AN ABSTRACT OF THE THESIS OF

Aakash Mankaney for the degree of Honors Baccalaureate of Science in Biology presented on May, 28 2010. Title: **Plant responses induced by Ptr ToxA and Ptr ToxB, host-selective toxins produced by *Pyrenophora tritici-repentis.***

Abstract Approved:

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Lynda Ciuffetti

Pyrenophora tritici-repentis (Ptr) is the causal agent of the disease tan spot of wheat, which can be responsible for the destruction of up to 50% of a wheat crop harvest. Tan spot disease results in chlorotic and/or necrotic lesions triggered by the pathogen's production of one or several host-selective toxins (HSTs). HSTs are diverse metabolites that dictate disease development only on susceptible hosts; therefore, they function as determinants of pathogenicity or virulence. Ptr produces two proteinaceous HSTs, Ptr ToxA (ToxA) and Ptr ToxB (ToxB). In toxin sensitive wheat cultivars ToxA and ToxB induce necrosis and chlorosis, respectively. The current model of ToxA mode-of-action involves binding of ToxA to an uncharacterized receptor on sensitive mesophyll cells that mediates ToxA internalization, allowing the toxin to localize to the chloroplast. This leads to an increase in the levels of reactive oxygen species (ROS) in the chloroplast. Because

less is known about ToxB mode-of-action, we initiated studies to compare and contrast plant responses to both toxins. Initially, we sought to determine whether ROS accumulation occurs in response to ToxB. Additionally, we measured the production of phenolic compounds and phenylalanine ammonia lyase (PAL) activity, important components of the defense-related phenylpropanoid pathway. Our results indicate that: 1) ROS accumulation occurs in the chloroplast after ToxB treatment of the toxin-sensitive cultivar; 2) antioxidant N-acetylecysteine decreases symptom development induced by ToxB, 3) both toxins induced accumulation of soluble phenolic compounds in the toxin-sensitive cultivar, and 4) PAL activity is elevated in ToxA and ToxB-treated sensitive leaves compared to water-treated controls. These data suggest that ToxA and ToxB induce similar responses in the sensitive wheat cultivar.

Plant responses induced by Ptr ToxA and Ptr ToxB, host-selective toxins produced by *Pyrenophora tritici-repentis.*

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INTRODUCTION

Tan spot of wheat is a foliar disease that is caused by the fungal plant pathogen *Pyrenophora tritici-repentis* (Died.) Drechs. [*Drechslera tritici-repentis* (Died.) Shoemaker. This disease was first discovered in 1940 in New York, USA (Strelkov and Lamari, 2003), has become a major concern worldwide (De Wolf et al. 1998, Strelkov et al., 2003), and its economical impact has gained importance in Australia and Central Asia (Rees, 1982). The disease can cause up to 50% damage in wheat (*Triticum aestivum* L.) growing areas such as the Northern Great Plains and South America (Hosford, 1982; Riede et al., 1996, reviewed in Ciuffetti and Touri, 1999). The severe yield losses caused by this disease are due to the reduction of the photosynthetic area of the leaves, which results in less grain fill, lower test weight, kernel shriveling, and lower number of kernels per head (De Wolf et al., 1998). Tan spot can also discolor the kernel, which negatively affects the wheat quality (De Wolf et al., 1998).

In the wheat fields, the rapid spread of the disease is due to both the resilience of the pathogen, as well as the increasing popularity in zero or minimal tillage farming. Additionally, residue retention and shorter crop rotations have increased the severity of the tan spot epidemic (Hosford 1982; Strelkov and Lamari, 2003). Current disease management techniques include burning or burring infested

straw and stubble to destroy the disease inoculum (De Wolf et al. 1998). The application of foliar fungicides is effective to control the pathogen, although expensive and detrimental to the environment (Tekauz et al. 1983, Lamari and Bernier 1989). Other effective alternative means to control the disease are the use of resistant cultivars, in combination with crop rotation (De Wolf et al. 1998; Strelkov and Lamari, 2003).

Pyrenophora tritici-repentis

Pyrenophora tritici-repentis (Ptr) is a homothallic ascomycete (Hosford 1971; Krupinsky 1982, 1992 Ali and Francl, 2003) that undergoes both sexual and asexual reproduction by producing ascospores and conidiospores, respectively (reviewed in Ciuffetti and Touri, 1999). However, the ascospores, are the source of primary inoculum for the spreading of the fungus (reviewed in Ciuffetti and Touri, 1999). The ascospores are stored in pseudothecia, which contain sac-like structures known as an ascus. Each ascus typically contains eight ascospores (Fig. 1a). Given that Ptr is able to grow as a saprophyte, its lifestyle favors the spreading of the disease. Survival of the Ptr on dead host tissue, i.e. wheat stubble, allows the formation of hundreds of pseudothecia, each containing ascospores that disseminate periodically (Strelkov et al 2003; reviewed in Ciuffetti and Touri, 1999).

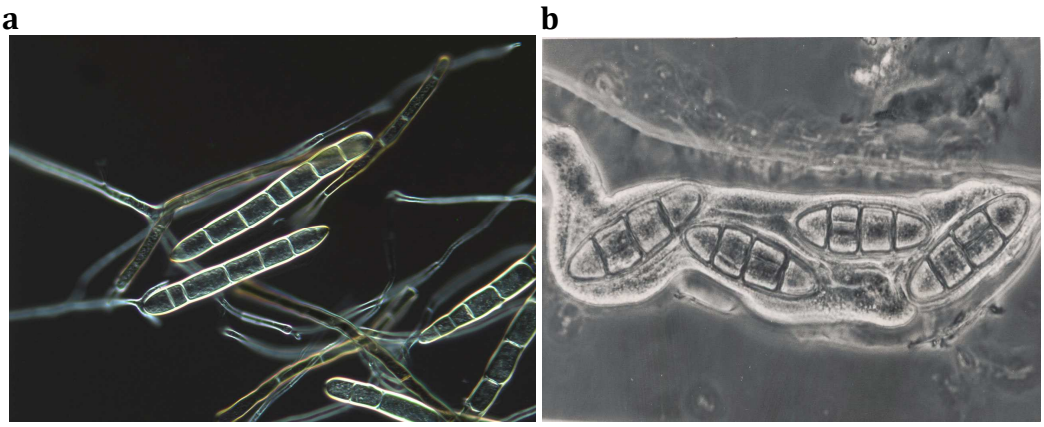


Figure 1. Sexual and asexual spores of Ptr. (**a**) Mycelia with conidiospores (products of meitosis). (**b**) Ascus containing four of the eight ascospores (products of meiosis). Courtesy of the Ciuffetti Laboratory.

