AN ABSTRACT OF THE THESIS OF

Robert P. Tuori for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on April 2, 1998. Title: Molecular Characterization of a Protein Toxin Involved in the *Pyrenophora tritici-repentis*/Wheat Interaction.

Abstract approved: Redacted for Privacy

Lynda M. Ciuffetti/ Thomas J. Wolpert

Pyrenophora tritici-repentis is the causal agent of the economically important disease tan spot of wheat. To facilitate the analysis of pathogenicity in the *Pyrenophora tritici-repentis*/wheat interaction, a host-selective toxic protein, Ptr ToxA, was purified from cultures of this fungus. Ptr ToxA is a 13.2 kD heat-stable protein that induces necrosis in sensitive wheat cultivars. Western analysis and indirect immunoprecipitation indicated that polyclonal antibodies raised against Ptr ToxA were specific for this protein. Bioassays of immunoprecipitated protein and Ptr ToxA protein eluted from polyacrylamide gels indicated that the protein is the toxic agent. Other less abundant necrosis-inducing components that are chromatographically and immunologically distinct from Ptr ToxA were also detected in cultures of the fungus.

In a previous report, we described the isolation of the gene that encodes Ptr ToxA, and confirmed that this gene functions as a primary determinant of pathogenicity in the *Pyrenophora*-wheat interaction (Ciuffetti *et al.*, 1997, *The Plant Cell* 9: 135-144). Amino acid sequencing of internal peptide fragments and from Ptr ToxA following treatment with pyroglutamate amino peptidase resulted in the determination of a significant amount of protein sequence and indicated the nature of the N-terminal blockage. A full length cDNA

molecule was constructed that was useful for the heterologous over-expression of the toxin. The occurrence of the *ToxA* gene in isolates of *P. tritici-repentis* and *P. teres* was assessed through PCR analysis. Secondary structure analyses of the deduced precursor protein indicate that the protoxin is composed of two distinct structural domains.

Finally, a cultivar-specific active form of Ptr ToxA was produced from a heterologous system. A polyhistidine-tagged, fusion protein consisting of both the N- and C-domains of the precursor protein, approximately 10-20% as active as native toxin, was produced from *Escherichia coli*. A C-domain fusion protein displayed only trace amounts of activity. These data suggest that: 1) the N-domain is necessary for correct folding of Ptr ToxA; 2) the N-domain does not function to eliminate activity of the protoxin; and 3) post-translational modifications of Ptr ToxA are not essential for full activity. The fusion protein consisting of the N- and C-domains will be useful for receptor binding studies as well as screening for disease susceptibility.

Molecular Characterization of a Protein Toxin Involved in the *Pyrenophora tritici-repentis*/Wheat Interaction

by

Robert P. Tuori

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirement for the
degree of

Doctor of Philosophy

Presented April 2, 1998
Commencement June, 1998

Doctor of Philosophy thesis of Robert P. Tuori presented on April 2, 1998

APPROVED:

Redacted for Privacy

Co-Major Professor, representing Botany and Plant Pathology

Redacted for Privacy

Co-Major Professor, representing Botany and Plant Pathology

Redacted for Privacy

Head of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

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

Redacted for Privacy

Robert P. Tuori, Author

Acknowledgement

I am very grateful to my co-major professors, Drs. Lynda M. Ciuffetti and Thomas J. Wolpert for scientific advice, enthusiastic discussions, patience, and guidance during the past several years.

I would like thank my committee members, Drs. Dallice Mills, Terri Lomax, and Kate Field for their efforts and contructive criticisms.

I am indebted to Janey Gaventa for invaluable assistance with infiltrations, and maintenance of fungal cultures; Judy Bononi for help with protein purifications; and Sean Ottum for assistance with construction of expression vectors. Thanks, also, to others in the Ciuffetti and Wolpert labs who have offered support and advice: Jenny Lorang, Roy Navarre, Dan Moore, Linda Hardison, Sadie Curry, and Amnon Lichter.

I would also like to thank Blaine Baker for photographic assistance, the Center for Gene Research and Biotechnology, Central Services Laboratory, for protein and DNA sequencing, the Mass Spectrometry lab at OSU, and Janet Andrews at The Monoclonal Antibody Facility of The Center for Gene Research and Biotechnology for help with the production of antibodies. This research was supported by grants from the U.S. Department Agriculture, the National Science Foundation, and a U.S. Department of Agriculture National Needs Fellowship awarded to the author.

Contribution of Authors

Drs. Lynda Ciuffetti and Tom Wolpert were involved in the design, analysis and editing of the three manuscripts of this thesis.

Table of Contents

Table of Contents (continued)

List of Figures

List of Tables

Molecular Characterization of a Protein Toxin Involved in the *Pyrenophora tritici-repentis*/Wheat Interaction

Chapter 1

Introduction

Host-Selective Toxins

A major goal of molecular plant pathology is the identification of factors influencing the specificity of microbes for their hosts. In some plant-fungal pathogen interactions specificity is determined by host-selective toxins (HSTs) (Yoder, 1980; Walton and Panaccione, 1993). Most HSTs are low-molecular-weight, secondary metabolic compounds and include cyclic peptides, polyketides, terpenoids, and saccharides, with a single known exception being a polypeptide. HSTs reproduce the symptoms of disease caused by the toxin-producing fungi and their production is strictly correlated with pathogenicity. They have high biological activity and selectivity for specific genotypes of plants. Host sensitivity to these toxins strictly correlates with susceptibility to the pathogen. Consequently, HSTs are readily identifiable pathogenicity or virulence factors and therefore, are of considerable interest to plant biology and plant pathology for both applied and basic reasons (Yoder, 1980). Several HST-producing fungi cause economically important diseases and the HSTs produced by these fungi are now appreciated to have been essential to their destructiveness: peritoxin produced by *Periconia cercinata* in *Periconia* blight of Sorghum; victorin produced by *Cochliobolus victoriae* in Victoria blight of oats; HS-toxin produced by *Cochliobolus sacchari* in leaf

spot of sugarcane; and T-toxin produced by *Cochliobolus heterostrophus* in southern corn leaf blight. Because HSTs are believed to play a causal role in plant pathogenesis, they provide the opportunity to study mechanisms of fungal pathogenicity and the molecular basis of disease susceptibility and resistance that could lead ultimately to novel disease control measures. They also provide insight into secondary metabolic pathways and the evolution of new pathogenic races in fungi.

Genetic Analysis of Toxin Production

A fundamental goal in the genetic analysis of toxin production would be to test or confirm the role of toxins in disease development (Scheffer, 1983; Yoder, 1980). Because all HSTs described to date, except one, are secondary metabolites and presumably therefore the products of multifunctional enzymes or complex biochemical pathways, the genetic analysis of toxin production is very difficult. This difficulty is exemplified with the analysis of three HSTs produced by the closely-related species of *Cochliobolus*: T-toxin, a family of linear longchain (C35 to C41) polyketides produced by *C. heterostrophus* (Kono *et al.*, 1981); HC-toxin, a cyclic tetrapeptide produced by *C. carbonum* (Gross *et al.*, 1982); and victorin, an unusual chlorinated cyclic peptide produced by *C. victoriae* (Wolpert *et al.*, 1985). In these three species of *Cochliobolus*, three single but different genetic loci appear to control production of their respective HSTs (Bronson, 1991). These genes have been designated *TOX1*, *TOX2*, and *TOX3*, and control the production of T-toxin, HC-toxin and victorin, respectively (Yoder *et al.*, 1989). Many of the other HST-producing fungi, including *Periconia circinata* and certain

pathovars of *Alternaria alternata,* do not have known sexual stages so are not amenable to conventional genetic analysis.

It has long been puzzling how a single genetic locus could be responsible for the production of a complex secondary metabolite such as the aforementioned HSTs. There are, however, several reasons why the production of a secondary metabolite that is likely the product of a complex locus or loci would appear to segregate as a single Mendelian "gene" in crosses of producers and nonproducers. These include: 1) tight genetic clustering of secondary metabolic pathway genes; 2) a single locus encoding a large multifunctional polypeptide responsible for production of the metabolite; 3) presence of the biosynthetic gene(s) on a dispensible chromosome in the producing strain; and, 4) suppression of crossing over in a chromosomal region containing biosynthetic genes. All of these scenarios would result in a complex trait appearing to be conditioned by a single Mendelian locus, and many have been implicated in the genetics of HST production. In addition, the loss or absence of a gene, gene cluster, or an entire chromosome in a nonproducing strain would also lead to a complex locus or loci appearing to segregate as a single Mendelian "gene" in crosses of producers and nonproducers.

HmT -Toxin

Hmt-toxin, or T-toxin is produced by race T of *C. heterostrophus*, a virulent pathogen of maize containing Texas male-sterile (T) cytoplasm. Race O of the fungus, a minor pathogen of both normal and T cytoplasm maize, does not make T-toxin. Susceptibility to *C. heterostrophus* and sensitivity to T-toxin in maize is controlled cytoplasmically by the mitochondrial gene *T-urf13* (Dewey *et al.*, 1988). Although the

polyketide structure of T-toxin suggested that multiple enzymatic functions would be required for its biosynthesis, the ability to synthesize T-toxin was shown to segregate in a simple 1:1 ratio in progeny obtained from crosses of race O and T isolates, indicating a single locus conditioning T-toxin production (Lim and Hooker, 1971). This locus was later designated *TOX1* (Yoder *et al.*, 1989). *TOX1* is tightly linked to a chromosomal translocation breakpoint (Bronson, 1988; Tzeng *et al.*, 1992) and race T isolates appear to have an insertion of 1.2 Mb of DNA that is linked to *TOX1*, but it is missing in tox^- isolates. The tight association of the translocation and insertion(s) with T-toxin production suggests that chromosome rearrangements may have been involved in the evolution of race T and *TOX1* (Chang and Bronson, 1996). Restriction enzyme-mediated integration mutagenesis (REMI) procedures were used to tag the *TOX1* locus (Lu *et al.*, 1994). The DNA recovered from the insertion site of one mutant encodes a 7.6-kb open reading frame that predicts a multifunctional polyketide synthase (*PKS1*) (Yang *et al.*, 1996). *PKS1* exists in the genome as a single copy surrounded by highly repetitive DNA. Another gene necessary for T-toxin biosynthesis, *Tox1B*, not linked to *PKS1* but found on the other translocated chromosome of $Tox1^+$ isolates, encodes a putative decarboxylase (Turgeon *et al.*, 1995). Yang *et al.* (1996) suggested that because race O strains lack a detectable homolog of *PKS1*, race T may have acquired *PKS1* by horizontal transfer of DNA rather than by vertical inheritance from an ancestral strain. PKS1 and Tox1B appear to segregate as a single locus due to the translocation. In crosses of isolates homozygous for the translocation, they segregate as two unlinked loci, both necessary for T-toxin biosynthesis.

