Small Secreted Proteins of *Pyrenophora tritici-repentis* and their Role in Wheat Infection

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Abstract

Host-selective toxins (HSTs) secreted by the pathogenic isolate of *Pyrenophora tritici-repentis*, BFP, are major pathogenicity factors in tan spot disease of wheat. Along with characterized HSTs such as Ptr ToxA (ToxA), BFP secretes at least two more uncharacterized toxic components. In an attempt to identify these additional components, 12 candidate genes predicted by comparative genomics were further analyzed by comparing their gene expression with ToxA. Four candidate genes (PTRG-B, -C, -D, and -E) were identified and cloned. PTRG-C was successfully expressed using *Pichia pastoris* expression system and its protein product shown to be a secreted protein.

Introduction

Wheat is an essential staple food and provides more than 20% of the world's caloric intake. *Pyrenophora tritici-repentis*, or *Ptr*, is a necrotrophic pathogenic fungus that causes tan spot of wheat and leads to wide spread crop loss (Ciuffetti et al. 2014). During pathogenesis *Ptr* expresses host-selective toxins (HSTs) that kill host cells to facilitate growth. HSTs such as *Ptr* ToxA (ToxA) are commonly small (less than 200 amino acids) secreted proteins that cause symptoms in susceptible cultivars of wheat. BFP is a pathogenic isolate of *Ptr* that secretes ToxA and at least two additional uncharacterized toxic components. The genome of BFP has been sequenced and genome annotation has provided insight into the genes that may encode these additional toxic components (Manning et al. 2013). Based on these and additional bioinformatics studies, 12 potential HST genes of BFP were described. We analyzed these 12 genes based on the hypothesis that uncharacterized HSTs similar to known HSTs, such as ToxA should be small, secreted, and expressed during infection on wheat.

Methods and Materials

Identifying and Cloning Candidate Genes

Putative HST genes were selected based on size (predicted protein less than 200 aa) and whether the proteins they encode are predicted to be secreted, and present only in pathogenic isolates (Manning et al. 2013). Gene expression patterns of the 12 genes (L.M. Ciuffetti and I. Pandelova, unpublished) were analyzed 30, 72, and 102 hours (hr) post inoculation (hpi) by BFP, for three live cultivars of wheat, Glenlea, 6B635, and Auburn, as well as dead Auburn.

To clone candidate HST genes, RNA was extracted with the RNeasy kit (Qiagen) from BFP tissue grown for 72 hr in Potato Dextrose Broth (Beckton, Dickinson, and Company) and RNA converted to cDNA with the iScript ™ cDNA Synthesis Kit (BioRad). Specific primers for Polymerase Chain Reaction (PCR) were designed based on the start and stop codons of each gene (Table 1). PCR was performed with the following parameters: 95º C for 2 minutes, 29 cycles of 95º for 25 sec, 58º for 25 sec, 72º for 25 sec, followed by 1 cycle of 72º for 7 min and a 4º hold. Amplification products were ligated into the cloning vector pGEM®-T Easy (Promega Corporation), transformed into *Escherichia coli* strain XL-1 Blue, and cultured overnight for 18 hours at 37º on low salt LB agar plates with IPTG and X-Gal for blue-white screening. White colonies that were predicted to contain the amplification product were grown overnight in Luria low salt broth. Plasmids were then isolated, digested with the restriction enzyme EcoRI, ran on a 1% agarose gel, and presence of the correct size PCR product determined. Plasmids that contained the proper sized insert were sequenced at the Center for Genome Research &

Biocomputing at Oregon State University. Cloned coding sequences PTRG-B, -C, -D, and -E within the pGEM®-T Easy plasmid were named pCJH1 through 4, respectively.

Protein Expression

PTRG-B, -C, and -D were sub-cloned into the expression vector pPICZ A (Thermo Fisher Scientific Inc.) and named pCJH5 through 7, respectively. The pCJH-6 vector was transformed into *Pichia pastoris* X-33 cells with the EasyComp™ transformation kit (Thermo Fisher Scientific Inc.) following manufacture instructions. A 5 ml X-33 cell culture with pCJH-6 in buffered minimal glycerol media was incubated overnight at 30 ºC with vigorous shaking, then separated by centrifugation, and suspended in 50 ml buffered minimal methanol media and incubation continued as above for 5 days. On days 2-4, 2.5 ml of 10% methanol was added to the culture. One ml samples were collected each day, centrifuged and the supernatant frozen at -20 ºC and later resolved on a SDS-PAGE.