Figure 2. Typical tan spot symptoms on a sensitive wheat cultivar. The browning of the leaf represents the necrosis, while the yellowing of the leaf represents the chlorosis. (Wikipedia)

Once Ptr reaches the wheat leaf, it forms an appresorium that allows the fungus to gain entry into the leaf tissue. The disease Tan spot of wheat is characterized by both necrotic and/or chlorotic symptoms (reviewed in Ciuffetti and Tuori, 1999) (Fig. 2). The symptoms develop initially as brown flecks along the leaf, which eventually grow out into larger elliptical legions. Necrosis is given by black and dark brown lesions along the surface of the leaf, whereas chlorosis, refers to the yellowing of the leaf tissue (Agrios, 2005).

The necrosis and chlorosis characteristic of tan spot are due to the pathogen's production of host-selective toxins (HST) (Ciuffetti and Touri, 1999; Strelkov and Lamari, 2003). HSTs are diverse metabolites that dictate disease development only on susceptible hosts. HSTs function as determinants of pathogenicity as it has been demonstrated that they can confer the ability to cause disease when expressed in non-pathogenic strain (Ciuffetti et al. 1997, Walton 2000, Wolpert et al 2002). In toxin sensitive plants, HSTs reproduce disease symptoms in the absence of the pathogen that produced them. There are eight Ptr races that have been characterized, based on the production of three HSTs, Ptr ToxA, Ptr ToxB, and Ptr ToxC, and a limited number of differential wheat cultivars (Strelkov and Lamari 2003). Ptr ToxA (ToxA) (Ciuffetti et al. 1997) and Ptr ToxB (ToxB) (Martinez et al. 2001) are two proteinaceous host-selective toxins that induce necrosis and chlorosis, respectively (Fig. 3). ToxC appears to be a polar, nonionic, low molecular weight compound that induces spreading chlorosis in different cultivars than ToxB (Effertz et al. 2002)

Figure 3. The effects of ToxA and ToxB on sensitive and insensitive wheat cultivars. Sensitive (Katepwa) and insensitive (Auburn) cultivars were infiltrated with either 2 μM ToxA or 20 μM ToxB. Black dots in the leaves delimit the toxins' infiltration zones. Courtesy of the Ciuffetti Laboratory.

ToxA and ToxB differ not only by symptoms (necrosis and chlorosis, respectively) they produce on a sensitive cultivar (Fig. 3), but also differ in the speed at which they produce visible symptoms. ToxA has a strong effect on the sensitive wheat cultivar and symptoms can be visualized at 14 hours post infiltration (Pandelova et al. 2009), while ToxB appears to be less severe than ToxA and the symptoms triggered by this toxin take approximately 48 hours to appear (Strelkov et al. 1999; Kim & Strelkov, 2007). Research in the Ciuffetti Laboratory has been focused on characterization of ToxA and ToxB. While great progress has been made towards understanding ToxA site- and mode-of-action, many questions about ToxB remain to be answered.

Ptr ToxA

ToxA gene encodes a 23 amino acid (aa) signal peptide (Ballance et al. 1996, Ciuffetti et al. 1997) and a 4.3-kDa pro-domain required for proper folding, both of which are cleaved before the secretion of the mature ToxA protein (13.2-kDa) (Touri et al. 1995). ToxA is a single domain protein with a β-barrel fold that exhibits a solvent-exposed loop containing an Arg-Gly-Asp (RGD) motif (Sarma et al. 2005). The RGD motif is found within a 10 aa sequence that is 60% identical to the RGD-containing region of the mammalian extracellular matrix protein, vitronectin (Manning et al. 2005). Nine out of 10 of these aa, including the RGD motif, are important for full toxic activity (Meinhardt et al. 2002; Manning et al. 2004). ToxA has been

demonstrated to enter the ToxA-sensitive plant cell and localize to the chloroplast (Manning and Ciuffetti 2005). Crystallography studies of ToxA confirmed the similarity of ToxA with the classic mammalian RGD-containing domain (Sarma et al. 2005). This raises the possibility that ToxA gains entry to the plant cell via binding to an integrin-like receptor in the host plant. Although, the mechanism by which ToxA is internalized is still unknown it depends on the presence of the RGD motif (Manning et al. 2008). ToxA activity is light-dependant, and it disrupts the photosynthetic process in the wheat cell. ToxA also elevates the level of reactive oxygen species (ROS) in a sensitive wheat cultivar (Manning et al. 2009).

The current working model to describe ToxA site- and mode-of action (Fig. 4). proposes that 1) ToxA initially binds to a receptor via an RGD-containing, solvent exposed loop (Manning et al. 2008); 2) upon binding, ToxA is internalized into the plant cell by an endosome-like structure; 3) ToxA disassociates from the receptor; 4) ToxA localizes to the chloroplast and interacts with ToxABP1, which is a chloroplast protein, that is required for the proper functioning of photosystem II (Manning et al., 2007); 5) the photosynthetic complex is altered and ROS accumulate in the cell; 6) cell death occurs.