HC-Toxin

HC-toxin is produced by race 1 of *C. carbonum* while race 2 of the fungus does not produce the toxin. Resistance to *C. carbonum* and insensitivity to HC-toxin in maize is dominant and is controlled by the nuclear gene *Hm1* (Johal and Briggs, 1992). Like T-toxin, HC-toxin production segregates in a simple 1:1 pattern, suggesting the involvement of a single genetic locus (Scheffer *et al.*, 1967), designated *TOX2* (Yoder *et al.*, 1989). Upon original molecular characterization, *TOX2* appeared to be a cluster of at least 54 kb encoding, in part, two copies each of a multifunctional enzyme, HTS1 (HC-toxin synthetase) (Panaccione *et al.*, 1992), and another gene *TOXA*, which encodes a presumed HC-toxin efflux pump protein (Pitkin *et al.*, 1996). *HTS1* encodes an enormous 15.7 kb open reading frame with domains having similarity to other large peptide antibiotic synthetases (Scott-Craig *et al.*, 1992). The entire region segregates as a single locus because race 2 isolates lack DNA homologous to *TOX2*. The region containing both copies of *HTS1* and *TOXA* is flanked by a repetitive element that is present in race 2 DNA and encodes a putative transposase (Panaccione *et al.*, 1996). Another gene required for HC-toxin biosythesis, *TOXC*, was found by analyzing regions of DNA novel to race 1 (Ahn and Walton, 1997). *TOXC*, presumed to be involved in the production of an unusual amino acid component of HC-toxin, is present in three copies in the race 1 genome, linked to *HTS1* and *TOXA*. The copies of *HTS1*, *TOXA* and *TOXC* are distributed over a 540 kb region on a 3.5 Mb chromosome. In some $TOX2^{+}$ isolates these genes are located on a 2.2 Mb chromosome indicating that a reciprocal translocation may have occurred

(Ahn and Walton, 1996). Like T-toxin, the genetic basis for HC-toxin production is extremely complex, dependent on multiple genes including a large multifunctional enzyme and apparently involving a translocation event. The capacity to produce HC-toxin, however, because it is so complex likely did not evolve by any single, simple mechanism such as the horizontal transfer of foreign DNA that has been suggested for T-toxin (Walton, 1996).

Victorin

Considerably less is known about the genetics of production of victorin, the HST produced by *C. victoriae*. Isolates of *C. victoriae* that make victorin are pathogens on oats that carry the dominant allele at the *Vb* locus. Sensitivity to victorin in oats correlates with susceptibility to *C. victoriae* and is dominant. This gene is either closely linked to or the same as the *Pc-2* gene, which confers resistance to *Puccinia coronata* (Rines and Luke, 1985). Matings between toxin producing isolates of *C. victoriae* and *C. carbonum*, gave progeny which produced victorin, HC-toxin, both toxins or neither toxin, in a 1:1:1:1 ratio indicating two different single loci conditioning the production of these toxins (Scheffer *et al.*, 1967). The locus controlling victorin production, *TOX3*, is hypothesized to encode a cyclic peptide synthetase similar to HTS of *C. carbonum*. Molecular genetic analyses of victorin production in *C. victoriae* are apparently in progress.

The Pyrenophora tritici-repentis HST

In contrast to other known HSTs, a toxin produced by *Pyrenophora tritici-repentis*, a pathogen of wheat, is a protein (Ballance *et al.*, 1989; Tomas *et al.*, 1990;

Tuori *et al.*, 1995) and therefore, very likely a direct gene product. This presents a significant experimental advantage over other HSTs in many regards. Firstly, toxin purification was simplified by the ability to detect and characterize the toxin protein with standard protein biochemistry techniques. Detection and isolation of the toxin protein was additionally facilitated by immunological procedures, because antisera were raised against the protein. Secondly, cloning of the gene(s) conditioning toxin production and functional complementation of the gene(s) was significantly more straightforward than with other HSTs. Lastly, future biochemical and ultrastructural studies of the site and mode of action of this toxin should be simplified due to the size and proteinaceous nature of this toxin.

Tan Spot of Wheat

Pyrenophora tritici-repentis (syn. *P. trichostoma*), anamorph: *Drechslera tritici-repentis* (syn. *Helminthosporium tritici-repentis*), is a homothalic ascomycete that causes the foliar disease tan spot of wheat (Hosford, 1971). Tan spot is an economically significant disease that has been reported worldwide and may account for yield losses of up to 40 % (da Luz and Hosford, 1980; Dublin, 1983; Hosford, 1971; Hosford and Bush, 1974; Raymond *et al.*, 1985; Shabeer *et al.*, 1991; Sykes and Bernier, 1991; and Watkins *et al.*, 1978). *P. tritici repentis* also causes leaf spot of several other gramineous hosts (Hosford, 1971; Krupinsky, 1992; Morrall and Howard, 1975), and is believed to have the widest host range of *Pyrenophora* species (Krupinsky, 1982; Shoemaker, 1962). The importance of tan spot has increased with the recent shift from conventional to

conservation tillage, in which significant amounts of crop residue are left on the soil surface (Pfender *et al.*, 1988; Schuh, 1990).

P. tritici-repentis reproduces both asexually by conidia and sexually by ascospores. The pathogen survives between growing seasons as pseudothecia on straw and stubble of wheat, from which ascospores, the primary source of inoculum, are released in the spring (Hosford, 1971). Conidia produced in the lesions are the secondary source of inoculum (Hosford, 1972). The fungus has also been shown to be transmitted by seed (Schilder and Bergstrom, 1995).

Toxins Produced by *Pyrenophora tritici-repentis*

Evidence Implicating a Toxin in Pathogenesis

The involvement of a toxin(s) in pathogenesis was suggested by both the symptomology of tan spot and the method of fungal colonization of host tissue. The initial symptoms of tan spot in susceptible wheat include the development of small yellow-brown spots that develop into larger, tan-to-brown lesions expanding rapidly and apparently ahead of the colonized area, indicating that a toxin may be involved in disease development (Hosford, 1971). Necrosis often begins near the tip and progresses toward the base of the leaf. These symptoms were observed only in some wheat cultivars in response to infection by certain isolates of the fungus (Lamari and Bernier, 1989a; Lamari and Bernier, 1989b), suggesting virulence results from a specific interaction between isolates of the fungus and the appropriate host genotype. Resistant plants respond to infection with the development of small spots which remain restricted. That a toxin could

be causal to these symptoms was further suggested by the similarity of these observations with that of disease symptoms of closely-related fungi of the genera *Cochliobolus*, many of which produce HSTs.

Cytological studies providing evidence that hyphae grow intercellularly without penetration of mesophyll cells (Larez *et al.*, 1986; Lamari and Bernier, 1989b; Loughman and Deverell, 1986) contributed to the hypothesis that a toxin was involved in pathogenesis. An early study of the infection process revealed fungal ingress through host epidermal cells from an appresorium that subsequently developed a penetration peg and an intracellular vesicle (Larez *et al.*, 1986). Secondary hyphae form from the vesicle and invade the mesophyll intercellularly. The lack of obvious structural mechanisms for restriction suggested a molecular basis for resistance to fungal development. Another cytological study of compatible and incompatible interactions between *P. tritici-repentis* and wheat indicated that resistance was not expressed until the fungus had colonized the intercellular space of the mesophyll (Lamari and Bernier, 1989b). Again, penetration of mesophyll cells was not observed. No major cytological differences were observed between compatible and incompatible interactions regarding germination of conidia, formation of appresoria, penetration of epidermal cells or vesicle formation. In both resistant and susceptible plants no damage could be detected to mesophyll cells until 72 hours after infection, when progressive damage was seen in mesophyll cells at the cytological level corresponding to visible tan necrotic lesions in the susceptible plant (Lamari and Bernier, 1989b).

Pathotypes of Pyrenophora tritici-repentis

Isolates of *P. tritici-repentis* were grouped into three pathotypes based on the reaction of differential wheat cultivars to infection (Lamari and Bernier, 1989b). Pathotype 1 induces either tan necrosis or extensive chlorosis on the differential cultivars and was denoted as $nec^{+}chl^{+}$, because it was presumed to make both necrosis and chlorosis-inducing toxins. Pathotype 2 induces tan necrosis only and was denoted as $nec^{+}chl^{-}$ because it was presumed to make only a necrosis-inducing toxin. Pathotype 3 induces extensive chlorosis only and was denoted as $nec^{-}chl^{+}$, because it was presumed to make only a chlorosis-inducing toxin. Avirulent isolates, pathotype 4 ($nec^{-}chl^{-}$), penetrate the epidermal cell but do not progress into the mesophyll and were described as presumed nontoxin producers. Later, chlorosis-inducing isolates were identified from Algeria that could be distinguished from pathotype 3 through the response of differential wheat cultivars (Lamari *et al.*, 1995). Based on this finding, pathotypes 1, 2 ,3 and 4 were designated race 1-4 and the new chlorosis-inducing isolate was designated race 5 (chl+nec+).

Ptr-Necrosis Toxin(s)

The initial report of *P. tritici-repentis* producing a toxin in culture (Tomas and Bockus, 1987) indicated that the toxin was cultivar-specific and mimicked typical tan spot necrosis symptoms. In addition, sensitivity to the toxin correlated with susceptibility to the fungal pathogen, suggesting the involvement of toxic compound(s) in the development of tan spot disease. Partial purification of the toxic compound(s) indicated that it is produced only by necrosis-inducing isolates of the fungus and is active only

against those wheat cultivars which respond to infection with tan necrosis. It is not produced by non-necrosis-inducing isolates of the fungus, nor is it active against those cultivars which respond to infection with chlorosis only (Lamari and Bernier, 1989c).

A necrosis-inducing toxin(s) from *P. tritici-repentis* culture filtrates was purified by two groups (Ballance *et al.,* 1989; Tomas *et al.,* 1990) employing slightly different procedures. Although both reports described a polypeptide of similar molecular weight, amino acid composition and sensitivity to reduction, discrepancies regarding heat stability and specific activity of the toxin exist. Ballance *et al.* (1989) reported that the toxin was a heat-labile protein (designated Ptr necrosis toxin) with an average minimum active concentration of 0.2 nM. Tomas *et al.* (1990) described the toxin as a heat-stable protein (designated Ptr toxin) with an average minimum active concentration of 90 nM. The difference in reported specific activity could be due to differential reactions by the different wheat cultivars used. Differences in specific activity, and heat lability suggest that multiple toxins or multiple forms of the toxin may be produced by *P. tritici-repentis.* Although conventional genetic analyses with *P. tritici-repentis* are difficult because the fungus is homothallic (Hosford, 1971), biochemical evidence regarding the proteinaceous nature of the major necrosis-inducing toxin produced by this fungus provides strong indications that it is the product of a single gene.

Ptr-Chlorosis Toxin(s)

Although isolates of race 1 and 3 have been reported to induce extensive chlorosis in particular wheat lines, a constituent from cell-free culture filtrates capable of inducing similar chlorosis has never been identified (Orolaza *et al.*, 1995). A cultivar-specific

chlorosis toxin, however, was identified from race 5 isolates in culture and in *planta* (Orolaza *et al.*, 1995). This toxin was described as being between 3.5 and 10 kD and heat labile. Another chlorosis-inducing compound from cultures *of P. tritici-repentis* has been reported (Brown and Hunger, 1993). Based on its predicted molecular weight of between 800 and 1,800 D and its lack of host specificity (it affects barley) it was presumed to be a different molecule (Orolaza *et al.*, 1995).

