AN ABSTRACT OF THE THESIS OF

Kara B. Miles-Rockenfield for the degree of Honors Baccalaureate of Science in Microbiology presented on May, 28 2009. Title: Mutagenesis and Crystallization of Ptr ToxB, a host-selective toxin of *Pyrenophora tritici-repentis.*

Abstract Approved:

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Pyrenophora tritici-repentis (Ptr) is the fungal causal agent of the disease tan spot in wheat and causes necrosis and chlorosis in infected leaves. These symptoms are caused by host-selective toxins (HSTs), molecules that serve as the sole determinants of pathogenicity. Ptr has three known HSTs, the proteinaceous Ptr ToxA and Ptr ToxB, and the non-proteinaceous Ptr ToxC. Ptr ToxB causes chlorosis on sensitive cultivars and has a protein homolog, toxb, which is present in a non-pathogenic race of Ptr. Using mutagenesis, six chimeric proteins were created from the *ToxB* and *toxb* genes. The study of the six chimeric proteins allowed us to investigate which specific regions of ToxB were responsible for the protein's induction of chlorosis in sensitive wheat cultivars. The presence of a higher molecular weight band on SDS-PAGE of toxb and chimeras containing the N-terminus of toxb suggest that this region in toxb is responsible for improper folding. The assessment of activities of the chimeras and site-directed mutants by bioassay further suggest that this region is also important for the lack of protein activity seen in toxb. Crystallization with the goal of determining the tertiary structure solution of ToxB has despite much effort proven unsuccessful.

Mutagenesis and Crystallization of Ptr ToxB,
a host-selective toxin of *Pyrenophora tritici-repentis*

by

Kara B. Miles-Rockenfield

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Kara B. Miles-Rockenfield, Author

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INTRODUCTION

The ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem.) is the fungal causal agent of the disease tan spot in wheat. The fungus propagates both asexually by conidia and sexually by ascospores (reviewed in Ciuffetti and Tuori 1999). The fungus is a diurnal sporulator with conidia that is disseminated by the wind. Pseudothecia from *Pyrenophora tritici-repentis* (Ptr) can develop on infected wheat residue and give rise to ascospores that are generally thought to be the source of primary inoculum. Infected kernels are called red smudge and can affect seedling emergence, seedling vigor, yield, and grain quality, as well as provide inoculum for further propagation of the disease (Ciuffetti and Tuori 1999). The organism was first described in 1902 on wheat and grasses, but large-scale epidemics have only been recorded since the early 1970s, and are increasing world wide (De Wolf et al. 1998, Strelkov et al 2003). Since Ptr is a stubble-born pathogen, the incidence of tan spot has increased over recent years due to an increase in farming practices such as zero and minimum tillage and shorter crop rotations (Strelkov et al. 1999). These practices leave crop residue on the surface, allowing the pathogen to survive from season to season and increase over time (Strelkov et al 2003).

The first occurrences of the disease in the United States were in 1940 in New York and 1947 in Kansas. It has been reported as the fastest-spreading disease in the Southern

Cone region of South America, a major leaf disease in northern wheat-growing areas of Australia, and one of the main wheat diseases in Central Asia. Tan spot also has a major economic impact on wheat growing areas worldwide, including the U.S., and can result in yield losses ranging from 3% to 50% (Rees et al. 1983). Foliar fungicides have been effective in the control of the disease, but in some areas costs are prohibitive (Lamari and Bernier 1989).

Tan spot symptoms are described as yellow to tan-brown elliptical necrotic lesions with a brown to black point at the center surrounded by a chlorotic border, often spreading to cover the entire leaf (reviewed in Strelkov and Lamari 2003). These symptoms, necrosis and chlorosis, are caused by host-selective toxins (HSTs), molecules that serve as the sole determinants of pathogenicity and are only known to be produced by plant pathogenic fungi (Walton 2000, Wolpert et al 2002). HSTs are the main determinants of pathogenicity as they are toxic only to susceptible hosts, and, toxin production by the pathogen is correlated with the disease of a susceptible host (Ciuffetti and Tuori 1999, Strelkov and Lamari 2003).

Initially, four pathotypes of Ptr were determined based upon the ability of an isolate to produce necrosis and/or chlorosis in susceptible wheat. Pathotype 1 isolates produce both chlorosis and necrosis, pathotype 2 produces only necrosis, pathotype 3 produces only chlorosis, and pathotype 4 was determined to be nonpathogenic and lack either symptom (Strelkov and Lamari 2003). After the discovery of isolates that produce different combinations of these symptoms, these four pathotypes became races 1 through 4, with

new races being classified as new isolates are identified. Eight different races are currently characterized (with other races and toxins known) (Table 1), with the classification system only being limited by the uniqueness and number of differential wheat cultivars used (Strelkov and Lamari 2003). Races 1, 2, 7, and 8 produce Ptr ToxA (ToxA), races 5, 6, 7, and 8 produce Ptr ToxB (ToxB), and races 1, 3, 6, and 8 produce Ptr ToxC (ToxC). The nonpathogenic race 4 has not yet been shown to produce HSTs, and therefore no symptoms are attributed to this race (Strelkov and Lamari 2003).

Ptr has three known HSTs, the proteinaceous ToxA and ToxB, and the non-proteinaceous ToxC. ToxA causes necrosis on sensitive wheat cultivars and is encoded by a single gene, *ToxA* (Ciuffetti et al. 1997). ToxB causes chlorosis on sensitive cultivars but unlike ToxA, it is encoded by multiple copies of a single gene, *ToxB* (Martinez et al. 2001, 2004). Similarly, ToxC, a polar, nonionic, low molecular weight compound, also induces chlorosis, but in different cultivars than ToxB (Effertz et al. 2002). The gene(s) responsible for the production of ToxC have not yet been characterized.

ToxA was the first HST from Ptr to be described (Tomas et al. 1990, Tuori et al. 1995, Ballance et al. 1989) and therefore is the best characterized. The *ToxA* gene encodes a protein that contains a pre-region composed of a 23 amino acid residue secretion signal (Ciuffetti et al. 1997, Ballance et al. 1996), followed by a pro-region of a 4.3 kDa anionic domain that is required for proper folding of the protein (Ciuffetti et al. 1997, Tuori et al. 2000). The pro-region is cleaved from the mature peptide and the active toxin is a small, 13.2 kDa protein (Tuori et al. 2000) that causes necrosis when infiltrated into leaves.

Table 1: Sensitivity of differential wheat lines to host-selective toxins of races of *Pyrenophora tritici-repentis* (From Strelkov and Lamari 2003).

	Race							
Differential line or cultivar	4	2	1	8	7	5	6	3
'Glenlea'		**Ptr ToxA**						
6B662				**Ptr ToxB**				
6B365			**Ptr ToxC**				**Ptr ToxC**	
Number of toxins produced*	0	1	2	3	2	1	2	1

Note: Shaded areas under each race indicate the toxin produced for the respective cultivars or differential lines. A blank indicates that no toxin is produced. 'Glenlea' is sensitive to Ptr ToxA; lines 6B662 and 6B365 are sensitive to, respectively, Ptr ToxB and putative toxin or pathogenicity factor Ptr ToxC.

*The eight races follow a 1:3:3:1 distribution for the production of 0, 1, 2, and 3 toxins, respectively: race 4 (0 toxin) : races 2, 3, 5 (1 toxin) : races 1, 6, 7 (2 toxins) : race 8 (3 toxins).

Structural studies of ToxA, including site-directed mutagenesis and x-ray crystallography, have brought to light the inner workings of this once-mysterious HST. The structure of ToxA was determined through x-ray crystallography as a single domain protein having a β-sandwich fold with two antiparallel β-sheets composed of four strands each (Sarma et al. 2005). ToxA contains a solvent-exposed loop which includes an Arg-Gly-Asp (RGD) sequence, the motif that may interact with a receptor on a plasma membrane in order to facilitate toxin internalization (Manning et al. 2008). Formation of a disulfide bond was also shown to be required for the stability of the protein (Tuori et al. 2000). ToxA is structurally similar to the fibronectin type III domain, which suggests a possible evolutionary relationship and a hypothetical means of entrance into a host cell through an integrin-like receptor (Sarma et al. 2005). Site-directed mutagenesis of ToxA allowed us to recognize that it associates with a high-affinity binding site on wheat mesophyll cells through the RGD-containing loop, which leads to toxin internalization and eventual cell death (Manning et al. 2008). After ToxA is internalized it localizes to the chloroplasts and alters their function (Manning et al. 2005, 2009). ToxA ultimately causes cell death due to the accumulation of reactive oxygen species (Manning et al. 2009).

ToxB is present in multiple copies in races 5, 6, 7, and 8 which produce chlorotic symptoms on the wheat cultivar Katepwa (Martinez et al. 2001, Strelkov et al. 2002). There are an estimated nine copies of the *ToxB* gene in the Ptr race 5 genome, which produce an additive effect on symptoms and increase the transcript level of *ToxB* compared to isolates with fewer copies of the gene (Martinez et al. 2009, Amaike et al.