Results and Discussion

Determining Potential Host Selective Toxin Genes

In addition to the well-characterized HST ToxA, the pathogenic *P. tritici-repentis* isolate, BFP, is known to produce at least two uncharacterized toxic components. Comparative genomics predicted 12 pathogen-specific genes encoded by BFP whose protein products are predicted to have the characteristics of HSTs, i.e. less than 200 amino acids (aa) and secreted. Additionally, we expected that their expression patterns would be similar to ToxA. To test this, we examined gene expression patterns of ToxA and the 12 potential HST genes *in planta* at 30, 72, and 102 hours post inoculation (hpi) with BFP. Inoculations included three live cultivars of wheat, Glenlea, 6B635, and Auburn, as well as dead Auburn. Seven of the 12 genes of interest exhibited very low or no expression levels in all inoculations (data not shown). ToxA exhibited high expression levels at 30 hpi with reduced levels at 72 and 102 hpi within live cultivars (Figure 1a, blue, green and pink), while expression in dead Auburn (red) remained low over time. As compared to ToxA expression, PTRG-A (Figure 1b) was expressed at relatively constant levels over time within cultivars 6B365 (green) and live Auburn (blue), and at higher levels at 72 and 102 hpi within Glenlea (pink) and dead Auburn (red). PTRG-B, -C, -D, and -E (Figure 1c, 2a, 2b, and 2c, respectively) were expressed similar to ToxA. The expression patterns of ToxA, PTRG-B, -C, -D, and -E are consistent with the function of a HST, which is needed early in the infection process to induce cell death; after tissue death, during later time points, they are no longer needed. Expression of HSTs in dead leaves (in this study Auburn) is not necessary, as the fungus can grow saprophytically. Given these data, PTRG-B, -C, -D, and -E were chosen for further analysis.

Cloning the Candidate Genes

Comparative genome analysis and gene expression data provided putative candidate HST genes PTRG-B, -C, -D, and -E. Further investigations were conducted to determine if the proteins produced by each gene are HSTs, i.e. treatment of a sensitive wheat cultivar by the purified protein leads to symptom development. Large amounts of protein can be obtained through heterologous gene expression, which is the expression of a cloned gene within a host organism that does not naturally contain that gene. For heterologous expression, we used cDNA synthesized from BFP RNA as the template for cloning. The coding sequences of PTRG-B, -C, -D, and -E were cloned and the presence of the proper PCR insert was confirmed through enzymatic restriction digest followed by DNA electrophoresis and DNA sequencing. Sequence confirmation indicated that the protein coding regions of our putative HSTs were available to be sub-cloned into other vectors for heterologous expression.

Protein Expression

The *Pichia pastoris* yeast heterologous expression system has been used successfully to produce small, secreted proteins (Touri 1995, Andrie 2011, Betts 2011). Benefits of this system include similarity to *Ptr* protein secretion and processing, as both are eukaryotes, and easy manipulation to secrete large amounts of protein by gene induction with methanol. During transformation of *P. pastoris* cells, coding sequences that were cloned downstream from a methanol inducible promoter into an expression vector are integrated into the *P. pastoris* genome through homologous recombination at the methanol inducible promoter region. Growth of the cells in methanol–containing media leads to induction of the promoter and production of protein from the cloned coding sequences. If these proteins contain a secretion signal, they will be secreted into the media and can be purified for further investigation.

PTRG-B, -C, and -D were successfully sub-cloned into pPICZ A, but due to time constraints only -C was transformed and expressed. To determine if PTRG-C is a secreted protein, the X-33 strain of *P. pastoris* was transformed with the pCJH-6 (PTRG-C in pPICZ A) expression plasmid, grown, and induced with methanol as described above. Media samples were taken at 0, 24, 48, 72, and 96 hours post induction. The five samples of pCJH-6 were resolved by SDS-PAGE and proteins detected by silver stain (Figure 3). Based on gene annotation, it is predicted that the mature protein PTRG-C would be ~ 8.1 kDa in size. On the protein gel, a band of ~ 8 kDa (directly above red arrow) appears over time. This suggests that PTRG-C is a small, secreted protein. Due to time constraints, we were unable to purify and bioassay the protein on BFP susceptible cultivars of wheat. Therefore, we could not conclude that PTRG-C is a HST.

From the initial 12 potential HST genes encoded in the pathogenic *Ptr* isolate, BFP, eight were eliminated because of low expression *in planta* or dissimilar expression patterns to ToxA. Their coding sequences were successfully cloned into pGEM and sub-cloned into the pPICZ A plasmid vector. The protein product of PTRG-C was successfully expressed and shown to be a secreted protein. Further work is required to determine whether these candidate genes are HSTs.