Genetics of Resistance to Tan Spot

Genetic resistance to tan spot is a highly desirable method of control of tan spot that would be both environmentally sound and economically practical. Identification of the number of loci conditioning resistance and their dominance relationships and linkages would be the first step in developing a successful breeding strategy. Determining resistance and susceptibility relationships of wheat to isolates of *P. tritici-repentis*, however, is not a simple task given that there are possibly several HSTs produced by this fungus, and the genetics of sensitivity to these toxins is conditioned by different loci which express both alleles either dominant or recessive for toxin sensitivity (Lamari and Bernier, 1991, see below). The genetic data for at least the necrosis-inducing toxin produced by *P. tritici-repentis*, however, like other HSTs, strongly indicates its role in pathogenicity. Segregation analysis of progeny from crosses between cultivars resistant and susceptible to tan necrosis indicated qualitative inheritance of a single dominant gene conferring sensitivity to the toxin and susceptibility to toxin-producing isolates of the fungus (Lamari and Bernier, 1989c; Lamari and Bernier, 1991; Sykes and Bernier, 1991). Resistance to infection by *P. tritici-repentis* has also been described as polygenic

(Cantrell *et al.*, 1985; Elias *et al.*, 1989), however, it is not known what toxins, if any, were produced by the isolates tested. The single nuclear recessive gene, designated as *tsn1*, conferring insensitivity to the necrosis toxin and resistance to necrosis-inducing isolates of the fungus was mapped to the long arm of wheat chromosome 5B by RFLP and aneuploid analysis (Faris *et al.*, 1996). Another group independently confirmed this finding by substitution and F2 monosomic analyses (Stock *et al.*, 1996).

Different genes apparently condition the reaction of wheat lines to both chlorosis–inducing isolates of races 1 and 3 (Lamari and Bernier, 1991) and chlorosis-inducing isolates of race 5 (Orolaza *et al.*, 1995). While susceptibility to fungal races 1 and 3 and sensitivity to the chlorosis-inducing toxin made by these races was attributed to a single recessive gene (Lamari and Bernier, 1991), susceptibility to race 5 and sensitivity to its chlorosis-inducing toxin was suggested to be conditioned by a single dominant gene (Orolaza *et al.*, 1995).

The fact that a single gene conditions sensitivity of wheat to the *P. tritici-repentis* necrosis toxin provides the basis for our hypothesis that there is a specific receptor for this HST in sensitive wheat cells, the product of the gene conditioning sensitivity. Given that this HST is a protein, and very likely too large to enter the wheat cell unprocessed, the likely location of this putative receptor is on the wheat cell surface. Specific binding to a receptor is also indicated by the dominant nature of sensitivity to the necrosis toxin, implying toxin recognition is an active function. These assumptions, if correct, should facilitate future studies to identify the site and mode of action of this toxin.

The objectives of this work were to 1) biochemically, immunologically and molecularly characterize the major toxin produced by *P. tritici-repentis*; 2) determine the

role of this HST in tan spot of wheat; and 3) produce biologically active derivatives of the toxin protein which could be used to study the site and mode of action of the toxin. The approach that was taken was to: 1) purify the toxin protein; 2) perform immunological analyses of toxic protein fractions from culture filtrates; 3) clone and sequence the gene encoding the toxin; 4) transform the toxin gene into a non-toxin producing, nonpathogenic isolate of the fungus and assess the effect on pathogenicity; and 5) express the toxin gene in *E. coli* as a fusion protein. The work describing these objectives are presented in this dissertation.

Chapter 2

Purification and Immunological Characterization of Toxic Components from Cultures of *Pyrenophora tritici-repentis*.

Robert P. Tuori, Thomas J. Wolpert and Lynda M. Ciuffetti

Published in *Molecular Plant-Microbe Interactions*
January, 1995

Abstract

To facilitate the genetical analysis of pathogenicity in the *Pyrenophora tritici-repentis*/wheat interaction, a host-selective toxin (HST) protein, Ptr ToxA, was purified from culture filtrates of this fungus. Ptr ToxA was shown to be a 13.2-kD heat-stable protein which induces visible necrosis on sensitive wheat cultivars at an average minimum concentration of 60 nM. Polyclonal antibodies raised against Ptr ToxA were shown by western analysis and indirect immunoprecipitation to be specific for this protein. Bioassays of immunoprecipitated protein and Ptr ToxA protein eluted from polyacrylamide gels indicated that the Ptr ToxA protein is the toxic agent. Other less abundant necrosis-inducing components that are chromatographically and immunologically distinct from Ptr ToxA were also detected in culture filtrates of *P. tritici-repentis*. These components were found in cationic and anionic protein fractions, and like Ptr ToxA, induced cultivar-specific necrosis.

Introduction

Pyrenophora tritici-repentis (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoem.) is the causal agent of tan spot of wheat (*Triticum aestivum* L.), an economically significant disease that has been reported worldwide (Hosford, 1982). Certain isolates of *P. tritici-repentis* were shown to produce in culture a cultivar-specific toxic compound or compounds that induced typical tan spot necrosis upon infiltration into susceptible wheat cultivars (Tomas and Bockus, 1987). Host sensitivity to the toxic compound or compounds was shown to correlate strongly with susceptibility to the disease (Tomas and Bockus, 1987; Lamari and Bernier, 1989c), suggesting an involvement of the toxin in pathogenesis.

The toxic compound(s) from *P. tritici-repentis* culture filtrates has been purified and partially characterized by two groups employing slightly different procedures. Both groups concluded that the toxin was a low-molecular weight protein (Ballance *et al.*, 1989; Tomas *et al.*, 1990). Ballance *et al.*, (1989) reported that the toxin was a heat-labile protein of M_r 13,900 (designated Ptr necrosis toxin) with an average minimum active concentration of 0.2 nM. Tomas *et al.*, (1990) described the toxin as a heat-stable protein of M_r 14,700 (designated Ptr toxin) with an average minimum active concentration of 90 nM. Comparison of these proteins showed only minor differences in amino acid content. Differences in molecular weight, specific activity, and heat lability suggest that multiple toxins may be produced by *P. tritici-repentis*.

This work describes the purification and immunological characterization of the major toxin, which we have designated Ptr ToxA, from cultures of *P. tritici-repentis*. To determine whether multiple toxins are produced by *P. tritici-repentis*, we developed

polyclonal antibodies against the Ptr ToxA protein and used them to perform an immunological analysis of toxic components from culture filtrates of *P. tritici-repentis*. Characterization of the Ptr ToxA protein and other toxins will facilitate the molecular genetical analysis of the corresponding toxin gene or genes.

Methods

Fungal cultures and plant material

Isolates of *P. tritici-repentis* were obtained from Dr. William Bockus, Kansas State University. Isolate Pt-1C exhibited high toxin production as determined by symptom expression on susceptible wheat cultivars. Single conidia of this isolate were inoculated onto the susceptible host, and mycelium from lesions was cultured in modified Fries medium as described by Tomas and Bockus (1987). Filtrates from these cultures were tested for toxic activity by leaf infiltration assays. A fast growing subculture of Pt-1C with a distinctive pink coloration was named Pt-1C-BFP. Conidia of Pt-1C-BFP were isolated as described by Hunger and Brown (1987) and used as an inoculum source for liquid cultures and toxin purification. Six-ml of a conidial suspension of approximately 3 X 10^4 conidia per ml were inoculated into 500 ml flasks that contained 100 ml of modified Fries medium and incubated in standing cultures for 17-21 days at 25°C under constant fluorescent light. Several other isolates of *P. tritici-repentis* were obtained from Dr. Gary Buchenau, South Dakota State University, and were designated by us as isolates SD-1 through SD-21.

Wheat plants used for bioassay were grown at 25oC with a 16-hr photoperiod. Plants were grown to the four-leaf stage, and samples were infiltrated into the third or

fourth leaves with a Hagborg device (Hagborg, 1970). Toxin activity was assayed in the wheat cultivars TAM-105 (susceptible) and Auburn (resistant). Plants were maintained at the temperature and photoperiod previously described until necrosis developed (3-5 days). Necrosis-inducing toxic activity from anionic protein fractions was detected by infiltration into the wheat cultivars Norkan (obtained from Dr. Warren Kronstad, Oregon State University) Auburn and Arkan (obtained from Dr. Greg Shaner, Purdue University) as well as TAM-105 (obtained from Dave Marshall, Texas A & M University).

Protein purification

Toxin was purified by a modification of the procedures established by Ballance *et al.* (1989) and Tomas *et al.* (1990). CCF was filtered through four layers of cheese-cloth, and proteins were precipitated from the filtrate with ammonium sulfate at 75 % saturation at 4° C overnight. Proteins were pelleted by centrifugation for 20 min at 8,000 X g and the supernatant was centrifuged again to further recover proteins. The pellets were combined, resuspended in water, centrifuged at 10,000 X g for 20 min to remove debris, and the supernatant was applied to a 100 cm X 2.5 cm gel filtration column of Sephadex G-50 (Sigma). The column was equilibrated and eluted with 10 mM sodium acetate, 100 mM sodium chloride, pH 4.8 at 0.5 ml/min and 6-ml fractions were collected every 12-min over 16-h. Fractions were assayed for toxic activity and active fractions were combined and concentrated approximately 20-fold by rotoevaporation at 40° C. Concentrated material was desalted on a disposable PD-10 Sephadex G-25 desalting column (Pharmacia) equilibrated with 10 mM sodium acetate, pH 4.8 (buffer A).

Proteins were separated by FPLC on a Mono-S cation-exchange column (Pharmacia) equilibrated in buffer A. The sample was applied to the column by pumping

at 1.0 ml/min in buffer A. Proteins were eluted from the column at 0.5 ml/min with a 60-min linear gradient of 0 to 300 mM sodium chloride in 10 mM sodium acetate, pH 4.8. One-ml fractions were collected, assayed for toxic activity *in planta*, and analyzed by gel electrophoresis. The total yield of purified protein was determined by a modification of the Folin Assay (Peterson, 1977).

Anionic proteins (those which were not retained on the Mono-S column and eluted in the void volume, designated Mono-S flow-through) were separated on a Mono-Q anion-exchange column (Pharmacia) equilibrated in 30 mM Tris, pH 9.0. The Mono-S flow-through was concentrated ten-fold by rotoevaporation at 40° C, dialyzed against 30 mM Tris, pH 9.0, in a 3500 MW cut-off membrane and applied to the Mono-Q column at 1.0 ml/min. Proteins were eluted from the column at a flow rate of 0.5 ml/min with a 30-min linear gradient of 0 to 1 M sodium chloride in 30 mM Tris, pH 9.0. One-ml fractions were collected and assayed for toxic activity *in planta.*

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a vertical mini-gel apparatus (C.B.S. Scientific Co., Dual Vertical Minigel Unit). Proteins were resolved with a 17% resolving gel and 5% stacking gel in the buffer system described by Laemmli (1970). Gels were fixed for 1 hr in an aqueous solution containing 7% glacial acetic acid in 40% methanol (v/v) and stained with Brilliant Blue G-Colloidal Concentrate (Sigma). Nondenaturing gel electrophoresis was performed with the low pH discontinuous buffer system described by Hames (1981) with a 10% resolving gel and 4.6% stacking gel. Samples were loaded on the gel at the anode and electrophoresed toward the cathode for 3.5 hr at a constant current of 30 mAmps. Native gels were silver-

stained as described by Heukshoren and Dernick (1985). Ptr ToxA protein was extracted from SDS-gels following electrophoresis according to the protocol described by Tomas *et al.* (1990), and extracted protein was assayed by infiltration *in planta.*

Pronase treatment of Ptr ToxA

Purified Ptr ToxA protein (20 µg) was treated with Pronase E (Sigma), 1µg/µl, in 10 mM Tris, 10 mM EDTA, and 0.5% SDS at 37° C for 18 hr. After the Pronase treatment, 5µg was infiltrated into TAM-105 and Auburn leaves after being diluted six-fold in distilled water so that the final SDS concentration was 0.08% (150 µl final volume). The other 10 µg was analyzed by SDS-PAGE.