2008). Six of the nine copies were cloned and analyzed (Martinez et al. 2004). It was found that all six copies had an identical 261 bp open reading frame (ORF) and encode the same form of *ToxB*. The majority of loci flanking these sequences are associated with retrotransposons and transposon-like sequences. There is no sequence similarity between *ToxA* and *ToxB*, suggesting that the two proteins may use different mechanisms for promoting pathogenicity. Other forms of *ToxB* are also present in several races lacking toxin activity, including isolates from races 3 and 4 (Strelkov and Lamari 2003, Martinez et al. 2004). DNA sequencing of the *ToxB* homologs from these isolates revealed differences in the promoter and signal peptide coding regions when compared with *ToxB*. The homolog from a non-pathogenic race 4 isolate, *toxb*, had differences that extended into the region of the mature protein, which may account for the absence of activity (Martinez et al. 2004).

ToxB has a 261-bp ORF that has no introns and encodes an 87 amino acid (aa) protein. The first 27 aa are a signal peptide that is cleaved, and the remaining 64 aa represent the mature form of ToxB (Martinez et al. 2001). Our laboratory has shown that the inactive homolog, toxb, found in non-pathogenic isolates of Ptr, does not induce symptoms when infiltrated into a ToxB-sensitive wheat cultivar. *toxb* is 86% identical in sequence to *ToxB*, has one additional amino acid, and unique flanking sequences (Martinez et al. 2004).

The overall objective of this research is to determine which region(s) of the structure of ToxB are responsible for the toxicity of the protein. This goal was achieved by using

mutagenic techniques to design chimeric proteins using the genes *ToxB* (active form) and *toxb* (inactive form). After narrowing down the region we believe to be the main source of ToxB's toxicity, we introduced point mutations to further discover the specific amino acids that cause changes in toxicity. Circular dichroism and protein crystallization methods were employed to learn more about the overall structure of ToxB.

MATERIALS AND METHODS

Purification of ToxB from Pyrenophora tritici-repentis

The following procedure for the purification of ToxB has been adapted and modified from the procedure presented in Strelkov, et al. 1999.

Plates with ToxB producing isolate of Ptr DW7 were grown in the dark for five to seven days. Plugs cut from around the edge of the mycelial growth from three plates were inoculated into 1L of Modified Fries media (15 g sucrose, 1 g ammonium nitrate, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.13g CaCl, 0.1 g NaCl, 1 g yeast extract/liter of H_2O). The cultures were grown in an incubator at 24°C for 21-28 days in constant light.

The fungal mass was filtered through a double layer of cheesecloth and Whatman #1 filter. The collected crude culture filtrate (CCF) was frozen and lyophilized to dryness. The lyophilized sample was re-suspended in 10-30 mL of water.

Large, unwanted proteins were precipitated using 20% ammonium sulfate. The ammonium sulfate was slowly added over time, and the sample left to precipitate at 4°C overnight. The sample was spun in a centrifuge for 30 min at 6000 rpm, and the supernatant saved. The remaining proteins from the CCF were precipitated with 80% ammonium sulfate and centrifuged as described above. The pellet was saved and re-suspended in 10-15 mL of water.

The sample was dialyzed for two nights against 2 L of water each night. The sample was loaded on to a weak cation exchange column (Waters, Accel Plus CM column) equilibrated with 10 mM sodium acetate pH 4.6. Flow through fractions were collected, the column was washed with 4 column volumes of equilibration buffer, and protein eluted in two steps with 0.1 M and 0.5 M NaCl in 10 mM Na acetate pH 4.6. The fractions were analyzed by a silver stained 14% acrylamide SDS-PAGE (Fling and Gregerson 1986) and Western Blot. Fractions that contained ToxB were combined, concentrated, and dialyzed against 20 mM Tris pH 8.0.

The sample was then purified on a strong anion exchange column (Waters Accel Plus QMA) equilibrated with 20 mM Tris pH 8.0. Flow through, washes and elutions with 0.5 M NaCl in 20 mM Tris, pH 8.0 were collected. The fractions were analyzed by a silver stained 14% acrylamide SDS-PAGE and Western Blot. Fractions containing ToxB were combined, concentrated to 2-5 mL, and dialyzed against 20 mM Na acetate pH 4.6.

The samples were applied on a High Performance Liquid Chromatography (HPLC) MonoS column (Waters) and purified using a linear gradient of 0 to 250 mM NaCl in 20 mM Na acetate pH 4.6. The fractions were analyzed by silver stained 14% acrylamide SDS-PAGE. Fractions containing purified ToxB were combined, dialyzed against water, and stored at -20°C for further use. Protein concentration was estimated using a Pierce BCA Protein Assay with Bovine Serum Albumin as a standard.

Preparation of Plasmids Containing ToxB/toxb Chimeric Open Reading Frames

In order to facilitate the synthesis of the chimeras, *Toxb* and *toxb* needed to be placed in a plasmid that had a unique combination of restriction sites so that the genes could be split into three regions. pBlueScriptII (pBSII) was chosen due to unique locations of the restriction enzymes *Bsa*I and *Bse*YI that were also present in *ToxB* and *toxb* and allowed the sequence to be divided into three regions (Figures 1 & 2). Region 1 contains a 143 bp sequence including plasmid DNA and the upstream end of the *ToxB/toxb* gene. Region 2 contains a 79 bp sequence in the middle of the *ToxB/toxb* open reading frame (ORF), and region 3 contains 124 bp sequence including the downstream end of *ToxB/toxb* and plasmid DNA. Colonies of *Escherichia coli* containing the plasmids pCM10 and pCM11, which include *toxb* and *ToxB* ORFs respectively, were isolated by streaking on LSLB (10 g tryptone, 5 g yeast extract, 5 g NaCl/liter of water) plates containing 25 µg/mL Zeocin. Overnight cultures of the *E. coli* containing pCM10 and pCM11 were grown in LSLB liquid media containing 25 µg/mL Zeocin. The cells were centrifuged and plasmids isolated according to the procedure for the Qiagen MiniPrep Kit. Isolated plasmids were cut with restriction enzymes *Eco*RI and *Not*I, and dephosphorylated using Shrimp Alkaline Phosphatase (SAP) enzyme and purified using the procedure from the Qiagen Gel Extraction Kit. The fragments were ligated into pBSII, which had also been cut with *Eco*RI and *Not*I, using T4 DNA ligase (Figure 2).

The ligations were transformed into XLI-Blue Competent cells (Stratagene) by adding 1 µL of each ligation reaction to thawed competent cells and incubating for 20 min on ice.

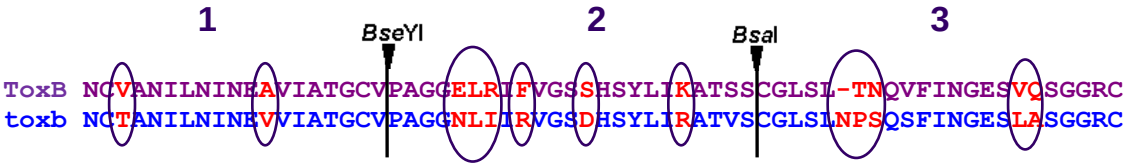

Figure 1: Restriction Sites of *Bse*YI and *Bsa*I in the *ToxB* and *toxb* ORFs. Cutting sites of the restriction enzymes *Bse*YI and *Bsa*I in the *ToxB* (top line, purple) and *toxb* (bottom line, blue) amino acid sequence. Using these cutting sites, the sequences were divided into three regions. Region 1 contains the upstream end to the *Bse*YI site and two amino acid changes; region 2 contains the *Bse*YI site to the *Bsa*I site and six amino acid changes; and region 3 contains the *Bsa*I site and the downstream end and four amino acid changes and one insertion in the *toxb* sequence.

