were found to be enhanced with their expression upon infection.
5. This study suggests that enzymes with hydrolytic and catalytic activities play an important role in the transitioning the pathogen from biotrophic to necrotrophic phase. Expression of a large number of genes encoding enzymes with catalytic and hydrolytic activities during late infection stage suggests that cell wall degradation is involved in establishing the necrotrophic phase in this pathogen.

Bibliography

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