Ptr ToxB

ToxB is encoded by the multi-copy gene *ToxB* (Martinez et al. 2001, Strelkov et al. 2002). *ToxB* is a 261 bp-open reading frame and encodes a protein that contains a

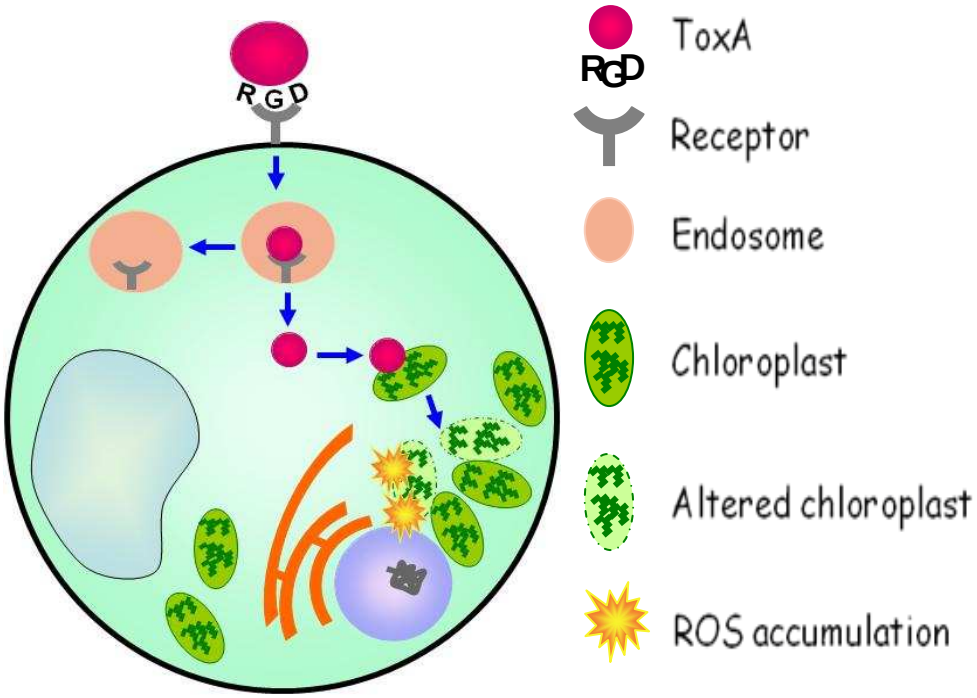


Figure. 4. Proposed model to describe ToxA site- and mode-of action. ToxA binds to a receptor via the RGD-containing, solvent exposed loop which facilitates ToxA internalization into the plant cell via an endosome. ToxA disassociates from the receptor and interacts with ToxABP1 in the chloroplast. ToxA treatment alterations in the photosynthetic complexes and accumulation of ROS. (Figure adapted from Manning et al. 2008).

secretion signal peptide of 23 aa and the remaining 64 aa constitutes the 6.5 kD mature protein (Martinez et al. 2001; Strelkov et al., 1999). A *ToxB* homolog, *toxb*, was identified in the non-pathogenic Ptr race 4 and in spite of its 86% similarity to *ToxB*, *toxb* encodes an inactive protein (Martinez et al. 2004). Other homologs of ToxB have been found in other ascomycetes, including *Pyrenophora bromi*, which is the causal agent of brown leaf spot (Andrie et al. 2008). ToxB inhibits photosynthesis by inducing chlorophyll degradation (Strelkov 1998). There is no sequence similarity between *ToxA* and *ToxB*, suggesting that the two toxins attribute pathogenicity in different manners, however like ToxA, ToxB activity appears to require light (Strelkov et al. 1998).

Plant defense

Plant defense is a critical component for the plant's survival and it is defined by various mechanisms that allow the plant to cope with abiotic or biotic stresses. In response to pathogens, plants express a number of defense responses. Some of these plant responses are dictated by a gene-for-gene recognition of the pathogen, that is explained by the classical gene-for-gene model (Flor 1971; Glazebrook 2005; Bent and Mackey 2007). This model states that for each resistance gene in the host there is a corresponding avirulence gene in the pathogen, and when both genes are present in a pathogen-host interaction the outcome is plant resistance (no disease). There is the “inverse” gene-for-gene interaction, which states that virulence/

pathogenicity factors, such as HSTs induce host susceptibility (plant disease) by the presence of a susceptibility gene in the host and a pathogenicity/virulence gene in the pathogen. ToxA and ToxB are two examples of an "inverse" gene-for-gene host-pathogen interaction since disease susceptibility to the toxin-producing isolates, depends on a single dominant gene in the host (Strelkov and Lamari, 2003; Wolpert et al. 2002). In the case of Ptr, ToxA sensitivity is due to the presence of *Tsn1* in the toxin sensitive cultivar (Gamba et al. 1998; Anderson et al. 1999).

While studies have provided plenty of data on the responses that occur during plant resistance governed by the classical gene-for-gene interaction, plant responses that occur during disease susceptibility are less understood. HSTs offer an excellent system to study the cellular events that take place during disease susceptibility because they have the ability to reproduce symptoms induced by the pathogen when infiltrated into sensitive plants. One of the advantages of working with these toxins, especially ToxA and ToxB is that they have been isolated and they can both be heterologously expressed in *Pichia pastoris* and *Escherichia coli,* respectively. The overall objective of this thesis is to characterize several plant responses that occur in ToxA and ToxB treated sensitive wheat.

A pathogen threat to the plant often triggers the production of a variety of secondary metabolites that are important for defense (Dixon et al. 1994; Bennett and Wallsgrove 1994, Glazebrook 2005). For example if a plant is exposed to a pathogen, the plant will elicit phenolics, that are precursors of compounds that will fortify the cell wall, in order to prevent further invasion. Apart from their role in plant-pathogen interactions, many of these secondary metabolites are necessary for

plant growth and development (Yu 2006). One group of secondary metabolites that play an important role in plant-pathogen interactions are the phenylpropanoids. These compounds are phenylalanine-derived chemicals that display large structural diversity (Noel et al. 2005). The most prevalent phenylpropanoid pathway across several organisms involves three enzymes that are required to transform phenylalanine into the Coenzyme A-activated hydroxycinnamoyl esters that enter other downstream pathways. The first reaction in the phenylpropanoid pathway is the deamination of L-phenylalanine by phenylalanine ammonia lyase (PAL). This reaction allows the non-oxidative elimination of ammonia from L-phenylalanine and results in the production of cinnamic acid. The cinnamic acid 4-hydrolase introduces a hydroxyl group in the phenyl ring of cinnamic acid leading to the production of coumaric acid. Finally, the hydroxycinnamate CoA ligase activates the carboxyl group of the coumaric acid by facilitating the formation of a thioester bond with CoA (Dixon and Paiva, 1995).