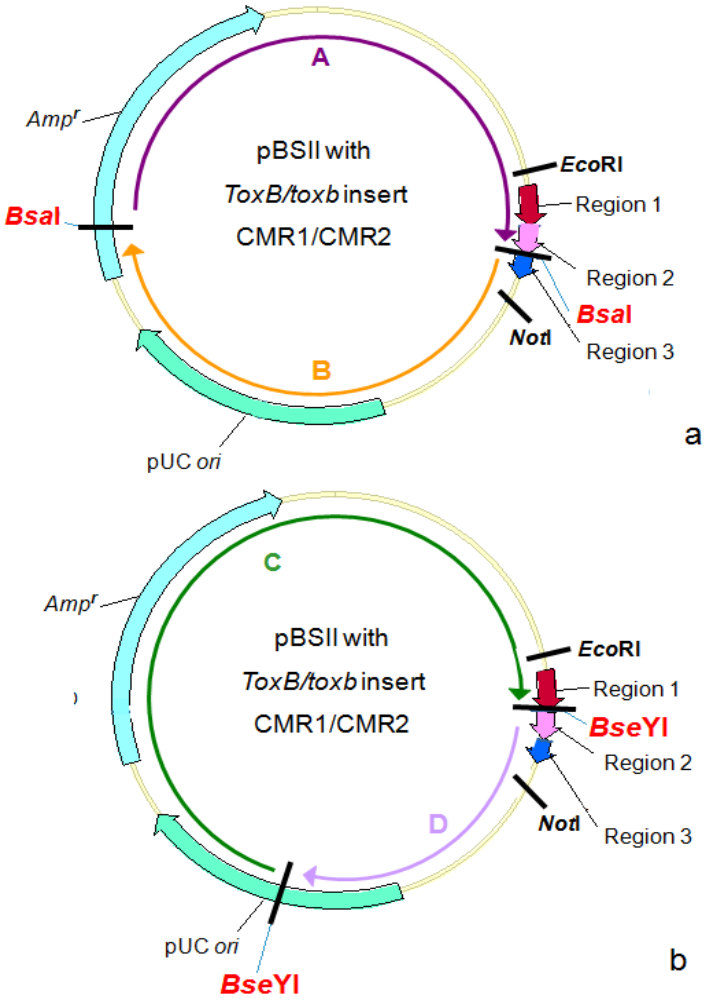

Figure 2: Plasmid maps of CMR1 and CMR2 with *Bse*YI and *Bsa*I. pBSII with either the *ToxB* or *toxb* gene was cut with either *Bsa*I or *Bse*YI to create two fragments that could be ligated in different combinations to create chimeric plasmids. **a)** pBSII shown with the restriction sites of *Bsa*I. The plasmid is divided into two fragments: A (dark purple arrow), which includes the C-terminal end of the *Amp*^{r} gene (light blue arrow) and region 1 (red arrow) and region 2 (pink arrow); B (orange arrow), which includes region 3 (blue arrow), the pUC *ori* (light green arrow), and the N-terminal end of the *Amp*^{r} gene. **b)** pBSII shown with the restriction sites of *Bse*YI. The plasmid is divided into two fragments: C (dark green arrow), which includes the C-terminal end of pUC *ori*, the entire *Amp*^{r} gene and region 1; D (lavender arrow), which includes region 2, region 3 and the N-terminal end of pUC *ori*.

The cells were placed at 42°C for 1 min and then incubated on ice for 2 min. After adding 900 µL of LSLB and shaking at 37°C for 1 h, the cells were plated on LSLB plates containing 100 µg/mL ampicillin, and incubated at 37ºC overnight. Plasmids were prepared from individual colonies and screened for an insert by cutting with *Eco*RI and *Not*I and analyzing on a 1% agarose gel. Clones with an insert were sequenced at the Center for Genome Research and Biocomputing (CGRB) lab at Oregon State University to confirm the proper insert, generating pCMR1 with the *toxb* insert and pCMR2 with the *ToxB* insert (Figure 2).

As mentioned above, pBSII was chosen for the use of its restriction sites *Bsa*I and *Bse*YI. Using these sites, the plasmids were broken into two pieces when cut with either *Bsa*I or *Bse*YI (Figure 2). When cut with *Bsa*I, fragment A contained regions 1 and 2 along with plasmid DNA including part of the Ampicillin resistance gene (*Amp*^{r}), and fragment B contained region 3 and plasmid DNA including the pUC origin of replication (*ori*) and the upstream end of *Amp*^{r}. Similarly, when cut with *Bse*YI, fragment C contained region 1 and plasmid DNA including the entire *Amp*^{r} and the downstream end of the ori, and fragment D contains regions 2 and 3 and the upstream end of the ori. Using combinations of these fragments from both pCMR1 and pCMR2, chimeric plasmids were created.

Plasmids were isolated from overnight cultures of pCMR1 and pCMR2 and purified using the procedure for the Qiagen MidiPrep Kit. pCMR1 and pCMR2 were cut with *Bsa*I by incubation at 50°C for 1 h to generate fragments 1A and 1B, and 2A and 2B, respectively. Fragments were purified as previously described. This process was

repeated with *Bse*YI and adding sodium dodecyl sulfate (SDS) to restriction reactions, to create fragments 1C and 1D (from pCMR1), and 2C and 2D (from pCMR2). Fragments 1A, 1C, 2A, and 2C were dephosphorylated by adding SAP enzyme and incubating at 37°C for 10 min, and incubating at 65°C for 15 min to inactivate the enzyme.

Fragment 1A was ligated with fragment 2B using T4 DNA ligase to create a chimeric plasmid, pCMR3. Fragment 2A was ligated with fragment 1B to create pCMR4, fragment 1C ligated with 1D to create pCMR5, and fragment 1D ligated with 2C to create pCMR6. The ligations were then transformed into XLI-Blue competent cells and plated on LSLB plates with 100 µg/mL Ampicillin. Individual colonies were screened for an insert by extracting the plasmids using the Qiagen MiniPrep Kit, cutting with *Eco*RI and *Not*I, and running on a 1% agarose gel. Sequencing to confirm the correct sequence was performed as described above.

In order to make the last two chimeras, pCMR7 and pCMR8, two chimeras previously made where utilized. First, pCMR4 was cut with *Bse*YI and pCMR6 with *Bsa*I. The fragment containing region 2 and region 3 from pCMR4 was ligated with fragment 1C to create pCMR7. The fragment from pCMR6 was ligated with fragment 2B to create pCMR8. The ligations were transformed, plasmids extracted and screened for insert from individual colonies, and sequence confirmed as previously described.

In preparation for transformation of the chimeric genes into *Pichia pastoris* for expression of the chimeric proteins, the chimeric genes from pCMR3, 4, 5, 6, 7, and 8

were ligated into a new vector, pPICZB. The chimeric plasmids were cut with *Eco*RI and *Not*I, the inserts isolated and ligated into pPICZB, which was also cut using *Eco*RI and *Not*I. The ligations were plated on LSLB plates with 25 µg/mL Zeocin. The ligations were transformed, plasmids extracted and screened for insert from individual colonies, and sequence confirmed as previously described.

Site-Directed Mutagenesis

Custom primers CMR2-F/CMR1-R (5'CTTAAACATCAACGAAGTGGTTATTGCGACTGG/GTTTAAGATATTGGCGGTGCAGTTGGCCGAAAC) and CMR2-R/CMR1-F (5'CCAGTCGCAATAACCACTTCGTTGATGTTTAAG/GTTTCGGCCAACTGCACCGCCAATATCTTAAAC) were made to generate point mutations of $Valine_{25}$ to Threonine (V_{25}/T) (SDM1) and $Alanine_{35}$ to Valine (A_{35}/V) (SDM2) in the *ToxB* ORF. The mutations were introduced by annealing the mutagenic primers to pCM11 (containing the *ToxB* ORF) and replicating the plasmid through PCR using PfuTurbo enzyme. Since the newly amplified DNA is not methylated and the parental DNA is, the parental DNA was removed by targeting methylated DNA as described in the Stratagene QuikChange Site-Directed Mutagenesis Kit. The parental methylated DNA was digested by adding 1 µL of *DpnI* restriction enzyme, mixing thoroughly, spinning in a microcentrifuge for 1 min, and incubating at 37ºC for 1 h. The reactions were analyzed on a 1% agarose gel to confirm amplification of DNA. The reactions were purified using Qiagen PCR Reaction Cleanup procedure transformed into XLI-Blue cells, screened for

insert, and sequenced as described above. Plasmids containing the mutations were used for subsequent expression.

Expression of proteins in Pichia pastoris

Three micrograms of plasmids were linearized with *SacI* restriction enzyme, purified using the Qiagen MinElute Kit and resuspended in 10 µL of 50% EB (10 mM Tris-Cl, pH 8.5) buffer. The samples were evaporated to dryness and resuspended in 5 µL of water. The samples were then transformed into *Pichia pastoris* using the procedure from the Invitrogen Pichia EasyComp Transformation Kit. The plasmids were linearized to facilitate integration into the *P. pastoris* genome by recombination. The transformed cells were plated on YPDS (10 g yeast extract, 20 g peptone, 182.2 g sorbitol, 20 g agar, 100 mL of 10% dextrose, 1 L water) plates with 100 µg/mL Zeocin and incubated at 30°C for two to three days.

To confirm the presence of the chimeric genes, proteins were precipitated from the media for small scale expressions of individual colonies (see below for details of expression). 100 µL of 0.15% deoxycholate was added to 1 mL of supernatant from spun down *P. pastoris* cells, mixed, and incubated at room temperature for 15 min. Next, 100 µL 72% trichloroacetic acid (TCA) was added to each sample, mixed, and centrifuged at 13,000 rpm for 15 min. The supernatant was decanted and the sample rinsed with 1 mL 75% ethanol, then centrifuged for 5 min at 13,000 rpm. The supernatant was decanted again and placed in a 37°C incubator for 5 min to remove the remaining ethanol. The samples

were resuspended in 100 µL "O" Buffer (0.0625 M Tris pH 6.8, 2.3% SDS) and placed in a 37°C incubator for 5-10 minutes for easy resuspension. The precipitated proteins were analyzed by 14% acrylamide SDS-PAGE and a Western Blot using anti-ToxB (1:1,000) as the primary antibody and rabbit-HRP (Sigma Chemicals) (1:40,000) as the secondary antibody to confirm the presence of *ToxB* ORF.

Because the chimeric genes had been recombined into the host genome, genomic DNA was extracted using the Zymo Research YeaStar Genomic DNA Kit to confirm the correct chimeric sequences. The isolated DNA was then amplified using a Polymerase Chain Reaction (PCR) with primers TB17 (5'TATAGAATTCCCTTGTTAG AAAATGGC) and TB16r (5'ATAGTTATGCGGCCGCTATACCTAATGTAGG). Amplified DNA was run analyzed on 1% agarose gel to confirm correct amplification, and the DNA was purified using the Qiagen PCR Reaction Cleanup Kit. The DNA was sequenced to confirm correct sequences as described above.