Heat stability of toxic components

The heat stability of Ptr ToxA was determined by incubating 1 µg samples diluted to 100 µl with distilled water for 10 min at 80° C or 100° C. The heat stability of cationic toxic activity fractions was also assessed by incubating undiluted samples for 10 min at 80° C or 100° C. The samples were cooled on ice and infiltrated into TAM-105 and Auburn leaves.

Western blotting

Western blotting of polyacrylamide gels and immunological detection of proteins was performed by a modification of the protocol described by Wolpert and Macko (1991). SDS-gels were immersed for 10 min in 25 mM Tris, 192 mM glycine and 20% methanol (v/v) (transfer buffer), and proteins were transferred to a nitrocellulose membrane (Millipore) at 1.0 mAmp/cm^2 of membrane for 1 hr with an electroblotting apparatus (Transblot, Bio-Rad). After transfer, blots were equilibrated for 1 hr in 10 mM

Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20 (TBST) and 3% nonfat dried milk. Blots were washed three times for 5 min each in TBST to remove nonfat dried milk, and incubated for 1 hr with polyclonal antisera diluted 1/4000 in TBST. Blots were washed three times for 5 min each in TBST and then incubated with alkaline phosphatase conjugated anti-mouse IgG (Sigma) at a 1/4000 dilution in TBST. Blots were again washed, and the color was developed as described by Wolpert and Macko (1991).

Slot blots were prepared with a Bio-Rad Bio-Dot SF apparatus as described by the manufacturer with Immobilon-P membranes (Millipore). Samples were adjusted to 20% methanol prior to loading in wells. Proteins were blotted and membranes were incubated in blocking solution and subsequently treated the same as western blots.

Indirect immunoprecipitations

Indirect immunoprecipitations were performed by mixing 400 µl of crude toxin preparation (concentrated and desalted active fractions from the Sephadex G-50 gel filtration column) containing approximately 200 µg of total protein with 1.6 ml of TBST and 12 µl of polyclonal antisera. Samples were vortexed and incubated for 16 h at 4° C. The following day samples were centrifuged in a microcentrifuge for 5 min and the supernatant was transferred to a fresh tube; 120 µl of a 1:1 suspension (w/v) of protein-A-agarose (Sigma) was added to each sample and the samples were incubated with shaking for 2 hr at room temperature. The protein-A-agarose had previously been washed and resuspended in 190 mM NaCl, 60 mM Tris-HCl (pH 7.4) and 6 mM EDTA (wash buffer) with 2.5% Triton X-100. Protein-A-agarose beads were pelleted in a microcentrifuge for 15 sec and the supernatant was removed and discarded. Pellets were washed five times with wash buffer (no detergent). For SDS-PAGE analysis, the final protein-A-agarose

pellet was resuspended in 62.5 mM Tris, 5% 2-mercaptoethanol (v/v), and 2.3% SDS (w/v), pH 6.8 (SDS loading buffer), boiled for 5 min, centrifuged and the supernatant was loaded onto a gel. For *in planta* bioassays, the final protein-A-agarose pellet was resuspended in 200 µl of water, boiled for 5 min, centrifuged, and the supernatant was infiltrated into both TAM-105 and Auburn leaves. Control immunoprecipitations with antisera from pre-immunized mice were conducted and analyzed identically.

Antibody production

Polyclonal antibodies were raised against purified Ptr ToxA in mice at the Monoclonal Antibody Facility of the Center for Gene Research and Biotechnology at Oregon State University. Mice were injected with 20 µg of Ptr ToxA protein and given two booster injections of the same amount of protein at 1-week intervals.

Amino acid analysis

The amino acid composition of purified Ptr ToxA was determined with a Beckman 126AA System Gold HPLC Amino Acid Analyzer at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University. Samples were hydrolyzed at 110° C for 20 hr in 6 N HCl containing 1% phenol. Ninhydrin derivatized amino acids were detected after separation on a Beckman Spherogel ion-exchange column.

Mass spectrometry

The molecular weight of purified Ptr ToxA was determined by mass spectrometry at the Mass Spectrometry Lab at Oregon State University. A custom-built time-of-flight mass spectrometer equipped with a frequency-tripled (355 nm) Nd:YAG laser was used

for positive ion MALDI mass spectrometric analyses (Jensen *et al.,* 1993). The time-of-flight spectra consisted of the summed data generated from fifty consecutive laser pulses. The data was analyzed with M over Z software provided by Dr. Ronald C. Beavis, Memorial University of Newfoundland. Samples were prepared by mixing 1 µl of protein solution into 2 µl of matrix solution (a 10 g/L solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid/acetonitrile (2:1)). A 1 µl sample of this mixture was applied to the mass spectrometer probe and dried under a stream of air. The sample was then rinsed with cold (4° C) water, dried, and inserted into the mass spectrometer. Equine cytochrome *c* (molecular weight 12,360) was used as an internal mass calibrant.

Results

Ptr ToxA purification

Gel permeation chromatography separated toxic agents from the majority of colored material and larger proteins found in crude culture filtrate (CCF) *of P. tritici-repentis*. Activity was typically found within the range of fractions 35 through 60, and did not correspond to a distinct UV absorbing peak. Further purification by cation-exchange fast protein liquid chromatography (FPLC) (Fig. 2.1A) resulted in the consistent isolation of a major peak (Ptr ToxA) which corresponded to a protein band with a molecular weight of approximately 14 kDa as determined by SDS-PAGE (Fig. 2.1B lane 2). Analysis of purified Ptr ToxA by mass spectroscopy indicated a molecular weight of 13.2 kDa. Nondenaturing-gel electrophoresis (Fig. 2.1C) further demonstrated the purity of the Ptr ToxA protein. No protein other than the 13.2 kD band was detected

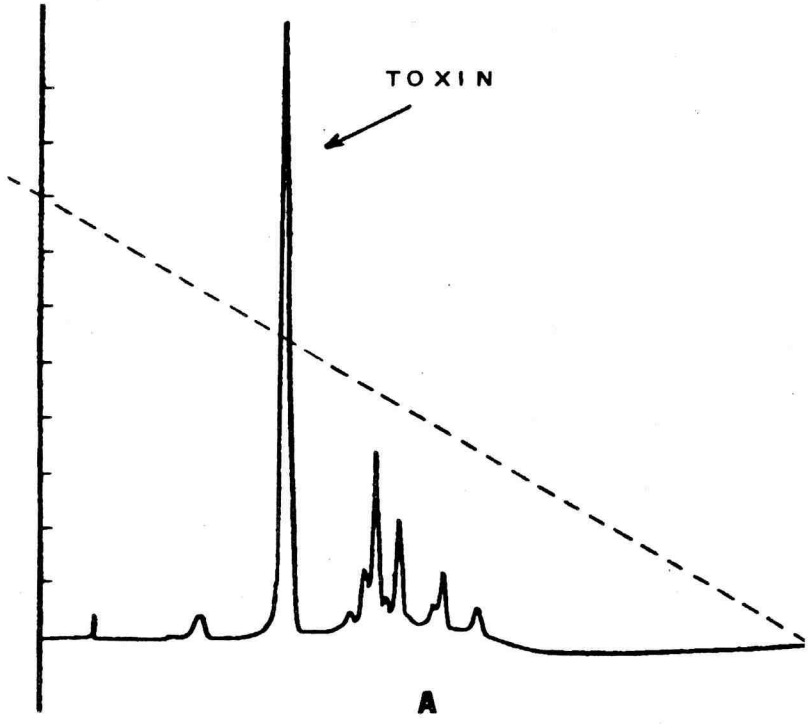
Figure 2.1 Chromatographic and Electrophoretic Analysis of Purified Ptr ToxA.

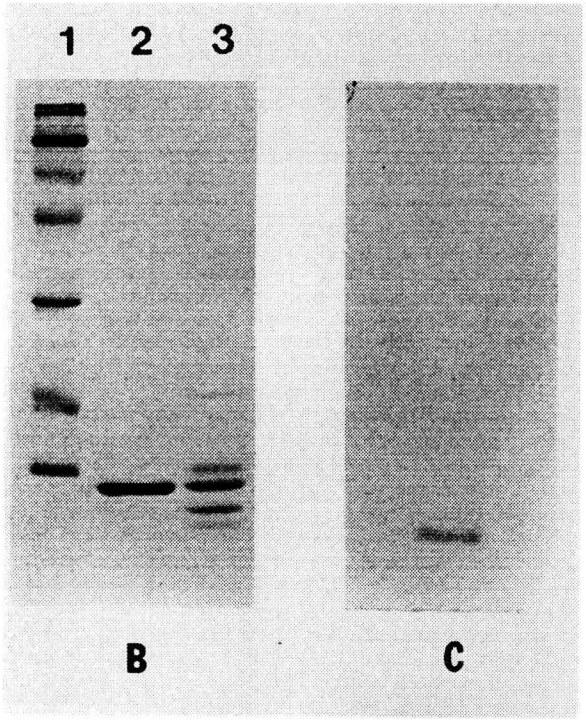
(A) Mono-S cation-exchange chromatography. Dashed line indicates increasing salt gradient (right to left) from 10 mM sodium acetate, pH 4.8 to 10 mM sodium acetate, 300 mM sodium chloride, pH 4.8 (Buffer B) over 60 min.

(B) Brilliant Blue G-Colloidal stained 14% SDS-polyacrylamide gel. Molecular weight markers of 14.3, 21.5, 30, 46, 69, 97.4, and 200 kD, respectively, from bottom to top (Lane 1). Purified Ptr ToxA (ca. 5 μg) from Mono-S cation-exchange chromatography (Lane 2). Concentrated active fractions from Sephadex G-50 column chromatography (Lane 3).

(C) Brilliant Blue G-Colloidal stained 10% native gel showing 5 μg purified Ptr ToxA.

Figure 2.1





when as much as 20 µg of purified Ptr ToxA was run on SDS-gels and stained with Brilliant Blue G-Colloidal stain. The 13.2 kD band was established as the toxic agent by bioassay of protein bands eluted from polyacrylamide-gel slices, and by bioassay of indirect immunoprecipitation products (see below). The amino acid composition of Ptr ToxA was determined (Table 2.1) and found to be similar to both previously published results (Tomas *et al.*, 1990; and, Ballance *et al.*, 1989).

This purification procedure resulted in the recovery of approximately 6.7 µg of Ptr ToxA per ml of CCF. The average minimum concentration of Ptr ToxA required to induce a visible tan necrosis in susceptible wheat plants was determined to be 80 ng per 100 µl infiltration (60 nM). Minimum active concentrations ranged from 50 ng per 100 µl infiltration (38 nM) to 120 ng per 100 µl infiltration (91 nM).

Heat and pronase treatment of Ptr ToxA

Treatment of Ptr ToxA with Pronase-E resulted in the loss of the 13.2 kD band when analyzed by SDS-PAGE and loss of all necrosis-inducing activity when the sample was infiltrated into the susceptible wheat cultivar. Infiltrations of buffer or Pronase-E alone did not result in damage to leaves, and Ptr ToxA incubated without Pronase retained activity. Ptr ToxA samples heated for 10 min at 80° C or 100° C retained activity.

Table 2.1 Mole Percentage of Detectable Amino Acid Composition of Toxins.