Microarray data generated in the Ciuffetti Laboratory shows that toxin sensitive wheat treated with either ToxA (Pandelova et al. 2009) or ToxB (Pandelova et al. in prep.) show an up-regulation in mRNA transcripts of enzyme PAL and other enzymes associated with the phenylpropanoid pathway. These data suggest that production of phenolic compounds are elicited by ToxA or ToxB. Therefore, the phenylpropanoid pathway will be subject of the studies presented in this thesis.

As stated earlier, ToxA induces the accumulation of ROS in toxin sensitive cells. In plant-pathogen interactions, ROS production, also referred as oxidative

burst, is one of the most common and rapid response to the pathogen (Dixon et al. 1994, Glazebrook 2005). ROS are usual byproducts of several biochemical pathways like photosynthesis and glycolysis and in the plant ROS have other functions such as cell elongation (Apel and Hirt, 2004). ROS production initiates a chain of subsequent oxidation reactions that give rise to free radicals in a cell. In-turn, free radicals are extremely harmful to the cell. Given that large quantities of ROS can severely damage the cell, well-tuned ROS producing and scavenging mechanisms are necessary for the plant's survival (Nande et al. 2010). Plants have enzymatic and non-enzymatic antioxidant mechanisms to prevent oxidative damage (Apel and Hirt, 2004). The anti-oxidants can prevent oxidative damage by being oxidized themselves and therefore preventing oxidation of cellular components (Sies, 1997).

To determine the significance of ROS in the ToxA-induced necrosis an anti-oxidant, N-Acetyl Cysteine, was directly applied to the area of the leaf that had been previously treated with ToxA. When the anti-oxidant N-Acetyl Cysteine (NAC) is administered to a sensitive wheat leaf treated with ToxA, the accumulation of ROS is significantly decreased and necrotic symptom development was delayed (Manning et al. 2008).

There are some similarities between ToxA and ToxB. Like ToxA, ToxB activity appears to be light-dependant, and it also appears to disrupt the photosynthetic machinery (Strelkov et al. 1998). In addition, ToxA treatment results in an accumulation of ROS. Based on these data, we hypothesize that ToxB could also increase intracellular levels of ROS in wheat. To address this hypothesis we will

carry out the same experimental approaches that were used to determine the presence of ROS in ToxA-treated leaves.

The objective of this research is to investigate defense responses triggered by ToxA and ToxB. To achieve this, we examined the effect of ToxB on ROS accumulation, and whether or not ToxA or ToxB activated the main components of the phenylpropanoid pathway.

MATERIALS AND METHODS

Bioassay

Plant growth, maintenance and toxin infiltration were performed as previously described (Manning et al. 2004). Approximately 20 µL of 20 µM of ToxB was infiltrated into secondary leaves of two-week old ToxB-sensitive Katepwa or ToxB-insensitive Auburn leaves. The plants were returned to the growth chamber (16 hour light and 8 hour dark) for the amount of time that each assay specified. To evaluate the activity of a toxin the plants were kept for five days in the growth chamber. After five days, the leaves were harvested and scanned with a flat bed Epson scanner (Epson America, Long Beach, CA, U.S.A). Each experiment was conducted at least three times.

Heterologous expression of ToxB

ToxB was expressed as described by Martinez et al. (2001) and Andrie et al. (manuscript in prep.). *Pichia pastoris* strain containing vector pCM11, containing *ToxB*, was grown overnight in a 50 ml-culture 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 L^{-1}], 2 ml 0.02% Biotin, 100 ml 10% glycerol L^{-1}) at 30°C while shaking at 250 rpm. The cells were harvested by centrifugation at 3,000 x g for 5 min at RT and four 250 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 L^{-1}) were inoculated to reach an OD600 of ~ 1.0. The BMM cultures were grown at 30°C shaking at 250 rpm for four days. On the second and third days, 12.5 ml of 10% methanol was added to each 250 ml-BMM culture. On the fourth day, cultures were centrifuged at 5,500 x g for 15 min at 4ºC and the supernatant was collected and concentrated to a volume of 100 ml using a rotary evaporator. Each sample was dialyzed overnight twice against four liters of Epure water at 4ºC and re-concentrated to 10 ml using a rotary evaporator. The samples were brought to 20 mM Tris (pH 8.0) and applied on a QMA anion exchange column (Waters). Fractions containing the protein of interest were pooled, reduced in volume using a rotary evaporator and dialyzed twice for 24 h against two liters of Epure water. The purified toxin fractions were analyzed by a silver stained 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.

ROS Detection

Production of superoxide O_2^- was detected using nitroblue tetrazolium (NBT) staining following the procedure described by Liu et al. 2007 with the modifications described in Manning et al. 2009. The NBT staining solution was prepared by dissolving 1 mg of NBT in 1 mL of 10 mM NaN_3 prepared in 10 mM potassium phosphate buffer (pH 7.8). Three leaves of the wheat cultivar Katepwa (ToxB-

sensitive) or Auburn (ToxB-insensitive) were infiltrated with either 20 μM ToxB or H_2O. ROS was detected at 14, 24 and 48 h after each treatment. For each time point, a three-centimeter region of the treated area was cut out. These treated leaf segments were placed in mini petri dishes, and 10 ml of NBT staining solution was added. In order to ensure the leaves are completely submerged in NBT solution, a thin layer of cheesecloth was draped over the leaves.

Immediately after treatment with NBT staining solution, the leaves were vacuum infiltrated for ten minutes, followed by overnight staining under bench light (15 umol m^2s^2). In order to prevent NBT precipitation in the petri dish, the petri dishes were placed on a shaker to ensure constant mixing. Following overnight staining, chlorophyll from the leaves was extracted by boiling them in 95% ethanol for ten minutes. Following extraction, the leaves were stored in 60% glycerol and examined using a microscope.

Extraction of cytosolic and cell wall bound phenolic compounds

Extraction of cytosolic and cell wall-bound phenolic compounds followed the protocol from Boyle and Walters (2006) with the modifications described below. The level of cytosolic and cell wall-bound phenolic compounds in Katepwa (ToxA and ToxB sensitive) and Auburn (ToxA and ToxB insensitive) leaves treated with 2 uM ToxA, 20 uM ToxB, or H_2O was quantified at 24 and 48 hours post treatment. For

each timepoint, a 4 cm-segment of the treated zone of each of the eight leaves was collected, weighed, and stored at -80º.