For protein expression, the *P. pastoris* chimeras were grown in 5 mL cultures of BMG (100 mL 1 M KH_2PO_4 pH 6.0, 100 mL 10% YNB [134 g yeast nitrogen base with $(NH_4)_2SO_4$ and without amino acids/liter of water], 2 mL 0.02% Biotin, 100 mL 10% glycerol/liter of water) overnight shaking at 30°C. The 5 mL cultures were spun down, and 100 mL cultures of BMM (100 mL 1 M KH_2PO_4 pH 6.0, 100 mL 10% YNB, 2 mL 0.02% Biotin, 100 mL 5% methanol/liter of water) were inoculated. The cultures were left shaking at 30°C for three days. On the second and third days, 5 mL of 10% methanol was added to each culture to induce the AOX1 promoter to express the chimeric ORFs.

On the last day, the cultures were spun at 6000 rpm for 15 min and the supernatant, containing the secreted protein used for protein purification, was saved. The supernatant from each chimera was brought down to a volume of about 10 mL using a rotary evaporator. Each sample was dialyzed twice against 1 L of water overnight. The volume of the dialyzed samples was brought down to 5-10 mL using a lyopholizer. The samples were run on a strong anion exchange column (Waters Accel Plus QMA) that had been equilibrated with 5 column volumes of 20 mM Tris pH 8.0. Flow through, washes of 20 mM Tris pH 8.0, and elutions of 20 mM Tris + 0.5 M NaCl were collected. The protein was eluted in the flow through and washes and these fractions were pooled and dialyzed as described above. The samples were lyophilized completely and then resuspended in an appropriate amount of water (0.3 mL to 1 mL). The concentrations of the chimeric proteins were determined as described above for purification of native ToxB. Equal amounts of protein were resolved and visualized by a silver stained 14% acrylamide SDS-PAGE.

Assessment of protein activity

Approximately 20 µL of 5, 10, and 15 µM of chimeric proteins were infiltrated into secondary leaves of two-week old ToxB-sensitive Katepwa wheat leaves. The plants were returned to the growth chamber for seven days in a 16 h light/8hr dark chamber. On the seventh day, the leaves were harvested and scanned on a flat bed scanner. At least three leaves were infiltrated with each protein preparation and each protein was produced and infiltrated at least three times.

Crystallization trials of ToxB

ToxB was heterologously expressed and purified as described above. Prior to crystallization, the protein was dialyzed twice against 2 L of water overnight and concentrated to 3-6 mg/mL. Initial crystallization screening of ToxB was carried out using both hanging and sitting-drop vapor-diffusion methods. Hanging-drop vapor-diffusion conditions were setup manually by mixing 1 µL of protein solution (3-6 mg/mL) and 1 µL of the reservoir solution. Commercial reservoir solutions Hampton Research Crystal Screens I and II and Emerald Biosystems Wizards I and II were used for a total of 192 different screening conditions. Crystallization drops were left to equilibrate at 295 K (22°C). Sitting-drop vapor-diffusion conditions were setup using the automated Art Robbins Instruments Phoenix system. Drops were prepared by mixing 0.2 µL of protein solution (3 mg/mL) and 0.2 µL of the reservoir solution and were left to equilibrate at 295 K (22°C). Commercial reservoir conditions used by the robotic Phoenix system, all in a 96-block format, were Hampton Research Crystal Screens HT I, II, and Index HT, and Emerald Biosystems I, II and Precipitant Synergy. A total of 384 different conditions were screened using the sitting drop vapor diffusion method. Crystals that formed were harvested, mounted in nylon loops and frozen in liquid nitrogen. The mounted crystals were screened using an X-ray diffractometer to check if the crystal was protein.

Circular Dichroism of ToxB

Circular dichroism (CD) experiments were conducted on a Jasco J-720 spectropolarimeter. Samples were heterologously expressed from *P. pastoris* and purified as described above. Concentrations were obtained using the NanoDrop ND-1000 UV-Vis Spectrophotometer at the CGRB lab. The concentration readings were taken at 220 nm, the absorbance at which peptide bonds absorb light, instead of the customary 280 nm since ToxB contains no tryptophan residues, which normally absorb light at 280 nm. Because there are few aromatic residues in ToxB, concentrations may not be accurate. Experiments were recorded in a 0.1 cm sample cell and spectra were scanned from 200 to 250 nm at temperatures of 25, 50, 70, and 80 °C.

RESULTS

Purification of ToxB from Pyrenophora tritici-repentis

Initially, the hope was that ToxB purified from Ptr would be used for crystallization and structure studies. The purification of native ToxB was previously described, but it was necessary to adapt and modify the protocol in order to achieve a highly-purified and concentrated product. The addition of a strong anion exchange column used in tandem with a weak cation exchange column and MonoS HPLC allowed us to ensure that the purified ToxB was free of any contaminating proteins. Prior to purification on the columns, the ToxB preparation contained many contaminating proteins at various molecular weights, with ToxB present at about 6.6 kDa (Figure 3.A, arrow). Mostly lower molecular weight bands (with the exception of ToxB) were removed by applying the sample on a weak cation exchange column (Figure 3.B), while a strong anion exchange column removed most mid-weight proteins and many higher molecular weight proteins (Figure 3.C). Finally, the HPLC MonoS removed all remaining contaminating proteins leaving a pure sample of ToxB (Figure 3.D, arrow) Although ToxB was successfully purified from Ptr only small quantities were obtained; one liter culture of Ptr yielded approximately 0.2-0.3 mg of pure ToxB protein. This method of purification could not yield a high enough concentration of protein in a timely manner in order to progress with the crystallization of ToxB. Thus, another more time-efficient method of ToxB purification needed to be developed.

Expression of Protein in P. pastoris

The *P. pastoris* expression system for the production of large amounts of heterologously expressed protein had previously been used for expression of other proteins from Ptr. In this system, ORFs are cloned into plasmids containing the methanol-responsive AOX1 promoter, which allows for the expression of the protein when induced with methanol. These plasmids are transformed into *P. pastoris*, recombined into the genome, and because they contain a secretion signal, are secreted into the media when the culture is induced. These proteins are relatively easy to purify as *P. pastoris* secretes only a few native proteins. Our laboratory had previously used this system for the production of ToxB, but the protein was neither purified nor quantified. Prior to purification, there are several proteins present in the media (Figure 4, lane 2), including ToxB (Figure 4, arrow). When these proteins are placed on a strong anion exchange column, all of the *P. pastoris* proteins bind to the column, and ToxB elutes in the flow through and the washes (Figure 4, lane 3). One liter of *P. pastoris* culture induced and purified by this method yields about 10-15 mg of pure ToxB.

Although the above purification scheme from *P. pastoris* yields pure ToxB, it was important to determine that the physical properties and activity of native ToxB and ToxB heterologously expressed in *P. pastoris* are identical. If the two are identical, the use of the heterologously expressed protein in place of the native-expressed can be justified. To determine if ToxB isolated from Ptr and ToxB heterologously expressed in *P. pastoris* are identical, the two proteins were first visualized on silver stained 14% acrylamide

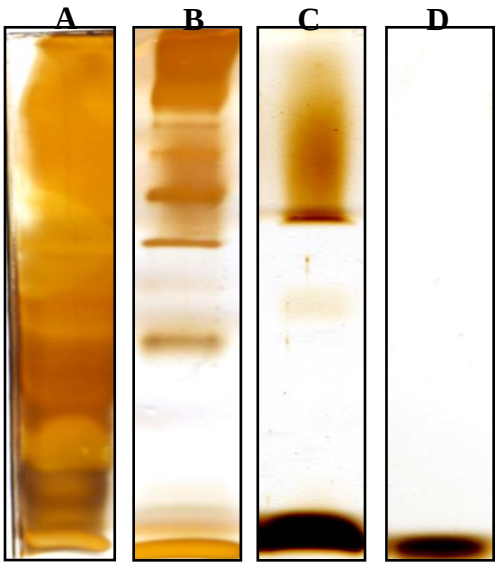

Figure 3: SDS-PAGE of native ToxB purification. **A:** Crude culture filtrate from Ptr containing ToxB (arrow) among other proteins of various molecular weights. **B:** Elution of proteins, including ToxB, from a weak cation exchange column. **C:** ToxB and contaminating high molecular weight proteins collected from the flow through and wash of a strong anion exchange column. **D:** Pure ToxB eluted from a HPLC MonoS column.

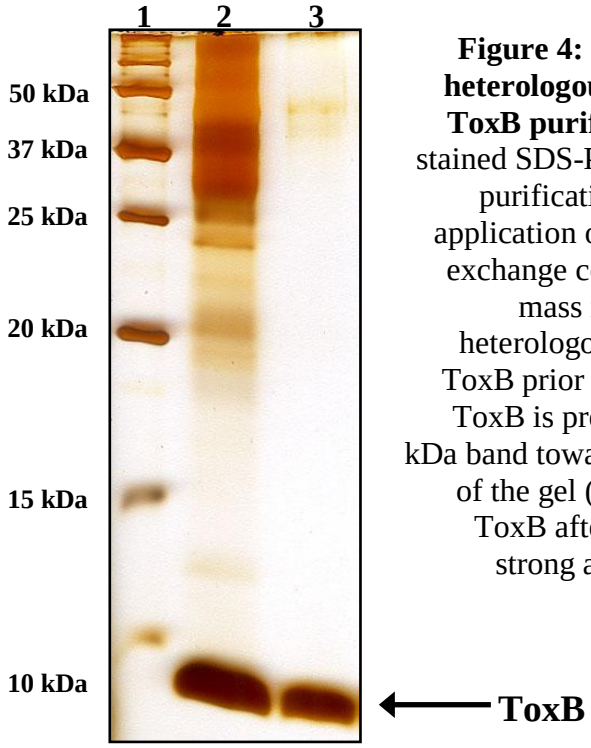

Figure 4: SDS-PAGE of heterologously expressed ToxB purification. Silver stained SDS-PAGE showing purification of ToxB by application on strong anion exchange column. Lane 1: mass marker; lane 2: heterologously expressed ToxB prior to purification. ToxB is present in the 6.6 kDa band towards the bottom of the gel (arrow); lane 3: ToxB after running on a strong anion exchange column.