Amino acid	ToxA[a]	Ptr toxin[b]	Ptr necrosis toxin[c]
Asp/Asn	17.7	18.7	17.2
Thr	7.7	7.0	7.9
Ser	6.3	10.7	7.3
Glu/Gln	7.9	9.2	8.1
Pro	5.3	8.4	3.0
Gly	11.0	10.5	11.2
Ala	3.2	4.3	3.1
Val	6.4	5.5	7.2
Met	1.6	0.7	1.3
Ile	9.8	4.3	10.3
Leu	6.9	4.9	7.0
Tyr	2.2	2.4	2.0
Phe	2.8	2.6	2.8
His	0.3	0.7	0.0
Lys	1.0	1.1	0.5
Arg	9.7	9.2	11.0

[a] Based on two independent determinations.

[b] Data obtained from Tomas *et al.* (1990).

[c] Data obtained from Ballance *et al.* (1989) has been converted to mole percentage of detectable amino acid. Values were calculated from nmole amounts of each amino acid detected divided by the sum total nmoles of all detectable amino acids.

Although the tryptophan and cysteine content of proteins can be calculated by other methods, as was done for both Ptr toxin and Ptr necrosis toxin, they are not detectable by standard amino acid analysis. These data were not determined for Tox-A, and thus, these values were not included for Ptr toxin and Ptr necrosis toxin.

Immunological characterization

Western analysis indicated that polyclonal antibodies raised in mice against Ptr ToxA reacted with and are specific to the 13.2 kD band associated with toxin activity (Fig. 2.2). No other protein bands were detected with the anti-Ptr ToxA polyclonal antibodies.

Indirect immunoprecipitations of crude toxin preparations confirmed the specificity of the antibodies for the 13.2 kD Ptr ToxA band and indicated that the 13.2 kD protein was the major toxic agent (Fig. 2.3 and 2.4). Electrophoretic analysis of immunoprecipitated products revealed a unique 13.2 kD protein band that co-migrated with purified Ptr ToxA on polyacrylamide gels present only when anti-Ptr ToxA antisera were used for the reaction (Fig. 2.3). Bioassay of the immunoprecipitated 13.2 kD band indicated specific necrosis-inducing activity when infiltrated into the susceptible wheat cultivar (Fig. 2.4). Infiltration of anti-Ptr ToxA antiserum (boiled and unboiled) did not elicit a response. The 13.2 kD toxin band did not react with control serum from preinoculated mice as determined by electrophoresis (Fig. 2.3) or bioassay (Fig. 2.4).

Other toxic components

Necrosis-inducing activity, distinct from the 13.2 kD Ptr ToxA band, was often detected when protein fractions from the Mono-S column were assayed by leaf infiltration. These components eluted in two fractions during chromatography: (1) in the flow-through, i.e., material which did not bind to the cation exchange resin (anionic toxic component, or AI); and (2) immediately following elution of the Ptr ToxA protein

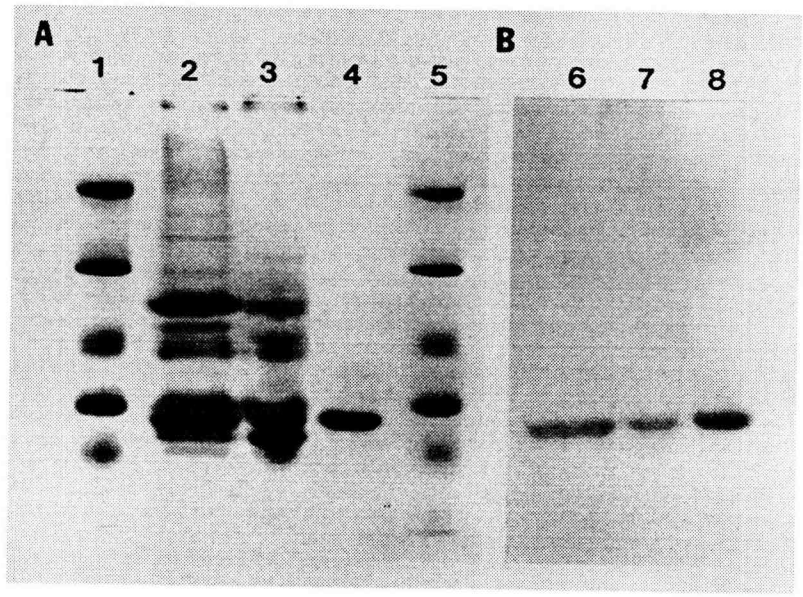


Figure 2.2 Western Analysis of Protein Purification Fractions.
(A) Brilliant Blue G-Colloidal stained 14% SDS-polyacrylamide gel.
(B) Western analysis. Molecular weight markers of 6.5, 14.3, 21.5, 30, and 46 kD, respectively, from bottom to top (Lanes 1 and 5). Concentrated crude culture filtrate of *Pyrenophora tritici-repentis* (Lanes 2 and 6). Concentrated active fractions from Sephadex G-50 column chromatography (Lanes 3 and 7). Ca. 10 µg purified Ptr ToxA from Mono-S cation-exchange chromatography (Lanes 4 and 8). Western analysis (Panel B) shows polyclonal antibodies specific for Ptr ToxA (lane 6, 7, and 8).

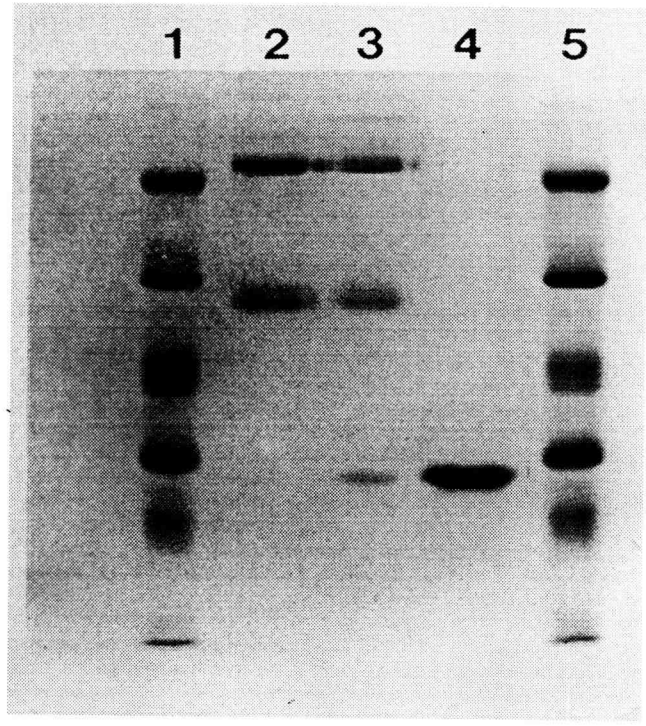


Figure 2.3 SDS-PAGE Analysis of Indirect Immunoprecipitation Products. Molecular weight markers of 6.5, 14.3, 21.5, 30, and 46 kD, respectively, from bottom to top (Lanes 1 and 5). Brilliant Blue G-colloidal stained 14% SDS-polyacrylamide gel of immunoprecipitation products from crude toxic fractions (Sephadex G-50 column enriched toxin preparations) (Lanes 2 and 3). Result of immunoprecipitation with normal (control, preimmunized) mouse serum (Lane 2). Result of immunoprecipitation of sample with polyclonal antibodies from mouse immunized with Ptr ToxA (Lane 3). Lane 4 shows ca. 10 μg purified Ptr ToxA.

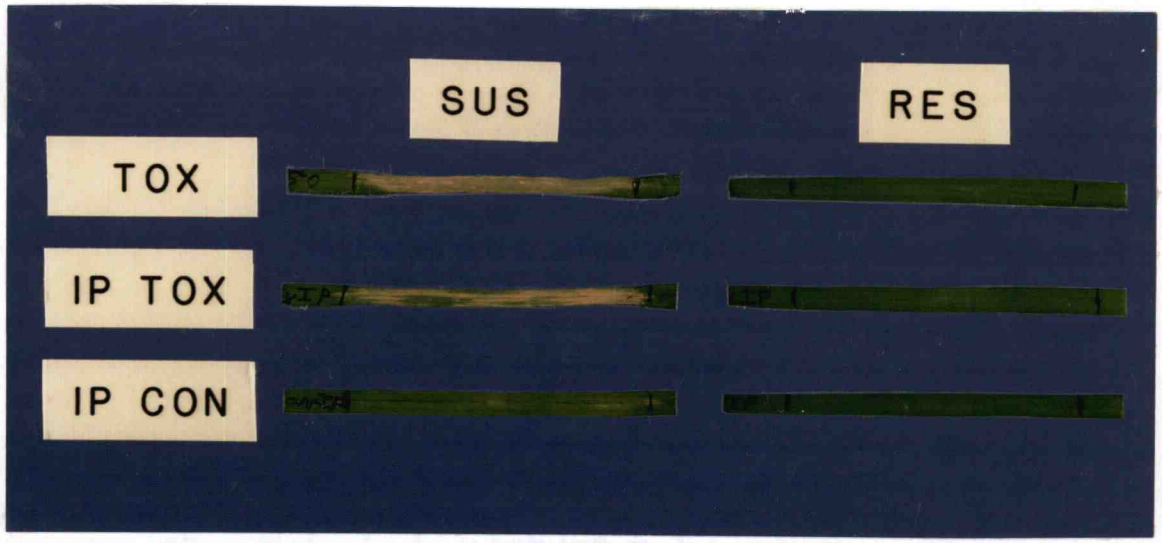


Figure 2.4 Leaf Infiltration Bioassay of Indirect Immunoprecipitation Products. Susceptible (TAM-105) and resistant (Auburn) wheat cultivars were infiltrated with purified Ptr ToxA (TOX), immunoprecipitated products obtained from anti-Ptr ToxA polyclonal antibodies (IP-TOX), or immunoprecipitated products obtained from control-mouse antisera (IP-CON). Typical toxin-induced tan-necrosis is shown on susceptible wheat cultivar by both the purified Ptr ToxA and the protein recognized by the anti-Ptr ToxA polyclonal antibodies. No necrosis is present on the resistant cultivar or on either wheat genotypes when immunoprecipitated products obtained from control-mouse antisera was used for the infiltration bioassay.

(cationic toxic component, or CI). Both cationic and anionic toxic components were present in relatively small quantities in comparison to the amount Ptr ToxA activity observed.

The CI fractions eluted in the Mono-S gradient within two to six fractions after the elution of Ptr ToxA and, thus, are chromatographically distinct from Ptr ToxA fractions. However, because of the similar retention times of CI and Ptr ToxA fractions, the CI fractions often contained detectable amounts of Ptr ToxA. Therefore, we determined whether the quantity of contaminating Ptr ToxA detectable by electrophoretic or immunological analysis could account for the biological activity in these fractions. Three representative CI fractions were analyzed biologically, electrophoretically, and immunologically (Fig 2.5). The three fractions analyzed were obtained from two different isolates (CI-1 and CI-2 from Pt-1C-BFP, and CI-3 from SD-21) and, thus, indicate that the CI fractions were not restricted to a particular fungal isolate. Similar necrosis-inducing CI fractions were also obtained from several other isolates. It is not known whether the necrosis-inducing activity of the different CI fractions is due to the same toxic component or to different components within these fractions. All CI fractions tested displayed cultivar-specific activity in TAM-105, and all were inactive when infiltrated into Auburn. SDS-PAGE of CI fractions revealed a predominant band migrating near Ptr ToxA and often with smaller bands running immediately below. Multiple bands were clearly resolved by native-PAGE (Fig. 2.5).