Leaves were removed from the freezer and immediately ground in liquid nitrogen; the ground tissue was transferred to a culture tube and dissolved in 500 µL of 50% methanol. Once dissolved, the mixture was transferred again to an eppendorf tube and the supernatant was used in the Folin-Ciocalteu assay, after undergoing centrifugation for 5 minutes at 3000 x g. The supernatant only contains cytosolic (free) phenolic compounds, while the remaining pellets contain esterified cell wall bound (CWB) phenolics. In order to extract CWB phenolics, the pellets were saponified with 150 µL of 0.5 M NaOH for 24 hours under dark conditions and at room temperature. After the 24-hour incubation period was completed, the mixtures were neutralized with 50 µL of 2N HCl and centrifuged for 5 minutes at 3000 x g. The supernatants were separated to carry out the Folin-Ciocalteau assay using 25 µl of the sample.

The Folin-Ciocalteau assay was performed by adding 50 µL of 2N Folin-Ciocalteu reagent. After a three minute incubation in dark conditions, 100uL of 20% sodium carbonate (Na_2CO_3) was added to sample; the sample was incubated again for twenty minutes at 37ºC. The absorbance of the samples was measured at 725 nm in a spectrophotometer. Phenolic content is then determined from a standard curve prepared with p-coumaric acid using the following concentrations: 0, 0.01, 0.025,0.05, 0.1,0.15, 0.25,0.5 mg/ml.

PAL Assay

The procedure was carried out as described by Southerton and Deverall (2006) with the some modifications. Leaves from wheat cultivars Katepwa and Auburn were infiltrated with 20 µM ToxB, or H_2O to assay the amounts of trans-cinnamic acid 24 and 48 h after treatments, and the activity of PAL was determined. For each time-point, a 3 cm-segment of the treated zone of each of six leaves were collected, weighed, and stored at -80ºC. The leaf tissue representing each treatment was ground on ice in 3 ml of 0.1 M sodium borate buffer using a few grains of sand. An aliquot of 1.25 ml of the sample was transferred to an oakridge tube containing 750 µL of supplemented borate buffer (11.2 mg EDTA and 2.4 µL 2-mercaptoethanol in 10 mL of 0.1M sodium borate buffer). After a brief vortex, each oakridge tube was centrifuged at 10,000 x g for 10 minutes, and the supernatant was transferred into a 1.5 mL eppendorf tube. It is critical to keep the supernatant separated from the pellet during the transfer, therefore, the oakridge tube was kept at a 45º degree angle. After isolating the supernatant, 300 µL was added to 600 µL of 0.1M sodium borate, 600 µM L-phenylalanine, or 600 µM D-phenylalanine. These samples were incubated in a 40ºC water bath for two hours. Following incubation, 100 µl of HCL followed by 1 ml of chloroform were added to each sample. After a brief vortex, the samples were centrifuged for 5 minutes at 1300 x g. Next, 500 µL of the lower aqueous phase was transferred into a 1.5 ml-eppendorf tube to be evaporated in a fume hood for approximately 5 hours. Following evaporation, the residue was resuspended in 1 ml of 0.1M sodium borate buffer by vortexing and mixing the

sample rigorously. The absorbance of the each sample was measured at 270 nm. At this point, the fresh tissue weight and absorption value to determine the accumulation of trans-cinnamic acid by a standard curve using the following concentrations: 0; 0.1;0.5; 1; 2; 4; 8; 9 µg/ml.

RESULTS

Heterologous Expression of ToxB in *Pichia Pastoris*

Genes are transcribed into mRNA, which are then translated into proteins. The heterologous expression of a protein refers to modification of the genetic background of the organism with the gene that encodes that specific protein. Previous work in the Ciuffetti Laboratory facilitated the heterologous expression of ToxB 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 and other organisms. The heterologous expression of ToxB was achieved by cloning the open reading frame (ORF) of the ToxB gene into a plasmid containing the methanol-responsive AOX1 promoter, which allows to drive the expression of ToxB by adding methanol to the growing *P. pastoris* cultures. The plasmid containing ORF of the ToxB gene was transformed into *P. pastoris* where it recombined into the genome, and because the plasmid contained a secretion signal, ToxB is secreted into the liquid media when the culture is induced. The crude culture filtrate from *P. pastoris* contains ToxB plus additional native proteins that need to be eliminated as they are contaminants. To purify ToxB the crude culture filtrate is placed on a strong anion exchange column where the native *P. pastoris* proteins bind while ToxB elutes in the flow through and the

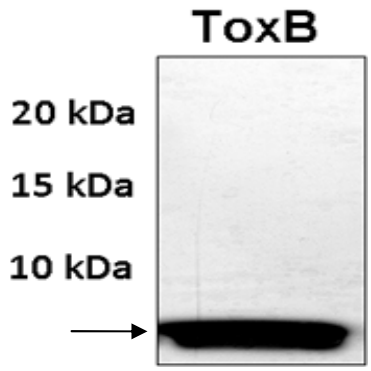


Figure 5. Silver stained SDS-PAGE of heterologously expressed and purified ToxB. The arrow points to the ToxB band that has a molecular mass of 6.5 kDa.

washes (Figueroa Betts et al. in prep). The efficiency of the purification process is analyzed in a silver stained 14% SDS-PAGE gel. ToxB has a molecular mass of 6.5 KDa, therefore it is expected to be visualized blow the 10 KDa molecular mass marker (Fig. 5).

Accumulation of the Reactive Oxygen Species

As shown previously in our laboratory, ROS levels are increased in response to ToxA treatment (Manning et al., 2009). To examine if ToxB induces responses similar to ToxA, we looked at accumulation of ROS in ToxB treated leaves. In order to detect ROS, we used the water-soluble compound nitroblue tetrazoliun (NBT), an aromatic molecule that forms a blue formazan deposit upon interaction with a superoxide (O_2^-) ion (Baehner et al. 1976; Armstrong et al. 2002). Oxidation of NBT by O_2^- and accumulation of the deposit can be visualized under the microscope.