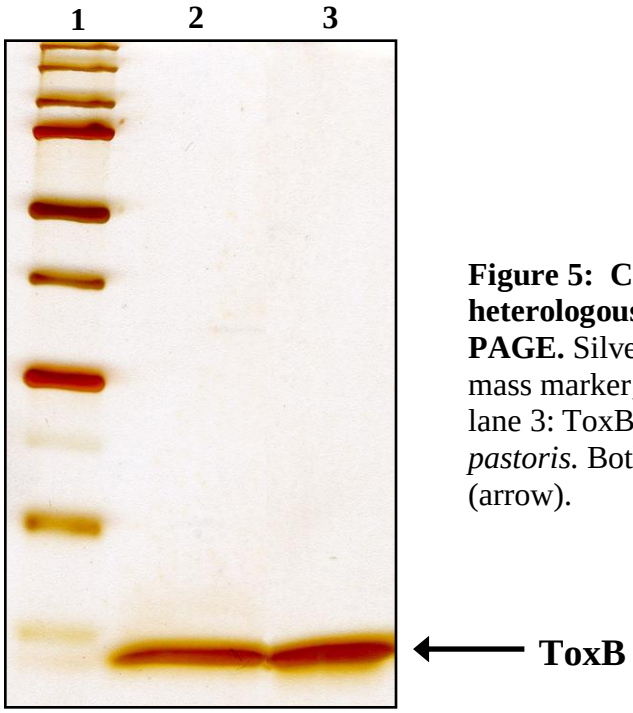

Figure 5: Comparison of native ToxB and heterologously expressed ToxB on SDS-PAGE. Silver stained SDS-PAGE of: Lane 1: mass marker; lane 2: ToxB purified from Ptr; lane 3: ToxB heterologously expressed in *P. pastoris.* Both proteins show bands at 6.6 kDa (arrow).

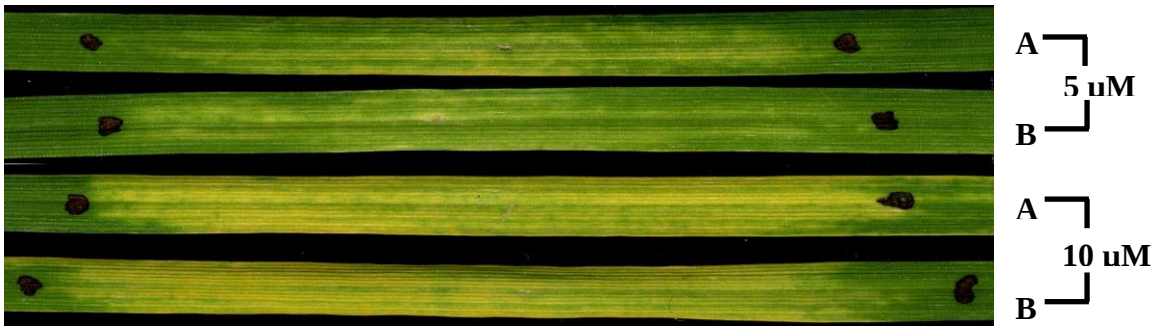

Figure 6: Bioassay of native ToxB and heterologously expressed ToxB. Infiltration of native ToxB (A) and ToxB heterologously expressed in *P. pastoris* (B) at 5 and 10 µM concentration in ToxB-sensitive wheat cultivar Katepwa. Leaves were harvested 7 days post infiltration.

SDS-PAGE (Figure 5, arrow) and were shown to have similar mobility correlating with their molecular weights at 6.6 kDa. It has been previously shown that the wheat differential Katepwa is sensitive to ToxB, which causes chlorosis in infiltrated leaves, thus native ToxB and heterologously expressed ToxB were tested for activity in a bioassay on Katepwa at concentrations of 5 and 10 µM (Figure 6). The assays were performed at two different concentrations to ensure accurate results as higher concentrations can sometimes saturate and blow-out symptom activity. The assay shows that both native ToxB and ToxB heterologously expressed in *P. pastoris* induce chlorosis at similar levels in a dose-dependent manner.

Comparison of ToxB and toxb

A homolog of Toxb, called toxb, is present in the non-pathogenic race 4 Ptr isolate. toxb was expressed heterologously in *P. pastoris* and purified as described above. To compare the differences between ToxB and toxb, the proteins were analyzed on SDS-PAGE and in bioassays. To determine the mobility of these proteins, silver stained SDS-PAGE was used. Both ToxB and toxb showed bands on the gel that migrate at a distance consistent with the molecular weight of each protein, 6.6 kDa (Figure 7). Along with the expected 6.6 kDa band, toxb also shows a higher molecular weight (HMW) band (Figure 7, box). To compare the activity of ToxB and toxb, 10 µM of each protein was infiltrated into Katepwa. The infiltrations of ToxB produced chlorosis in Katepwa while at the same concentration, toxb produced no noticeable chlorosis (Figure 8).

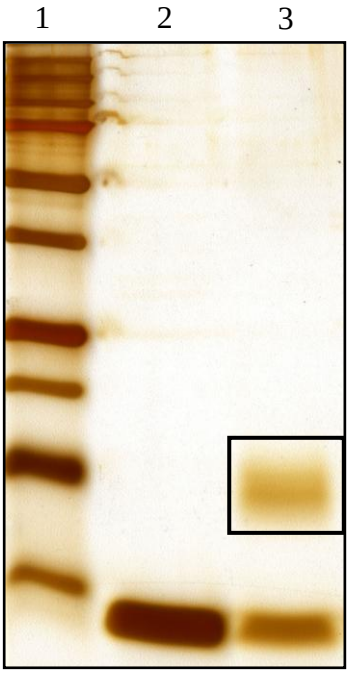

Figure 7: Comparison of ToxB and toxb on SDS-PAGE. Silver stained SDS-PAGE of: lane 1: mass marker; lane 2: ToxB with a 6.6 kDa band (bottom); lane 3: toxb with a 6.6 kDa band (bottom) and a higher molecular weight band (box). Equal amounts of ToxB and toxb were run on the gel, but the 6.6 kDa band of toxb (lane 3) is much lighter than the 6.6 kDa band of ToxB (lane 2) due to some of the protein forming the higher molecular weight band (box).

Figure 8: **Bioassay of ToxB and toxb.** Infiltration of ToxB (A) and toxb (B) into Katepwa. ToxB produces chlorotic symptoms, while toxb produces no symptoms. Leaves were harvested 7 days post infiltration.

Circular Dichroism of ToxB

Circular dichroism (CD) spectroscopy measures differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. In the context of proteins, CD spectroscopy can be used to discern secondary structure content, in specific, α-helices and β-strands. The intensity and wavelength of the peaks in a spectrum indicate the amount and type of secondary structure elements present. With increasing temperature, monitoring the CD signal at a specific wavelength (220 nm for proteins with high α-helix content, 215 nm for proteins with high β-strand content) gives information about the thermal stability of the protein; as the protein unfolds and secondary structure elements are lost, signal intensity decreases. To compare the secondary structures and stability of native ToxB and ToxB heterologously expressed in *P. pastoris*, as well as the inactive toxb, CD spectra were obtained.

CD spectra for all three proteins at 25°C were analyzed to determine the secondary structure elements present. Raw data is shown in Figure 9A. The intensity of the negative peak at ~215 nm indicates two things: 1) the sample concentration is high enough for a reliable signal for all samples and 2) all samples are composed of primarily β-strands with little to no α-helix content.