Three representative CI fractions and a range of Ptr ToxA standards were analyzed by leaf infiltration bioassay (Fig. 2.5A). The same quantity of CI-1 infiltrated (50 µl), was also run on native gels (Fig. 2.5B), and immunoassayed by slot blot analysis

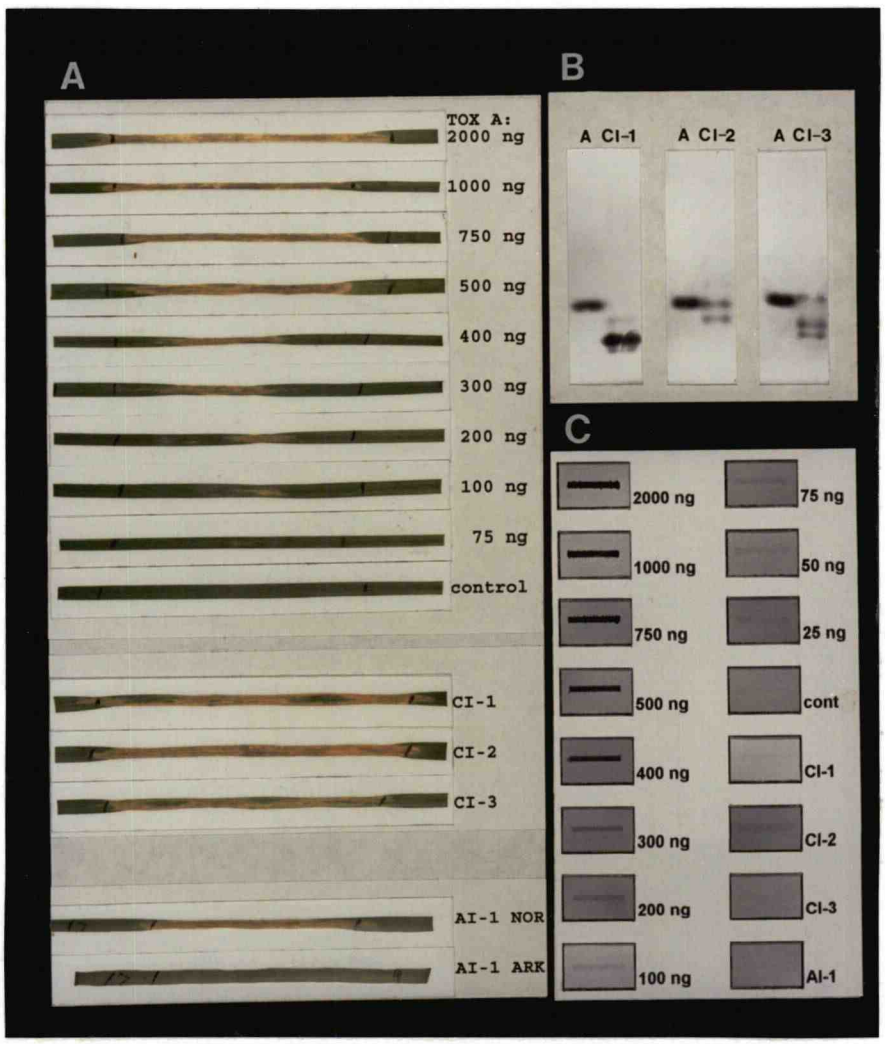
Figure 2.5 Biological, Electrophoretic, and Immunological Analyses of Cationic and Anionic Toxic Components.

(A) Leaf infiltration bioassay. Susceptible (TAM-105) wheat infiltrated with a range of purified Ptr ToxA standards, from 75 ng to 2000 ng, and, for comparison of activity levels, with 50 µl each of three representative CI fractions (CI-1, CI-2, and CI-3). Wheat genotypes Norkan and Arkan infiltrated with 50 µl of a representative AI fraction (AI-1). All infiltrations were brought up to a final volume of 100 µl with distilled water.

(B) Silver stained native gel with 500 ng of Ptr ToxA standard run next to 50 µl each of CI-1, CI-2 and CI-3.

(C) Slot blot immunological assay of a range of Ptr ToxA standards, from 50 ng to 2000 ng, and 50 µl each of CI-1, CI-2, CI-3 and AI-1. Control (cont) is 10 mM Na Acetate, pH 4.8 buffer.

Figure 2.5


A
TOX A:
2000 ng
1000 ng
750 ng
500 ng
400 ng
300 ng
200 ng
100 ng
75 ng
control
CI-1
CI-2
CI-3
AI-1 NOR
AI-1 ARK
B
A CI-1
A CI-2
A CI-3
C
2000 ng
1000 ng
750 ng
500 ng
400 ng
300 ng
200 ng
100 ng
75 ng
50 ng
25 ng
cont
CI-1
CI-2
CI-3
AI-1

(Fig. 2.5C). This process was repeated exactly for CI-2 and CI-3. Fraction CI-1 induced necrosis equivalent to a minimum of 500 ng Ptr ToxA (Fig 2.5A), yet no Ptr ToxA was detected by native gel electrophoresis (Fig 2.5B) or slot blot analysis (Fig 2.5C). Fraction CI-2 induced necrosis equivalent to a minimum of 1 μg of Ptr ToxA (Ptr ToxA amounts of 1 μg or greater induced complete necrosis of the infiltrated area), yet far less than 500-ng was detected by electrophoretic analysis and only approximately 100 ng was detected by slot blot analysis. Fraction CI-3 also induced necrosis equivalent to 1 μg or more of Ptr ToxA, yet slot blot analysis detected levels of Ptr ToxA no higher than that of the buffer control. Electrophoretic analysis of fraction CI-3 revealed a protein migrating slightly slower than Ptr ToxA, as well as two faster-migrating protein bands. The slot blot immunoassay was repeated three times with similar results. The results shown in Figure 2.5C are all from the same slot blot. When CI fractions were tested for heat stability by incubating for 10 min at either 80° C or 100° C, most necrosis-inducing activity was lost, whereas Ptr ToxA activity was stable under these conditions.

Chromatography of the AI fractions on a Mono-Q anion-exchange column yielded protein fractions that eluted during the gradient and induced cultivar-specific necrosis in the wheat cultivar Norkan. Ptr ToxA, an apparently cationic protein, does not bind to the Mono-Q column. AI fractions, such as the representative AI-1, consistently induced necrosis in cultivar Norkan (Fig. 2.5A) but not in the cultivars Auburn or Arkan (Fig. 2.5A). Inconsistent necrosis-inducing activity was observed when AI-1 was infiltrated into TAM-105. Fraction AI-1 could not be analyzed by the native gel system used for CI fractions because it contained anionic proteins. Fraction AI-1 showed no detectable reaction with the anti-Ptr ToxA polyclonal antibody when analyzed by slot

blot (Fig 2.5C). The necrosis induced by AI fractions showed a distinctive progression of symptoms. AI-induced lesions originally appeared chlorotic instead of the gray, collapsed appearance initially seen with Ptr ToxA infiltrations. Within 5-7 days, however, necrosis with pink to red margins was visible in most reactions.

Discussion

The purification procedure reported here resulted in ca. 6.7 µg purified Ptr ToxA protein per ml of CCF, with an average minimum active concentration of 0.8 µg/ml (60 nM). This concentration for activity is far higher (300-fold) than that obtained for Ptr-necrosis toxin (Ballance *et al.,* 1989), yet comparable to that obtained for Ptr-toxin (Tomas *et al.,* 1990).

We were unable to obtain sufficient quantities of purified toxin by the procedure of Tomas *et al.* (1990) or Ballance *et al.* (1989). With the protocol of Ballance *et al.* (1989), toxic compounds eluted during the gradient as reported. However, in two of the four attempts, toxic activity was also detected in fractions that eluted prior to the salt gradient. This activity of compounds eluting prior to the gradient could be the same as the anionic toxic components described here. In all four instances, the gradient fractions with necrosis-inducing activity contained the Ptr ToxA band as well as other contaminating protein bands when analyzed by SDS-PAGE.

The heat stability of Ptr ToxA is similar to that determined for Ptr-toxin (Tomas *et al.,* 1988), yet contrasts with the heat lability of Ptr-necrosis toxin (Ballance *et al.,* 1990), which lost most activity after treatment for 30 min at 70° C. This indicates that Ptr ToxA may be similar to or the same compound as Ptr-toxin. The loss of most activity of

cationic toxic components following a 10 min treatment at 80° C or 100° C indicates that these fractions represent a different toxin than Ptr ToxA, and suggests that the cationic activity is caused by the same compound as Ptr-necrosis toxin.

Polyclonal antibody raised in mice against Ptr ToxA was useful in characterizing the toxic agents produced by *P. tritici-repentis* in several ways: (1) The anti-Ptr ToxA antibodies were specific for this protein among all extracellular proteins produced *by P. tritici-repentis* as determined by western blot analysis and indirect immunoprecipitations. Western blot analysis showed that, even in lanes overloaded with crude protein preparations, only the 13.2 kD band reacted with anti-Ptr ToxA antibodies (Fig. 2.2). When these crude protein preparations were again used for indirect immunoprecipitations, only the 13.2 kD Ptr ToxA band eluted from the polyclonal antibodies and was detected on SDS-polyacrylamide gels (Fig. 2.3). (2) The bioassay of the immunoprecipitated products (Fig. 2.4) demonstrated most convincingly the causal nature of the Ptr ToxA protein in necrosis. The immunoprecipitation results, demonstration of antibody specificity, results of the bioassay of protein eluted from slices of polyacrylamide-gels, and the Pronase-E sensitivity of Ptr ToxA provide strong evidence that the 13.2 kD protein itself is a toxic agent. (3) Other toxic components were produced by *P. tritici-repentis* that are immunologically and chromatographically distinct from the Ptr ToxA protein.

Toxic components were isolated that either did not bind to the Mono-S column under the conditions used (AI) or bound to the column but eluted later in the gradient than Ptr ToxA (CI-1,2 and 3). Although these fractions contained several proteins, crude preparations of both the cationic (CI) and anionic (AI) active fractions were obtained

with little or no Ptr ToxA contamination. The CI fractions were the most difficult to resolve free of Ptr ToxA contamination. However, the necrosis that the three CI fractions induced, clearly cannot be accounted for by the amount of Ptr ToxA detected electrophoretically and immunologically.

The proteins in AI fractions have distinctly different chromatographic and immunological properties from Ptr ToxA. Because of the initial chlorotic appearance of AI-induced lesions, the possibility that these fractions cause the chlorotic response described by Lamari and Bernier (1989a) was investigated by infiltration of these fractions into the wheat cultivars Norkan and Arkan. The wheat response of extensive chlorosis to infection by *P. tritici-repentis* has been determined to be genetically distinct from the development of necrosis (Lamari and Bernier, 1991). Both Norkan (nec^+chl^+) and Arkan (nec^-chl^+) are susceptible to infection and react with extensive chlorosis to certain pathotypes of *P. tritici-repentis*. Norkan also shows a necrosis response similar to TAM-105 (susceptible, nec^+chl^-). AI fractions obtained from all isolates consistently induced necrosis when infiltrated into the cultivar Norkan and did not induce necrosis or chlorosis in the cultivars Auburn or Arkan. The AI toxin, therefore, is not responsible for the chlorosis observed by Lamari and Bernier (1989a). The AI fractions from some isolates, however, produced an inconsistent necrotic response in TAM-105 plants. AI fractions obtained from isolate Pt-1C-BFP produced necrosis in TAM-105 in approximately 50% of the infiltrations, while fractions obtained from isolate SD-8 produced necrosis in TAM-105 in all infiltrations. Ptr ToxA infiltrations into TAM-105, above the minimum active concentration of 60 nM, always resulted in a visible necrosis. These results suggest: (1) there is more than one toxic component in the AI fractions; (2)

the production of the different toxic components is isolate dependent; (3) the different AI components interact specifically with different wheat genotypes that are present within TAM-105; and, (4) there are additional genes in wheat that condition a necrotic response to toxic components from *P. tritici-repentis*. Clearly, the AI toxin(s) is significantly different from both Ptr ToxA and the previously described toxins, Ptr-toxin (Tomas *et al.*, 1990) and Ptr-necrosis toxin (Ballance *et al.*, 1989). Thus, the AI toxin or toxins can be considered a novel cultivar-specific toxins produced by this fungus.