ROS accumulation was examined at different time points and was not detected at 3 and 9 hours post treatment (hpt) but it was positively detectible at 24 hpt. Figure 6a illustrates the ROS accumulation in ToxB-treated sensitive leaves at 24 hpt. Additionally, formazan deposits were formed around chloroplasts in the ToxB-sensitive leaves. The intensity of the NBT stain was greater in ToxB-treated sensitive leaves at 48 hpt (Fig. 6b) and the cell shape is less organized than at 24

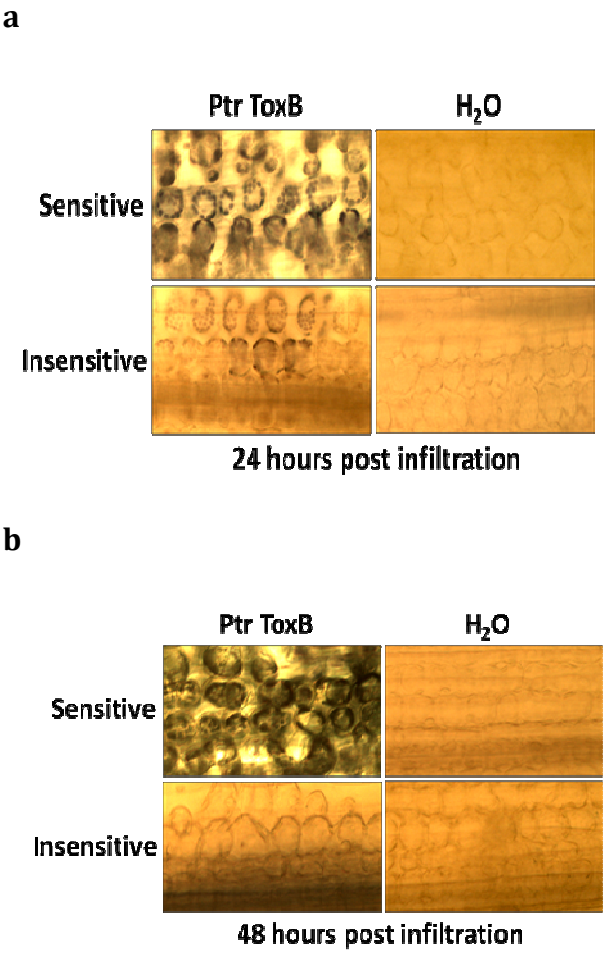


Figure 6. ToxB effect on ROS accumulation in sensitive and insensitive leaves. Sensitive and Insensitive leaves were infiltrated with either water or ToxB (20 uM). Leaves were collected and stained after 24 (a) and 48 (b) hpt with NBT stain and visualized under a microscope.

hpt. In some instances ToxB-treated insensitive leaves displayed minor traces of ROS at 24 hpt, however those traces disappeared at 48 hpt. The significance and reproducibility of these observations would need to be studied further. Neither water sensitive nor insensitive treated leaves have visible levels of formazan deposits neither at 24 nor at 48 hpt. To establish importance of ROS accumulation on symptom development induced by ToxB in sensitive wheat cultivar, we examined weather treatment with the antioxidant NAC would reduce the symptom development. We determined that treatment of ToxB–treated sensitive leaves with NAC after 3 h post toxin infiltration leads to reduction of the symptoms after 72 hpt (Fig. 8). Water and NAC-infiltrated leaves shown as negative control.

Figure 7. The effect of NAC on ToxB-treated sensitive leaves. Sensitive leaves treated with either ToxB (20 μM), ToxB and NAC (25 μM), or NAC only.

Phenylalanine Ammonia Lyase Activity

Two of the responses that help a plant to protect itself from pathogen invasion are strengthening of the cell wall and synthesis of antimicrobial compounds. To achieve that, the plant activates the phenylpropanoid pathway (Dixon and Paiva, 1995). The first step of the phenylpropanoid pathway involves the conversion of L-phenylalanine to trans-cinnamic acid, a reaction catalyzed by the enzyme phenylalanine ammonia lyase (PAL) (Fig. 8). Thus, activation of the phenylpropanoid pathway depends on an increase of PAL activity. A microarray study conducted in our laboratory showed significant up-regulation of PAL transcript in both ToxA and ToxB-treated leaves (Pandelova et al. 2009). To investigate if this up-regulation results in an increase of PAL activity, PAL was determined for both H_2O and ToxB-treated sensitive and insensitive wheat leaves. As shown in figure 9, PAL activity is elevated in ToxB-treated leaves compared to controls. Additionally, PAL activity is greater in ToxB-treated leaves 24 hpt compared to 48 hpt.