When comparing CD spectra of different proteins, the data is typically shown as molar ellipticity (ε) versus wavelength. Molar ellipticity corrects for differences in protein concentrations and allows for a direct comparison of the CD signals. Due to the difficulties in determining protein concentration (the BCA protein assay was not used to

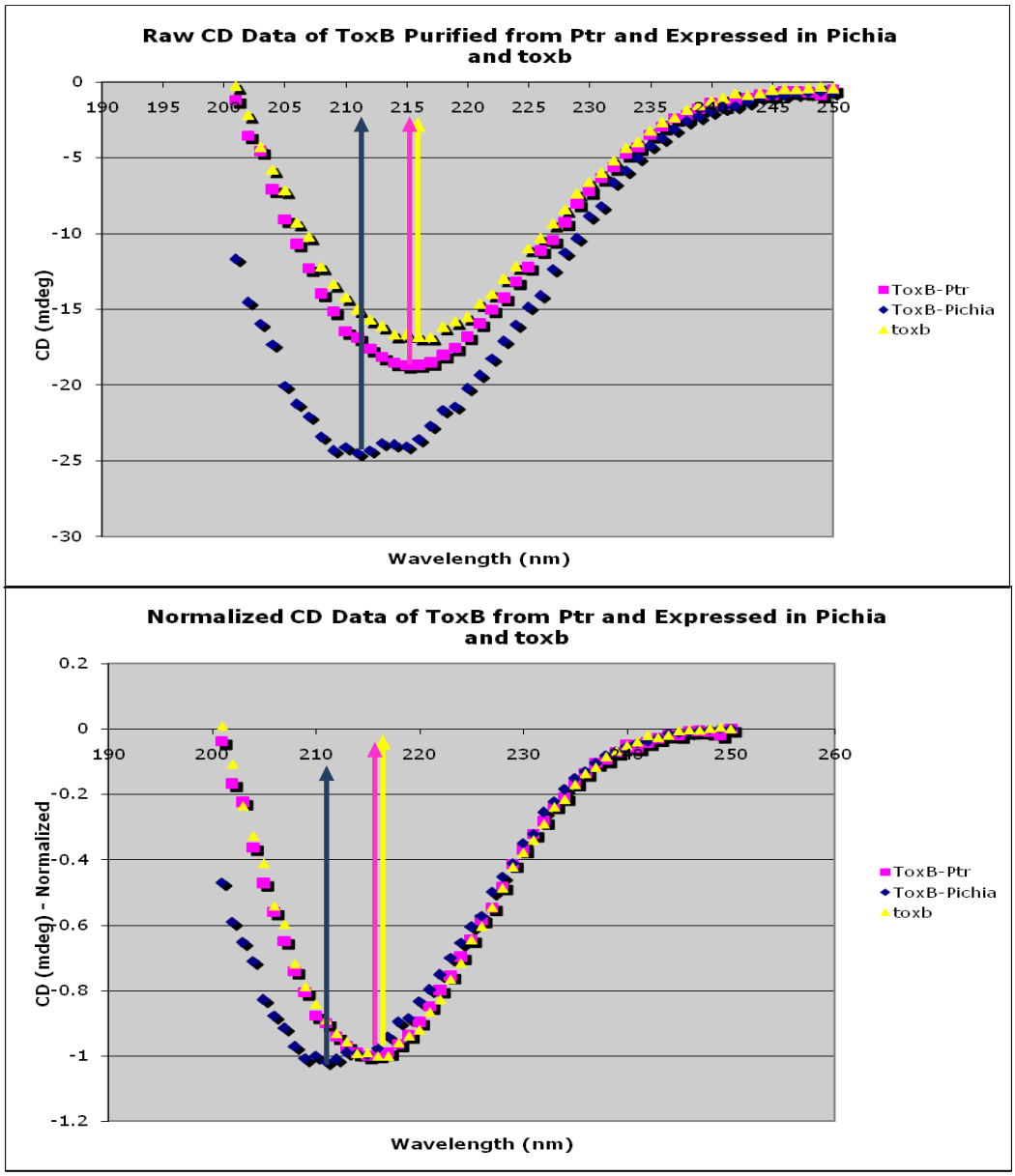

Figure 9: CD spectra for ToxB proteins. A) Raw CD data and **B)** normalized CD data for native ToxB, heterologously expressed ToxB and toxb indicate that all three proteins are composed primarily of β-strands. CD signal in millidegrees (y-axis) is plotted versus wavelength in nanometers (x-axis). Native ToxB (pink squares) and toxb heterologously expressed in *P. pastoris* (yellow triangles) have a minimum CD signal at 215 nm (pink arrow) and 216 nm (yellow arrow), respectively. ToxB heterologously expressed in *P. pastoris* (blue diamonds) has a minimum CD at 212 nm, indicating a slight decrease in secondary structure in comparison with the other two proteins. In graph **A**, the different concentrations of the proteins caused the differences in CD signal intensity. In graph **B**, the CD values are normalized so that the peak minimum occurs at -1.

calculate the concentrations for this experiment), ε could not be calculated. To better compare these spectra, data are normalized so that the maximum negative signal is equal to -1 (Figure 9B). Native ToxB shows a minimum at ~215 nm (pink arrows), while heterologously expressed ToxB (blue arrows) shows a minimum at ~212 nm. The toxb protein (yellow arrows) has a minimum at ~216 nm.

To monitor thermal stability, the CD signal of all three proteins, native ToxB, ToxB heterologously expressed in *P. pastoris*, and toxb, was monitored at 215 nm with increasing temperature. The CD signal at temperatures of 25, 50, 70, and 80 °C remained constant (data not shown), indicating no change in secondary structure elements. This suggests that all three proteins are very stable, even at high temperatures, and do not denature easily.

Study of Chimeric Proteins

In order to better determine what region(s) of ToxB is responsible for toxicity of the protein, six chimeric proteins were constructed using regions from both the *ToxB* and *toxb* ORF. The construction of these six chimeras was achieved by dividing the *ToxB* and *toxb* ORFs into three regions, region 1 (encoding the N-terminal end of the protein), region 2 (encoding the middle section of the protein), and region 3 (encoding the downstream end of the protein), based on the restriction sites *Bsa*I and *Bse*YI (Figure 1). In order to utilize these sites, the *ToxB* and *toxb* ORFs were ligated into pBSII as it had unique sites for both *Bsa*I and *Bse*YI, synthesizing pCMR1 and pCMR2, respectively. By

joining together different fragments cut with *Bse*YI and *Bsa*I from pCMR1 and pCMR2, six chimeric plasmids were created that each had a different set of regions from either *ToxB* or *toxb* (Table 2). Using these chimeras, we were able to investigate the region of the *ToxB* ORF is important for the toxicity of the protein.

Once the chimeric ORFs were produced in pBSII, they were subcloned into *P. pastoris* expression vector pPICZB and expressed in *P. pastoris* and purified. In order to properly test the activity of the chimeric proteins, their concentrations needed to be standardized before testing in a bioassay. Quantification of protein concentration for ToxB, toxb and the chimeras proved very difficult because there are no tryptophan residues present in the proteins to allow for quantification using spectrophotometric methods. Thus, in order to obtain accurate results, protein concentrations were first estimated using a BCA protein assay and then visually compared on an SDS-PAGE by loading equal amounts according to these estimates.

Equal concentrations of ToxB, toxb, and chimeric proteins, estimated using the BCA protein assay, were analyzed on silver stained SDS-PAGE. On the gel, the purified chimeras CMR3 (ttT), CMR5 (tTT), and CMR7 (tTt), which each have the *toxb* ORF in region 1, consistently show a HMW band similar to that seen with toxb (Figure 10, compare lane 3 with lanes 4, 6, and 8). This HMW band does not occur in CMR4 (TTt), CMR6 (Ttt), or CMR8 (TtT). In the chimeras with the HMW band, less protein is shown in the 6.6 kDa band than in the 6.6 kDa band of the chimeras without the HMW band.

Table 2: Chimeric proteins and the regions of the *ToxB* and *toxb* ORFs they contain.

Chimera	*Region 1*	*Region 2*	*Region 3*	*Code*
pCMR3	toxb	toxb	ToxB	ttT
pCMR4	ToxB	ToxB	toxb	TTt
pCMR5	toxb	ToxB	ToxB	tTT
pCMR6	ToxB	toxb	toxb	Ttt
pCMR7	toxb	ToxB	toxb	tTt
pCMR8	ToxB	toxb	ToxB	TtT

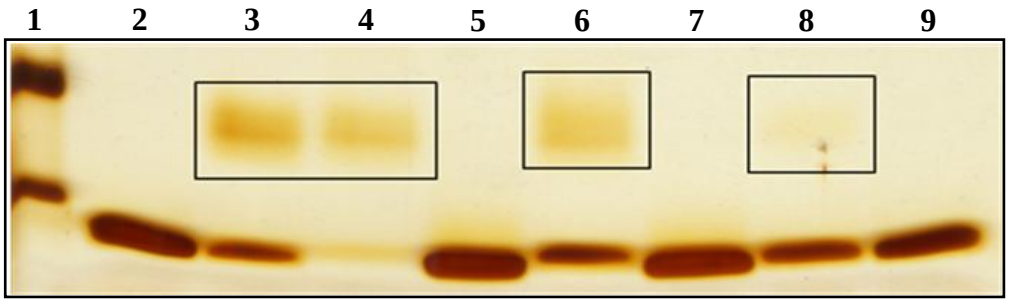

Figure 10: Silver stained SDS-PAGE of ToxB, toxb, and chimeric proteins. Boxes show higher molecular weight bands present with toxb, CMR3, CMR5, and CMR7. Lane 1: mass marker, lane 2: ToxB, lane 3: toxb, lane 4: CMR3, lane 5: CMR4, lane 6: CMR5, lane 7: CMR6, lane 8: CMR7, and lane 9: CMR8.