It is possible that the Ptr ToxA protein described here is distinct from both Ptr-toxin and Ptr-necrosis toxin. However, Ptr-toxin obtained as a gift from A. Tomas co-migrated on SDS polyacrylamide gels and reacted with anti-Ptr ToxA polyclonal antibodies on a western blot (data not shown). These data, along with the similar molar activities and heat stability, indicate that Ptr-toxin and Ptr ToxA are likely the same protein. Ptr-necrosis toxin and Ptr ToxA, however, are probably different proteins. Ptr ToxA appears distinct from Ptr-necrosis toxin in molecular weight as determined by mass spectrometry, heat stability, and molar activity. The cationic toxic component we observed was low molecular weight and heat-labile. Thus, the CI fractions could contain Ptr-necrosis toxin which may be produced at low levels by our isolate.

Chapter 3

Amino Acid Sequence Analysis, Construction of a Chimeric Full Length cDNA, Occurrence of the *ToxA* Gene in Isolates of *Pyrenophora tritici-repentis* and *P. teres*, and Secondary Structure Analyses of the Ptr ToxA Precursor Protein

Robert P. Tuori, Thomas J. Wolpert, and Lynda M. Ciuffetti

Abstract

In a previous report, we described the isolation of the gene that encodes the host-selective toxic protein, Ptr ToxA, produced by the fungus *Pyrenophora tritici-repentis*, and confirmed that this gene functions as a primary determinant of pathogenicity in the *Pyrenophora*-wheat interaction (Ciuffetti *et al.*, 1997, *The Plant Cell* 9: 135-144). This study describes supplementary work done concurrent with the prior report. Because Ptr ToxA is N-terminally blocked, internal peptide fragments of the protein were generated and the fragments were subjected to direct amino acid sequencing, resulting in the determination of approximately half the amino acid sequence of the toxin. Amino acid sequence information was also obtained from Ptr ToxA after treatment with the enzyme pyroglutamate amino peptidase, indicating the nature of the N-terminal blockage of this protein. A chimeric, full-length cDNA molecule was constructed that was useful for the heterologous over-expression of the toxin. The occurrence of the Ptr ToxA gene in several isolates of *Pyrenophora tritici-repentis* and *P. teres* was assessed through PCR analysis. Finally, secondary structure analyses of the precursor protein deduced from the *ToxA* open reading frame (ORF) indicate that the protoxin is composed of two distinct structural domains.

Introduction

The fungus *P. tritici-repentis* is the causal agent of tan spot of wheat, an economically significant disease that has been reported worldwide and may account for yield losses ranging from 2-40% (da Luz and Hosford, 1980; Shabeer *et al.*, 1991; Sykes and Bernier, 1991). Certain isolates of *P. tritici-repentis* have been shown to produce in culture a host-selective toxin (HST) that induces typical tan spot necrosis upon infiltration into tissue of susceptible wheat cultivars. Host sensitivity to the toxin is genetically dominant (Lamari and Bernier, 1989c) and is correlated with susceptibility to the pathogen (Tomas and Bockus, 1987; Lamari and Bernier, 1989b, 1989c), indicating a causal role of the toxin in pathogenesis. The toxin was purified and shown to be an approximately 13 kD protein (Ballance *et al.*, 1989; Tomas *et al.*, 1990; Tuori *et al.*, 1995).

To assess the role that the major necrosis-inducing toxin, designated Ptr ToxA, plays in the pathogenicity of *Pyrenophora tritici-repentis* on wheat, we previously described the molecular cloning of the gene encoding this protein and showed that it functions as a primary determinant of pathogenicity (Ciuffetti *et al.*, 1997). Poly(A)$^+$ RNA was prepared from mycelia of 6-, 7- and 10-day-old cultures and translated *in vitro* to confirm the integrity of the mRNA. In addition, to confirm the presence and to estimate the relative abundance of the toxin transcript, we performed indirect immunoprecipitation of total translation products with anti-Ptr ToxA antibody, demonstrating that *ToxA* is initially expressed as an approximately 19.5 kD precursor protein. Following confirmation of Ptr ToxA mRNA in total mRNA from 6- and 7-day-old cultures, a λgt11 cDNA library was prepared from this total mRNA fraction and screened with polyclonal anti-Ptr ToxA antibody. Antibody-positive recombinants were identified at a high frequency. The cDNA

clone was used as a probe to isolate a genomic copy of the *ToxA* gene with its endogenous promoter. A tox$^-$, hygromycin B-sensitive isolate of *P. tritici-repentis* was transformed with a vector containing the genomic copy of the *ToxA* gene and the hygromycin B resistance gene for use as a selectable marker. PCR analyses confirmed that the tox$^+$ transformants contain the *ToxA* gene and protein gel blot analyses indicated that the tox$^+$ transformants produced Ptr ToxA in culture. Infiltration bioassays indicated that culture filtrates from the tox$^+$ transformants caused typical tan spot necrosis in the sensitive wheat cultivar but not in insensitive wheat. The tox$^-$ recipient isolate and the tox$^-$ recipient transformed with the vector only did not produce the Ptr ToxA protein, and culture filtrates from these isolates did not induce necrosis in either wheat cultivar. Pathogenicity assays with tox$^+$ transformants resulted in typical tan spot lesions in susceptible wheat but not in resistant wheat, similar to the tox$^+$ control isolate (Fig. 3.1). The vector-only transformant and the tox$^-$ recipient did not develop tan spot symptoms in either wheat cultivar. Transformation clearly demonstrated that this gene is sufficient for pathogenicity of *P. tritici-repentis* on the toxin-sensitive wheat genotypes tested (Fig. 3.1). Nucleotide sequence analysis (Fig. 3.2) of the *ToxA* gene indicated that the ORF encodes a preproprotein, with an N-terminal signal peptide, followed by a dual-domain protoxin. The protoxin consists of a small, anionic N-domain, of unknown function and destiny, and a larger cationic C-domain that is the mature, secreted Ptr ToxA protein. No significant homology to other genes was observed in database searches. The deduce ORF encodes a protein with a calculated mass of 19,707 D.

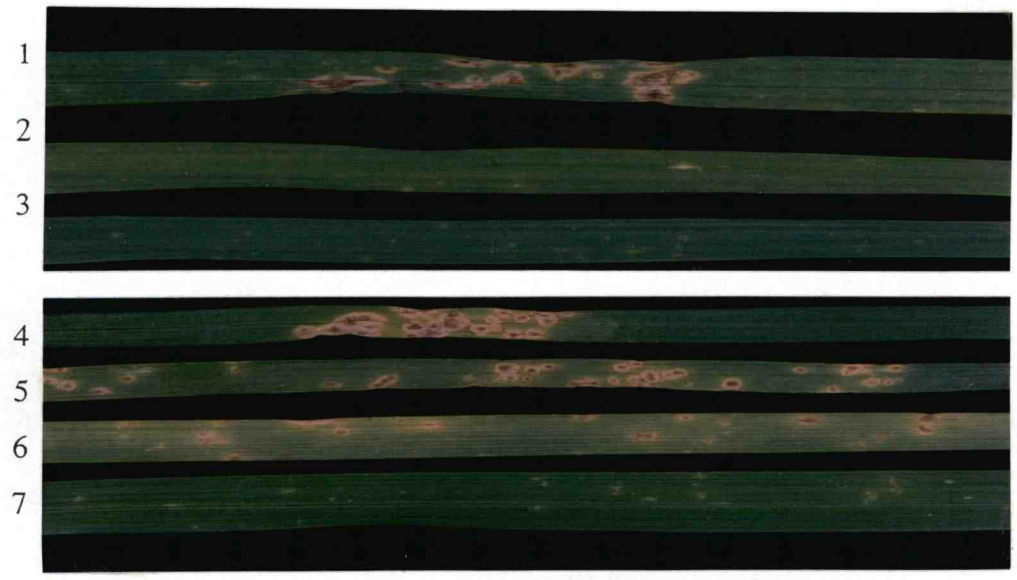


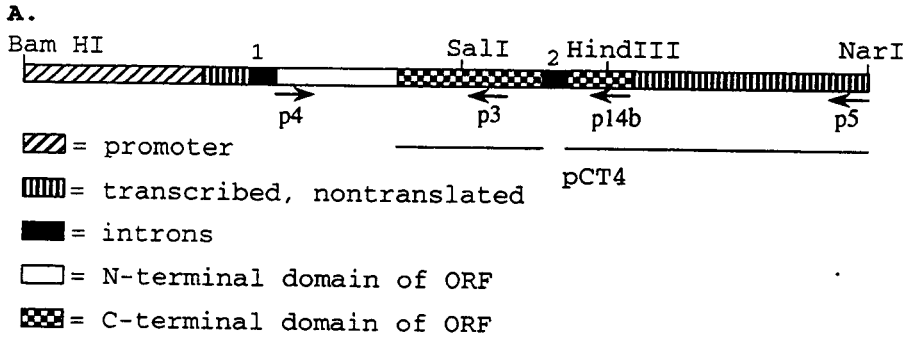
Figure 3.1. Pathogenicity Assay of Tox$^+$ Transformants. Conidial suspensions of the tox$^+$ control isolate BFP (leaf 1), the tox$^-$ recipient SD-20 (leaf 2), SD-20 transformed with the vector only, 6-2-2 (leaf 3), and tox$^+$ transformants 8-3-4, 9-3-1, and 9-3-2 (leaves 4 to 6) were inoculated onto susceptible (TAM 105, leaves 1-6) and resistant (Auburn, leaf 7) wheat. Typical tan spot lesions developed on leaves of the susceptible wheat cultivar in both the tox$^+$ control isolate and the tox$^+$ transformants. Typical tan spot lesions were not observed in the susceptible wheat cultivar inoculated with the tox$^-$ recipient or the tox$^-$ recipient transformed with the vector only. A typical resistant reaction developed in the resistant cultivar (Auburn) with all of the isolates tested. Results of inoculation with the tox$^+$ transformant 9-3-2 (leaf 7) illustrate a typical reaction of the resistant cultivar.

Figure 3.2. Sequence Analysis of the *ToxA* Gene.

(A) Genomic organization of the *ToxA* locus. Shown is the predicted 534-nucleotide ORF beginning with the first ATG codon containing the N- and C-domains. The N- domain encodes a 60- to 61-amino acid polypeptide, with the first 22 amino acids of this region showing homology to signal peptides. The putative signal peptide is followed by a 38-39 amino acid region with a theoretically determined anionic isoelectric point of 4.55. The C-terminal domain encodes the 117-118 amino acid Ptr ToxA region. The 278-nucleotide region 5' to the transcribed section of the gene functions as a promoter both in culture and in the plant. A 137-nucleotide transcribed, nontranslated leader sequence contains a 55-nucleotide intron (1). A second intron (2) is present in the C-domain of the ORF. A subgenomic library of *P. tritici-repentis* was constructed in the vector pHA1.3. The genomic copy of *ToxA* was identified with the ^{32}P-labeled cDNA insert of plasmid pCT4 (underlined in the genomic map). This probe contains the C-domain and 3' nontranslated region minus intron 2. Also shown are four primers used for PCR-amplification of *ToxA*: primer 4 (p4), primer 3 (p3), primer 14b (p14b), and primer 5 (p5).