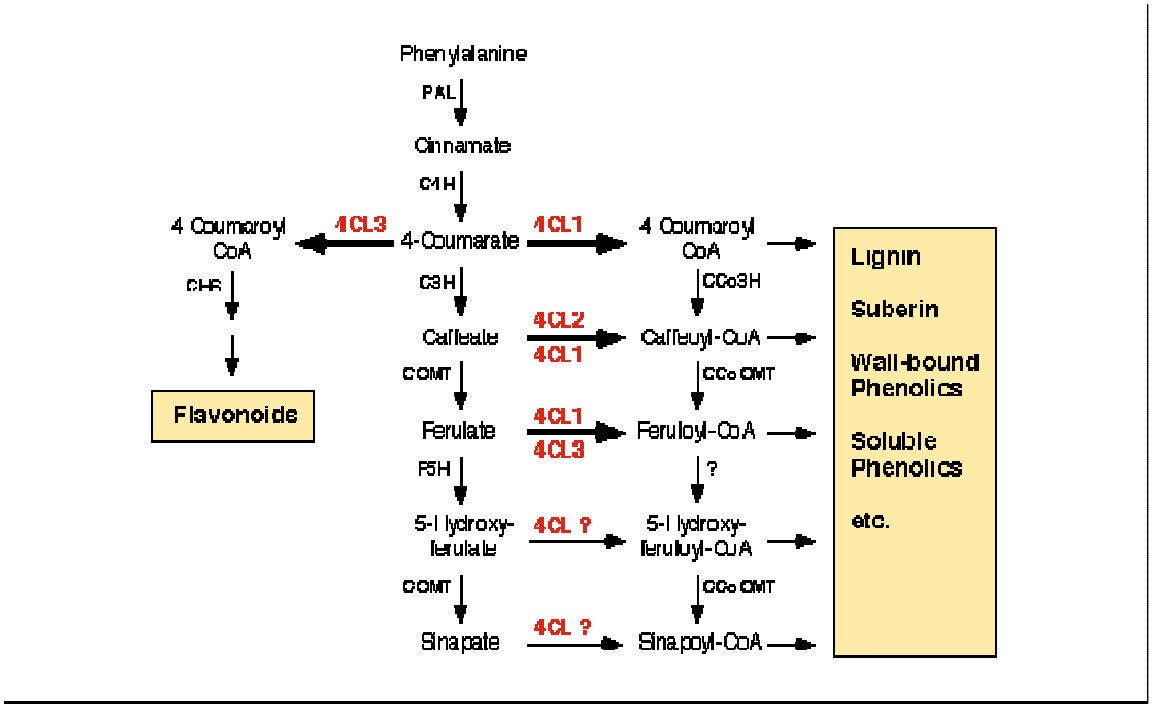


Figure 8. The general phenylpropanoid biochemical pathway. Enzymes are shown beside the arrows, and final products are shaded in yellow. PAL, phenylalanine ammonia lyase, C4H, cinnamate-4-hydroxylase; C3H, coumarate-3-hydroxylase; 4CL, 4-coumaroylCoA ligase; CQT, coumaroylCoA:quinate hydroxycinnamoyl transferase; CHS, chalcone synthase. (Erich Kombrink http://www.google.com/imgres?imgurl=http://www2.mpiz-koeln.mpg.de/schlef/pic/Kombrink_fig5.gif&imgrefurl=http://www2.mpiz-koeln.mpg.de/schlef/Kombrink_webpage.html&usg=__5IZgq0H_MWvlTQUvoUXRB947a54=&h=435&w=681&sz=8&hl=en&start=14&um=1&itbs=1&tbnid=ubcslEyMNcntZM:&tbnh=89&tbnw=139&prev=/images%3Fq%3Dphenylpropanoid%2Bpathway%26um%3D1%26hl%3Den%26sa%3DN%26rlz%3D1R2GGLL_enUS342%26tbs%3Disch:1)

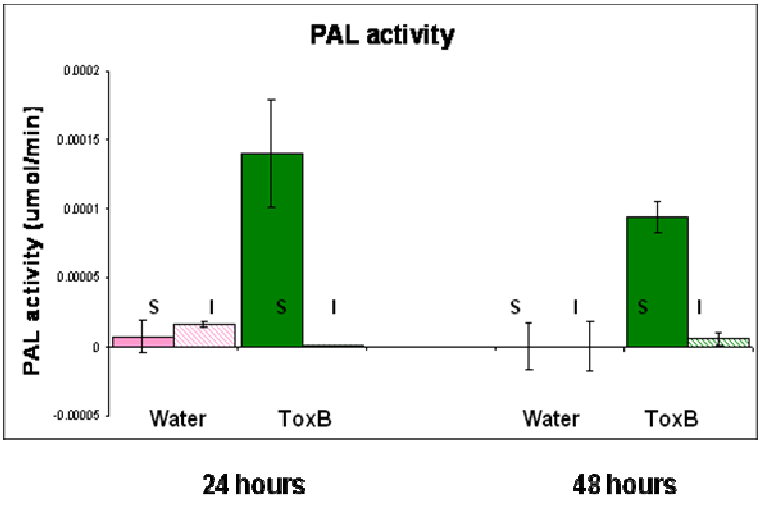
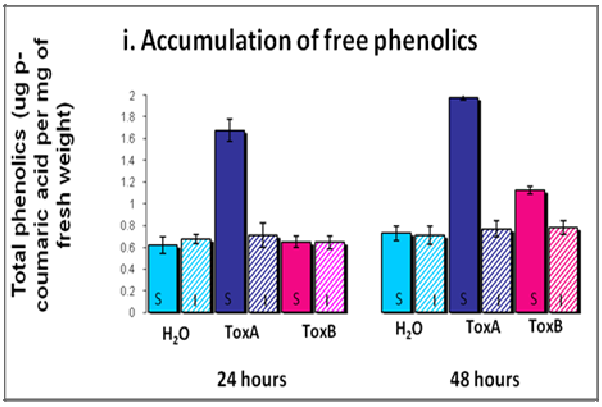


Figure 9. The effect of ToxB on PAL activity. Sensitive (S) and insensitive (I) wheat leaves were infiltrated with either water or ToxB (20 uM). Leaves were collected, and PAL activity was measured after 24 and 48 hpt. Presented data are based on at least three biological replicates.

Quantification of phenolic compounds

The production of antimicrobial compounds and physical reinforcement of cell walls are important components of the plant defenses against pathogens (Glazebrook, 2005). Lignin, which plays a role in reinforcing the cell wall, is produced by a branch of the phenylpropanoid pathway, and is a compound derived from phenolic compounds such as p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Yu, 2006). Figure 8 shows schematic of phenylpropanoid pathway steps examined in this study.

To determine if elevated levels of PAL lead to increase in the amount of phenolic compounds, we determined the concentration of p-coumaric acid at 24 and 48 hours for both sensitive and insensitive leaves treated with ToxA, ToxB, or water. Both free and cell wall bound phenolics were extracted and quantified after 24 and 48 hpt. In this experiment p-coumaric acid acted as a marker for the accumulation of phenolic compounds in the plant. ToxA treatment induced significant accumulation of both free and cell wall bound phenolics at 24 and 48 hpt compare to both water and insensitive wheat control. Alternatively, ToxB effect was not that considerable and no increase of phenolic compounds was detected at 24 hpt. At 48 hpt both types of phenolic compounds were increased compare to water and insensitive control, however, to a lesser extent than after ToxA treatment (Fig. 10).