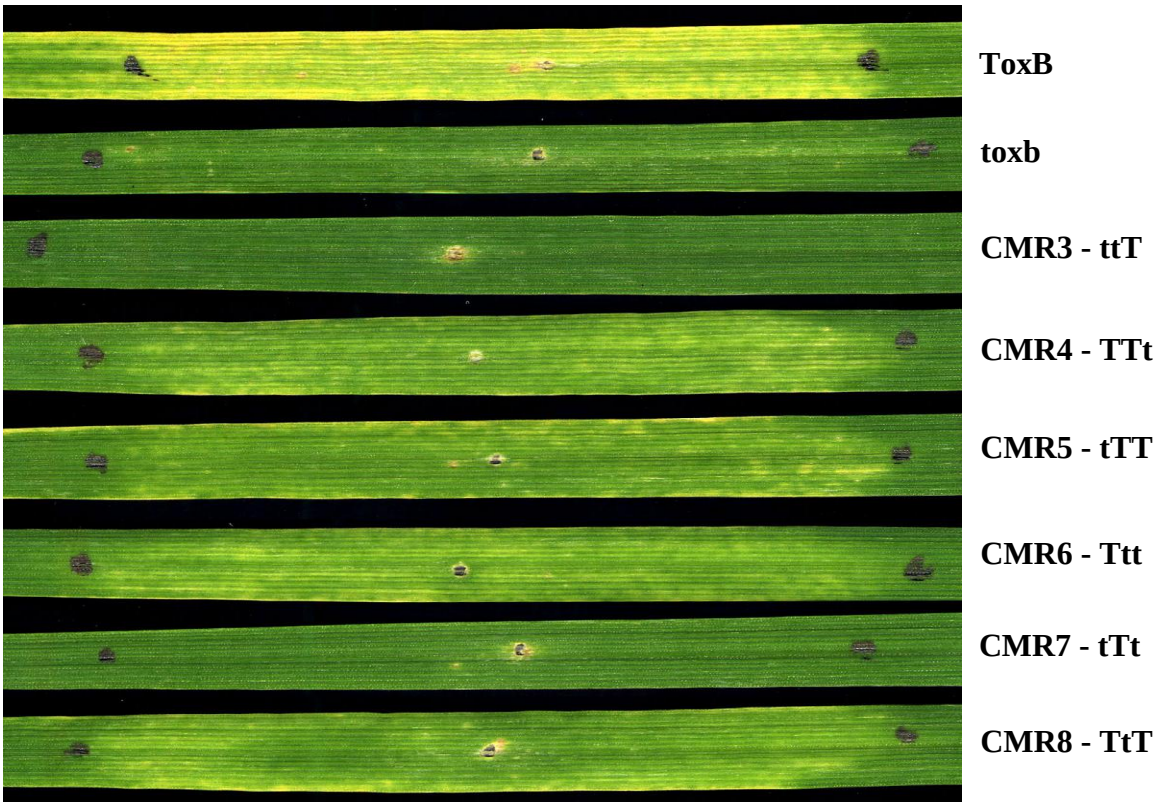

Figure 11: Bioassay of ToxB, toxb, and chimeras. Infiltrations of 15 µM of proteins into Katepwa. ToxB (top leaf) produces chlorosis while toxb (second leaf) produces no chlorosis. Leaves were harvested 7 days post infiltration.

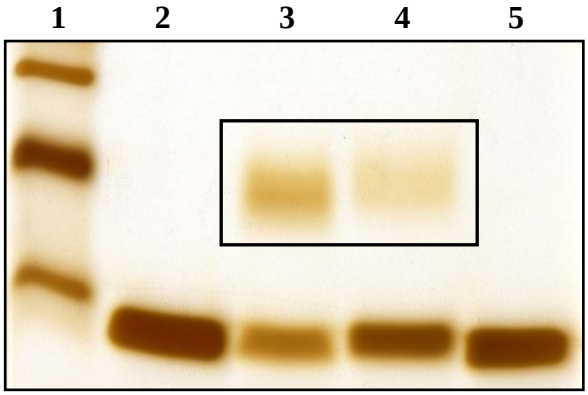

Figure 12: SDS-PAGE of site-directed mutants. Silver stained SDS-PAGE of: lane 1: mass marker; lane 2: ToxB; lane 3: toxb; lane 4: SDM1; and lane 5: SDM2. SDM1 and toxb contain a higher molecular weight band (box) and also have lighter 6.6 kDa bands than ToxB and SDM2.

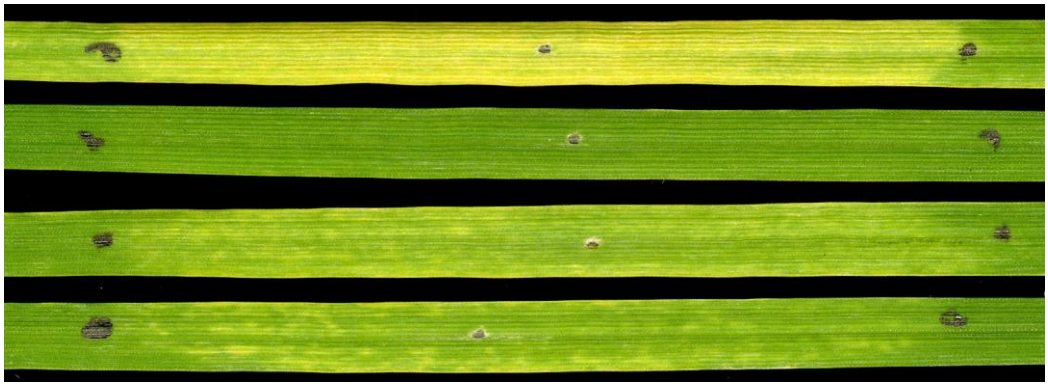

Figure 13: Bioassay of site-directed mutants. Infiltration of ToxB, toxb, SDM1 and SDM2 in Katepwa. Leaves were harvested 7 days post infiltration.

CMR3 (ttT) also consistently has an extremely light 6.6 kDa band even when compared with the other chimeras with HMW bands. Since ToxB causes chlorosis in Katepwa and toxb has no noticeable symptoms of chlorosis, the activity of the chimeric proteins is crucial for understanding how the amino acid changes in *toxb* may contribute to the lack of activity and what regions of *ToxB* are necessary for activity. Bioassays were performed by infiltrating 5, 10, and 15 µM of concentrated protein into Katepwa. An example of a bioassay performed with 15 µM is shown in Figure 11. Activity of infiltrated proteins ranges from full activity (ToxB) to no activity (toxb, CMR3 [ttT], CMR7 [tTt]). Chimeras CMR4 (TTt), CMR5 (tTT), CMR6 (Ttt) and CMR8 (TtT) had the greatest chlorotic activity after ToxB.

Site-Directed Mutagenesis of ToxB

According to the results shown above, amino acid changes in region 1 of ToxB compared to toxb are most likely the cause for the lack of toxicity of *toxb*, although other regions may play a role as well. Similarly, only chimeras with the *toxb* ORF in region 1 contained a HMW band, signifying that one of the amino acid differences in region 1 of *toxb* may be responsible for this band. There are two amino acid differences in region 1, $Valine_{25}$ to Threonine (V_{25}/T) and $Alanine_{35}$ to Valine (A_{35}/V), and either one or a combination of these amino acid changes may be directly responsible for the lack of toxicity of toxb and the presence of the HMW band. In order to investigate which amino acid change(s) may be the cause, point-mutations were created in the *ToxB* sequence of V_{25}/T to produce site-directed mutant 1 (SDM1) and A_{35}/V to produce site-directed mutant 2 (SDM2). These

chimeras were heterologously expressed in *P. pastoris* as described above and infiltrated into the ToxB-sensitive Katepwa wheat.

SDM1 (V_{25}/T) showed a HMW band on silver stained SDS-PAGE consistent with other proteins that had part of the *toxb* ORF in region 1, while SDM2 (A_{35}/V) did not contain this band, consistent with chimeras that had the *ToxB* ORF in region 1 (Figure 12, lanes 4 and 5). These results, along with the results above showing only chimeras containing region 1 from the *toxb* ORF have the HMW band, strongly suggest that the V_{25}/T mutation alone is responsible for the HMW band observed in chimeras with region 1 from the *toxb* ORF and toxb itself. To test the activity of the site-directed mutants, 15 µM of each protein was infiltrated into Katepwa. Both SDM1 (V_{25}/T) and SDM2 (A_{35}/V) have reduced chlorosis compared to ToxB (Figure 13), but similar levels of chlorosis when compared to the symptoms seen in CMR6 and CMR5 (Compare Figures 11 and 13).

Crystallization of ToxB

The technique of crystallization creates an environment where a supersaturated protein solution in the presence of various chemical compounds may nucleate and come out of solution in such as way that a protein crystal is formed. Crystal formation is a stochastic process that involves many variables. Each protein will react with different chemicals in different ways based on the structure and charge of the protein, so it is impossible to predict which chemical compound(s) will induce crystallization. To overcome this

unknown and obtain protein crystals, many different crystallization conditions are screened. As a drop containing protein and reservoir equilibrates, many options ranging from no visible changes to heavy precipitation are possible in addition to crystal formation.

Initial crystallization trials of ToxB were manually setup using the hanging-drop vapor-diffusion method using a heterologously expressed ToxB protein stock solution of ~3 mg/mL that had not been thoroughly dialyzed. Many screening conditions produced crystals which were frozen in liquid nitrogen and mounted on an X-ray diffractometer for screening. All of these crystals proved to be salt crystals, suggesting a high concentration of salt in the protein stock solution. As an additional indication that protein stock solution conditions should be changed, there was a high ratio of clear drops to drops containing precipitate. This suggested that the concentration of the protein was not high enough to support nucleation of the protein.