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References

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Figure Legends:

Figure 1. Gene expression patterns of BFP specific genes: a) HST ToxA, b) PTRG-A and c) PTRG-B.

Figure 2. Gene expression patterns of BFP specific genes: a) PTRG-C, b) PTRG-D, and c) PTRG-E.

Figure 3. SDS-PAGE of proteins secreted into supernatant of X-33 culture expression of PTRG-C. Lane 1 contains size marker (kDa), lanes 2-6 contain culture expression samples taken from 0, 24, 48, 72, and 96 hours, respectively. Band above red arrow indicates small, secreted protein of interest.

Table 1. Forward and reverse primers for PCR amplification of the coding sequences of putative HST genes.

Figures and Tables:

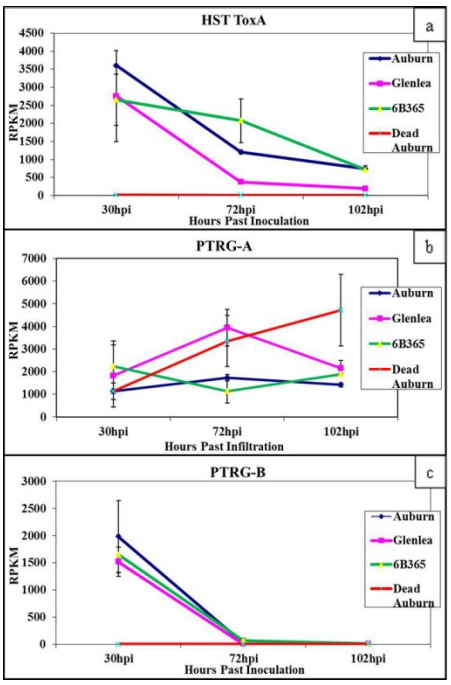

Figure 1

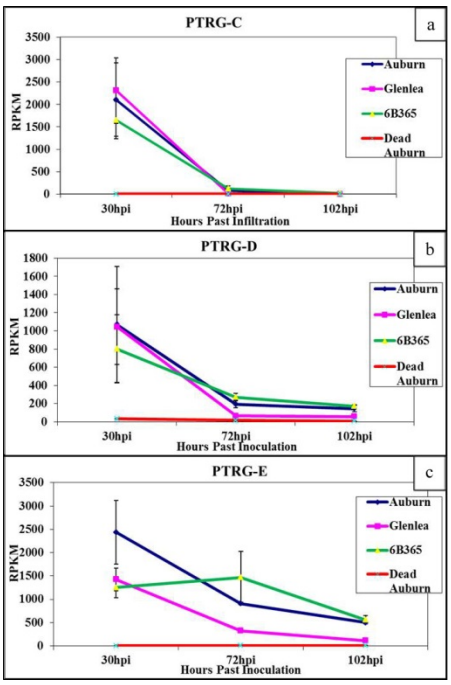

Figure 2

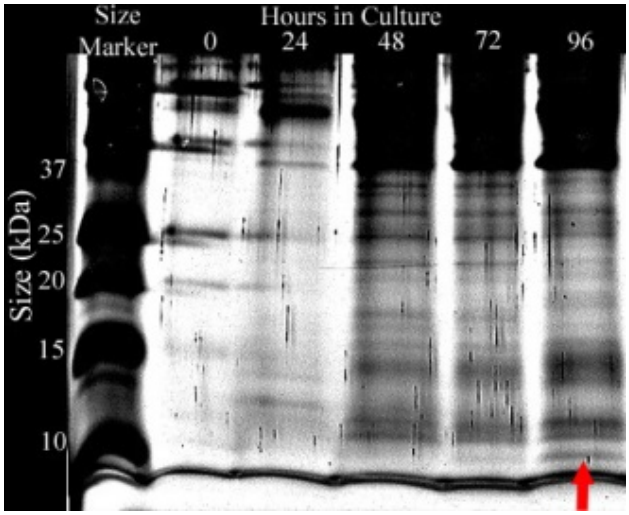

Figure 3

Gene	Coding Sequence	Forward/Reverse
PTRG_B	ATGAAGTTCCTAACTGCCG	Forward
PTRG_B	AAGCCCTAAGCCCACTC	Reverse
PTRG_C	AATGAAGGGTCTTGCC	Forward
PTRG_C	GCCCTAAATCCTAGCC	Reverse
PTRG_D	CACTATGCGCTTCTTTACTG	Forward
PTRG_D	CTCTAGCAAATTTCACCTCC	Reverse
PTRG_E	GCCCTTAAGATGAGGATCG	Forward
PTRG_E	GCATCTACGAGTCGCAGC	Reverse

Table 1