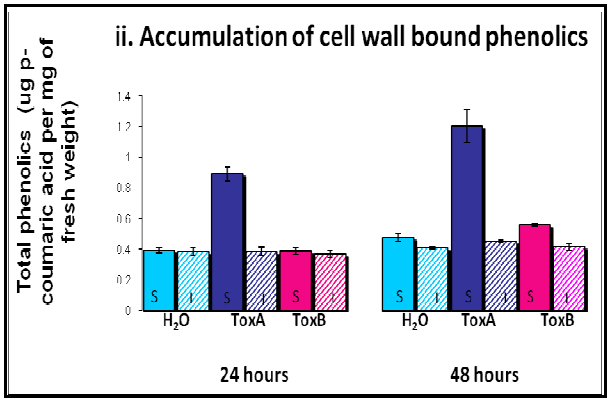


Figure 10. The effect of ToxA or ToxB on the accumulation of free and cell wall bound phenolic compounds. Toxin sensitive (S) and insensitive (I) leaves were treated with either H_2O, ToxA (2µM), or ToxB (20µM). Leaves were harvested at 24 and 48 hpt and phenolic compounds (free (i) and cell wall bound (ii)) were extracted and quantified.

DISCUSSION

Heterologous Expression of ToxB

In order to purify heterologous ToxB ($_{het}$ToxB) we applied an anion exchange column during the purification process. This method proved to be very effective in eliminating endogenous *P. pastoris* secreted proteins. This protocol results in high protein yields with limited processing time. As it has been demonstrated previously in the Ciuffetti Laboratory, the isolated $_{het}$ToxB has the same molecular mass and activity as native ToxB (Figueroa Betts et al. in prep). We verified the purity of the toxin by SDS-PAGE (Fig. 5). Production of large amounts of $_{het}$ToxB with equivalent activity to native ToxB is advantageous because the purification of native ToxB is difficult and time-consuming. Therefore, $_{het}$ToxB was used in all the subsequent experiments presented in this thesis.

Accumulation of ROS

Determining ROS accumulation can be a complicated procedure, because the production of ROS occurs quickly in response to any physical stress that the plant encounters. Therefore, a gentle manipulation of leaf tissue was crucial to obtain accurate and consistent results. We conducted at least 5 biological replicates of this experiment.

Our results indicate that a sensitive wheat cultivar treated with ToxB does accumulate a significant level of ROS after 24 hpt. The formazan deposits caused by the oxidation of NBT were concentrated around the edges of the mesophyll cells in the vicinity of the chloroplasts (Fig. 6a). Given these observations, we hypothesize that the ToxB mode-of-action, similarly to ToxA, involves impairment of chloroplast function. At 48 hpt, we observed a greater intensity of the NBT staining, which suggests that the there was more ROS accumulated in the cell (Fig. 6b). Additionally, at this point the mesophyll cells looked damaged and ROS was prevalent throughout the entire cell. Interestingly, the ToxB-treated insensitive leaves showed minor traces of ROS at 24 hpt (Fig. 6a). However, there were no visible traces of ROS at 48 hpt in the insensitive cultivar (Fig. 6b). This implies that ROS accumulation was transient, and the plant defense system was able to overcome the ROS accumulation.

To address the hypothesis that the accumulation of ROS is responsible for the symptom development, we treated ToxB infiltrated leaves with the antioxidant NAC (Fig. 7). Preliminary results indicate that the symptom development is reduced upon NAC treatment, which supports the hypothesis that ToxB induced ROS is part of the mechanism by which the toxin triggers cell death in the sensitive cultivars.

Phenylalanine ammonia lyase activity

The phenylpropanoid pathway is activated as a defense response to many pathogens (Dixon et al. 2002). Microarray data acquired in the Ciuffetti Laboratory indicated that some components of the phenylpropanoid pathway that lead to the synthesis of secondary metabolites and possibly lignin are activated in response to ToxA treatment (Pandelova et al. 2009). Because the microarray data showed that the transcription of *PAL*, the first enzyme of the phenylpropanoid pathway (Fig. 8) is elevated, we examined whether the activity of PAL is also increased in response to ToxA and ToxB. One biological replicate indicated that the PAL activity was increased in the ToxA-treated sensitive cultivar at 24 hpt compared to control (data not shown). Our results (Fig. 9) examining PAL activity in ToxB-treated sensitive leaves demonstrate that PAL is more active in response to this toxin at 24 hpt than at 48 hpt. Since this timing correlates with the onset of symptom development at this concentration of the toxin (Strelkov et al. 1999), lower PAL activity at 48 hpt could be a reflection of the start of the cell death process and exhaustion of plant resources. In the insensitive cultivar neither ToxA (data not shown) nor ToxB (Fig. 9) induced an increase in PAL activity.

Accumulation of phenolic compounds

Based on the previous results, it was important to establish whether the increase of PAL activity in ToxA and ToxB treated plants resulted in accumulation of phenolic compounds such as p-coumaric acid. P-coumaric acid is a precursor for p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol which are compounds that play a role in the synthesis of structural barriers in the cell wall such as lignin (Fig. 8). Our results indicate that the sensitive cultivar accumulated more p-coumaric acid in response to ToxA than to ToxB. Additionally, the p-coumaric acid accumulated faster in the ToxA treated leaves (Fig. 10). The slower accumulation of phenolic compounds in ToxB-treated leaves correlates with the delayed onset of symptom development. The difference in the amount of cell wall bound phenolic compounds accumulated in plants treated with either ToxA or ToxB may imply that more cell wall reinforcement occurs in response to ToxA.

Concluding remarks

Host-selective toxins provide a unique advantage to study the plant pathogen interactions that lead to susceptibility, in the absence of the pathogen that produces them. Because ToxA and ToxB are two major pathogenicity factors of *P. tritici-repentis*-wheat pathosystem, studying the plant responses triggered by these toxins will expand our understanding of the classical and inverse gene-for-gene model and other aspects of plant-pathogen interactions. Unraveling the steps of the susceptible interaction should provide valuable data to understand the course of disease development. Because ROS accumulation and activation of the phenylpropanoid pathway are both characteristic to resistance responses in plants, the results presented in this thesis support the notion that plant resistance and susceptibility share pathways and some responses. It is intriguing that although ToxA and ToxB have different molecular characteristics and cause different symptoms on the plant (ie. necrosis vs chlorosis) they still evoke similar responses in the plant. However, for ToxB treatment these responses are delayed compared to ToxA. The fact that these toxins are able to induce these type of plant responses in absence of the pathogen reinforces their primary role as major pathogenicity factors.

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