To improve the protein stock solution used in crystallization trials, another sample of ToxB was dialyzed and concentrated to ~6 mg/mL prior to crystallization set-ups. It was expected that the dialysis would remove the excess salt (and salt crystal formation) and higher protein concentration would increase the likelihood of nucleation, creating a lower ratio of clear wells to wells containing precipitate. This sample was set up only on the conditions that had previously created salt crystals in order to confirm that the sample had been thoroughly desalted. Upon examining these trays after 2 weeks, it was observed that no crystals, salt or otherwise, had grown. Additionally, the ratio of clear wells to wells

containing precipitate had lowered, indicating that the higher protein concentration had increased the number of nucleation events.

Utilizing the knowledge of a target concentration and desalting techniques, new crystallization trials were set up using the automated Phoenix system. However, due to a limited quantity of ToxB available, a concentration of 3 mg/mL was used. Preliminary results show a high ratio of clear well to wells containing precipitate, indicating that the protein concentration may still be too low. Eight conditions produced crystals. These crystals were harvested, mounted and frozen before X-ray diffraction screening. Collected data indicate that the crystals are salt.

DISCUSSION

Purification and Expression of ToxB

The purification of native ToxB proved to be time consuming and produced very low yields of pure protein. Thus, another method of purification of ToxB was utilized in order to gain a high enough concentration of protein in a timely manner. The *P. pastoris* system allowed us to purify large quantities of ToxB in a relatively short amount of time when compared to the native purification. This system was used to purify ToxB for crystallization as well as purify the chimeras for the mutagenesis studies.

As with any non-native expression system, there was the possibility that the structure or activity of ToxB had been altered in the expression system, thus we evaluated the properties of native ToxB and ToxB heterologously expressed in *P. pastoris*. When visualized on silver stained SDS-PAGE, both native ToxB and ToxB heterologously expressed in *P. pastoris* showed bands at the same molecular weight. This was our first indication that ToxB heterologously expressed in *P. pastoris* did not undergo major alterations that may have allowed it to form aggregates or dimers when secreted from the yeast. Bioassays of ToxB from the two different expression systems also showed identical symptoms of chlorosis, signifying that the activity of ToxB had not been affected from expression in *P. pastoris*.

Circular dichroism was performed to analyze the secondary structures of native ToxB and heterologously expressed ToxB. The spectra collected at 25 ºC from native ToxB and

heterologously expressed ToxB shows minima that are at a wavelength consistent with the formation of β-strands. A protein containing α-helices would normally show a minimum at 220 nm, and since there was no minimum at this wavelength, it can be assumed that there are no α-helices within ToxB from either expression system. There is, however, a slight shift between the two minima, which suggests that there may be a slight loss of β-strand structure in heterologously expressed ToxB compared with native ToxB. It should be emphasized however, that this is just a slight shift of only 3 nm, signifying that the structures are extremely similar, unlike a larger shift of 10 nm or more, which would show that there is a definite change in structure. Thermal unfolding of native ToxB and heterologously expressed ToxB indicated that both proteins are extremely resistant to denaturing by temperature, as their spectra remained the same. This resistance may be attributed to the small nature of the protein and the two disulfide bonds, which may all contribute to a tight conformation of the protein. Results from SDS-PAGE, bioassays, and circular dichroism confirm that native ToxB and heterologously expressed ToxB are highly similar. Thus, we believe the substitution of the heterologously expressed ToxB for the native ToxB is justified for structure/function studies.

Comparison of ToxB and toxb

In order to facilitate the study of the structure of ToxB as it relates to activity, toxb, a nonactive homolog encoded by a non-pathogenic race 4 Ptr isolate, was utilized. A study of the comparison of ToxB and toxb was performed by visualizing on SDS-PAGE and conducting bioassays with ToxB-sensitive Katepwa. On the SDS-PAGE, both ToxB and

toxb produced bands consistent with their molecular weights, but toxb also exhibited a HMW band. This band is most likely due to the multiple amino acid changes in *toxb* when compared with *ToxB*, that allow the protein to mis-fold and form aggregates that would migrate at a slower rate on the SDS-PAGE. The infiltrations of toxb also showed no chlorotic activity, once again most likely due to the amino acid changes relative to active ToxB. This would suggest that one or a combination of the amino acid changes present in the toxb gene is responsible for its lack of toxicity. Thus, by studying these amino acid changes, important information about the structure and a possible active site in ToxB could be obtained.

CD spectra of toxb are consistent with that of ToxB and indicate that the protein is composed of primarily β-strands and is stable at high temperatures. Interestingly, toxb's minimum was at ~216 nm, which is closer to the minima from native ToxB than to the minima of the heterologously expressed ToxB, despite its expression in the heterologous system. This indicates that toxb has a β-strand content that is more similar to native ToxB. Although only speculative, if the use of the heterologous expression system does cause a slight decrease in β-strand content, the shift in the toxb spectra may indicate an increase in β-strand content for the inactive form compared to ToxB that may be responsible for the change in actively. What is clear from these studies however is that CD is not a powerful enough tool to discern the subtleties in the structure/function relationships of the ToxB protein.

The study of the six chimeric proteins allowed us to investigate which specific regions of ToxB are responsible for the protein's induction of chlorosis in sensitive wheat cultivars. The chimeras were visualized on silver stained SDS-PAGE in order to compare them to ToxB and toxb, as well as each other. The presence of a HMW band in CMR3, CMR5, and CMR7 on silver stained SDS-PAGE may be caused by the aggregation of mis-folded protein. All of these chimeras have part of the *toxb* ORF in region 1, and since the N-terminus of any protein is very important for correct secondary and tertiary structure, any changes to this region may have a large affect on a possible active site. This upper band could signify that the first region in the *ToxB* ORF is responsible for proper folding, and thus, could cause a loss in toxicity if mutated.

To test the activity of these chimeric proteins, they were infiltrated into ToxB-sensitive Katepwa. There are three general statements that can be made about the activity of the chimeras. First, all the chimeras had less activity than wild-type ToxB. This indicates that no matter where the amino acid changes are in the ToxB gene, they have an effect on the protein's activity. Second, chimeras with two regions from the *ToxB* ORF have greater chlorotic symptoms than chimeras with only one region containing the *ToxB* ORF, except in the case of CMR5 (Ttt). This indicates that the fewer amino acid changes there are (i.e. only one region from the *toxb* ORF), the greater the activity. Thirdly, CMR6 (Ttt), which has only one region from the *ToxB* ORF, has comparable symptoms to the chimeras with two regions from the *ToxB* ORF. This indicates that region 1 is the most important in regards to activity of the protein. Region 2 and 3 seem to be less important for the activity of the protein as CMR3 (ttT) and CMR7 (tTt) showed no chlorotic symptoms.

To pinpoint the specific amino acid differences that caused *ToxB*'s loss of activity, site-directed mutants were created starting on the N-terminal end of *ToxB* because that region proved to be the most important for *ToxB*'s toxicity. Since SDM1 shows a higher molecular weight band on a silver stained SDS-PAGE and SDM2 does not, it can be safely assumed that the presence of this higher molecular weight band in SDM1 and some of the other chimeras is due to the mutation of $Valine_{25}$ to Threonine. Valine is a very hydrophobic amino acid with a hydropathy index of 4.2, while Threonine is more neutral with a hydropathy index of -0.7. Hydrophobic amino acids are often buried in the structure, and a switch from a very highly hydrophobic amino acid to a more neutral amino acid could change this part of the structure of ToxB. This change in structure could result in a mis-folding or aggregation of ToxB molecules resulting in the higher-molecular weight band. This mutation is also adjacent to a cysteine residue and could impact proper disulfide bond formation.

This mis-folding may very well be a cause for loss in toxicity as well. Since both SDM1 and SDM2 had decreased chlorotic-inducing activity, both amino acid changes of the N-terminal end of *toxb* are responsible for a loss in chlorotic activity. Only one amino acid change is needed to lose activity, but as seen in CMR5 (tTT), when both amino acids are changed, the activity still appears to be same as when only one amino acid is changed. This illustrates that the lack of toxicity of *toxb* is due to a combination of the amino acid changes in region 1, but either one can cause a lack of toxicity on its own. It also suggests that while this first N-terminal region of *ToxB* is the most important for loss in activity, amino acid changes in other regions can increase the loss of activity when paired with

these amino acid changes. Further site-directed experiments with more site-directed mutants could better shed the light on the role that the primary structure of ToxB plays in its toxicity.

Crystallization of ToxB

Despite the variety in crystallization conditions screened, no successful crystal leads have been obtained. This however is not unexpected when working with a novel protein and there are many variables in protein stock preparation that need to be optimized to increase the likelihood of crystallization success. From the crystallization trials that have already been setup, it appears that a target concentration for ToxB stock solutions should be at least 6 mg/mL. Anything lower than this concentration does not promote nucleation. This variable is difficult to optimize for ToxB as the protein tends to precipitate and come out of solution at these higher concentrations. To overcome this challenge, we need to find a more-suitable buffer that is not close to the pI of the protein for resuspension instead of water. This could help prevent the precipitation and allow higher concentrations of ToxB to be achieved. In addition, it is important to ensure that the ToxB sample has been thoroughly desalted as salt crystals are easily formed if the sample has not been sufficiently dialyzed. Keeping the above findings in mind, the crystallization of ToxB should still be pursued, especially since we now have access to the robotic Phoenix system which makes setting up screens incredibly time-efficient.

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