(B) The nucleotide and deduced amino acid sequence of the *ToxA* gene. Nonboldface numbers in the column on the left indicate nucleotide position. Boldface numbers indicate amino acid position. The promoter region consists of 278 bp (lowercase letters). A potential CAAT motif and TATA box, thought to be involved in initiation of transcription (Gurr *et al.*, 1987) (boldface), are located approximately 72 and 57 nucleotides, respectively, before the presumed transcriptional start site. The sequence found in the cDNA copy of the *ToxA* gene is shown (uppercase). Introns 1 and 2 are indicated by uppercase letters in boldface. Amino acid residues 1-22 comprise a putative signal peptide, with the probable signal peptide cleavage site shown (double arrow). Two sites of possible proteolytic cleavage that result in the mature Ptr ToxA protein are shown (single arrows). Approximately 50% of the mature Ptr ToxA protein sequence was determined through direct amino acid sequencing, and the sequences from all peptide fragments are found within the predicted ORF. Underlined amino acid sequences are those predicted by direct amino acid sequencing (the first sequence underlined was generated by cleavage with cyanogen bromide (CNBr), the remainder shown were generated by trypsin digest). Restriction enzyme recognition sites are underlined.

Figure 3.2



B.

Bam HI

```
   1  ggatccgatggaatccatggaggagttctgtacgcgcaattccgctctccgtaaggatgc
  61  ttcggaggtgcacatggtctcatacatgtaggcccgacgaggatcgagtcggttccgaag
 121  taggatcgtctcgattgttgggcatcattgcatggacattcagagggcctactgatacct
 181  ggaatccgcaccgtccggctacctagcaataagattctgtgtatataaagggctaaggtg
 241  tccgtccttgataaaaccaccaccctcaacaacttaccTCGACTATCAGCATCCCGTCCT
 301  ATCTAACAATCGTCCATCGGTATCCAACTCCAACTCTATTCGCAGGGTCCTAGAATCGTA
 361  AGTACACGCTTATATCTTGTTGCCAGCGATAGCTGACAATGAATGAATATAGGTCATGCG
   1                                                      M  R

 421  TTCTATCCTCGTACTTCTTTTCAGCGCCGCTGCTGTGCTTGCTGCCCCAACGCCTGAAGC
   3   S  I  L  V  L  L  F  S  A  A  A  V  L  A  A  P  T  P  E  A
                                                              ⇑
 481  CGATCCCGGTTACGAAATCGTTAAACTTTTCGAAGCCGCCAACTCTTCTGAACTCGACGC
  23   D  P  G  Y  E  I  V  K  L  F  E  A  A  A  N  S  S  E  L  D  A

 541  GCGCGGACTCTCTCTCGACTGGACCCTCAAACCGCGCGGACTCCTACAGGAGCGGCAGGG
  43   R  G  L  S  L  D  W  T  L  K  P  R  G  L  L  Q  E  R  Q  G
                                                        ↑  ↑
 601  AAGCTGCATGTCAATCACAATCAACCCTAGTCGTCCGTCTGTCAACAACATCGGCCAAGT
  63   S  C  M  S  I  T  I  N  P  S  R  P  S  V  N  N  I  G  Q  V
      Sal I
 661  CGACATTGACAGTGTTATACTCGGACGACCTGGTGCTATAGGCTCGTGGGAACTGAACAA
  83   D  I  D  S  V  I  L  G  R  P  G  A  I  G  S  W  E  L  N  N

 721  CTTTATTACCATTGGATTGAACCGCGTAAACGCCGATACAGTGCGAGTCAACATCCGAAA
 103   F  I  T  I  G  L  N  R  V  N  A  D  T  V  R  V  N  I  R  N

 781  CACCGGCAGGACTAATCGCCTCATTATTACTCAATGGGAATAAGTCTCCCCACCATTTTA
 123   T  G  R  T  N  R  L  I  I  T  Q  W  D

 841  TTAGATTACTTATTACTAACTTATACTACTAATACTGTCACTCGGGGGGACGTTTATGAG
 136                                  N  T  V  T  R  G  D  V  Y  E
                                                 Hind III
 901  CTTTTTGGTGATTACGCTTTAATTCAAGGTAGAGGAAGCTTTTGCCTTAATATACGATCT
 146   L  F  G  D  Y  A  L  I  Q  G  R  G  S  F  C  L  N  I  R  S

 961  GATACAGGTCGTGAAAATTGGAGAATGCAGCTAGAAAATTAGCGAGCAAGCACTTGTTCT
 166   D  T  G  R  E  N  W  R  M  Q  L  E  N  *

1021  GTATAACAGTGGCTTGTACTAAGAACATGGATTAGCCTATTAGGGAGTGAAGGATGCTCT
1081  TTTCAGTGTGCTTTAGGAGAGTAATCAGGAGGGGAATGATCAGGAGAGGAGTCGGATCGA
1141  GAAGGAGGAGCACAACCGGAACGGCACGTGTTGGCCACGGCATTCCCCACACTCATTTTG
1201  TCATTTTTCGCACTTCCACGCTGCACGCAACCCCCCAGTCAAAATCTTGTCTTCTACATC
                                                          Nar I
1261  TCATAGGTGGTAGCTTCCCATAGGTAGCTTTTTGAAATGAAACATTTATCACGGGCGCC
```

This paper describes: 1) the generation of internal peptide fragments of Ptr ToxA and the amino acid sequencing of these fragments; 2) the attempt to determine the N-terminal amino acid sequence from Ptr ToxA; 3) the construction of a chimeric, full length cDNA clone; 4) the occurrence of the Ptr ToxA gene in isolates of *Pyrenophora tritici-repentis* and *P. teres*, as determined by PCR-analyses; and 5) secondary structure predictions for the deduced Ptr ToxA precursor protein.

Amino acid sequencing of the protein was conducted with the intent to facilitate identification of putative toxin-encoding clones. Two other points in this paper were addressed because of possible importance for the heterologous over-expression of the Ptr *ToxA* gene. These include: 1) determination of the extreme N-terminal amino acid sequence of Ptr ToxA; and 2) construction a full length cDNA clone that could be used as a template for producing smaller truncated or domain-specific portions of the protoxin for expression.

The occurrence of the Ptr *ToxA* gene in isolates of *P. tritici-repentis* and a closely-related species, *P. teres*, was investigated with the intention of providing insight into both the evolutionary origin of the gene and also the race differentiation of the fungus. Previous reports indicated multiple toxins are produced by the fungus (Lamari and Bernier, 1989b; Orolaza *et al.*, 1995; Tuori *et al.* 1995) and that race differentiation is determined by the production of either chlorosis toxin(s), necrosis toxin(s), or both (Lamari and Bernier 1989a; Lamari *et al.*, 1995). In our prior report (Ciuffetti *et al.*, 1997), Southern blot analysis demonstrated that the *ToxA* gene was present only in tox^+ isolates of *P. tritici-repentis*, indicating that isolates not producing the toxin are completely missing the gene. The PCR-analysis included in this report was intended to

facilitate the testing of fungal isolates for the presence of the *ToxA* gene. In addition, PCR products could be cloned and/or sequenced. This could reveal possible sequence polymorphisms and hence, information regarding the origins of the gene and phylogenetic relationships between isolates and species.

Finally, secondary structure information on the protein deduced from the ORF could provide valuable information on the active site of the toxin. Information regarding amino acid residues which are likely on the surface of the three-dimensional structure of the protein should identify useful candidates for future studies involving site-directed mutagenesis and protein crosslinking to a putative receptor protein in wheat.

Methods

Fungal Cultures

Isolate BFP (Tuori *et al.*, 1995) of *Pyrenophora tritici-repentis* is a fast-growing subculture of Pt-1C obtained from W. Bockus (Kansas State University, Manhattan). *P. tritici-repentis* isolates SD-1, SD-2, SD-6, SD-8, SD-11, SD-13, SD-16, SD-19 and SD-20 were obtained from G. Buchenau (South Dakota State University, Brookings). *P. teres* isolates Pt-R3B, Pt-GR-91-33-31 and Pt-MN-85-36E3 were obtained from T. Peever and M. Milgroom (Cornell University). *C. fulvum* race 9, was obtained from J. Lorang (Oregon State University).

Generation and Sequencing of Internal Peptide Fragments of Ptr ToxA

Ptr ToxA was purified from culture filtrates of the fungus as described (Tuori *et al.*, 1995). Ptr ToxA was treated with trypsin (US Biochemical) at 1:50 (w/w) ratio of protein to enzyme with a final Ptr ToxA concentration at 1 mg/mL, in a reaction buffer of 100 mM

NH_4CO_3, 1 mM $CaCl_2$, pH 8.0, overnight at room temperature. Ptr ToxA was treated with CNBr (Sigma) at a final protein concentration of 0.1 mg/mL and a final CNBr concentration of 10 mg/mL. CNBr from a freshly opened bottle was dissolved in 90% formic acid at 12.8 mg/mL. Ptr ToxA was diluted with 933-μL of dissolved CNBr, 120-μL of 10X buffer (0.1 M Tris, pH 8.0, 3% SDS, 20 mM DTT) and brought up to 1.2 mL with the addition of water. The reaction was performed in a screw-capped tube overnight in the dark and was purged of oxygen with gaseous nitrogen for 2 min prior to incubation. The next day the tube was heated to 55 °C for 5 min and then the volume was reduced by 50% under a steady stream of nitrogen. The reaction volume was further reduced to approximately 30% of the original volume in a dessicator attached to a vacuum, and then dried completely in a speed-vac (Savant) with no heat. Peptides were solubolized in 30 mM Tris, pH 9.0, 0.5% SDS and 0.1 M DTT. Prior to HPLC purification trypsin-generated fragments were dried in a speed-vac, resuspended in 200-μL of water, sonicated for 5 min, and heated to 90 °C for 5 min. Peptide fragments generated from either trypsin digestion of CNBr cleavage were applied to a C-18 microbore reversed-phase-HPLC column (Beckman) equilibrated in water and 0.1% trifluoroacetic acid (TFA) and then eluted with a linear gradient of 0-70% acetonitrile with 0.1% TFA added. Peptides were detected at 210 nM, collected, lyophilized, and sequenced at the Central Services Lab at the Center for Gene Research and Biotechnology at Oregon State University.

Determination of the N-terminus of the Mature Ptr ToxA Protein

Purified Ptr ToxA (33 μg in 100 μL water) was treated with the enzyme pyroglutamate amino peptidase (Sigma) by diluting with 1 mL of reaction buffer (100 mM K_2HPO_4, 10 mM EDTA, 5% glycerol and 5 mM DTT with the pH adjusted to 8.0 with 1 M

HCl) and then adding 100 μL of enzyme (5 units) such that the final reaction volume was 1.2 mL. The reaction was incubated at 37 °C for 15 min and then concentrated in a Centricon-3 microconcentrator (Amicon) for 2 hr, spinning at 7500xg, 25 °C until the volume was reduced to approximately 250 μL. Four 50 μL aliquots (6.6 μg or 500 pmoles) were separated in a 17% SDS-polyacrylamide gel, electroblotted as described (Tuori *et al.*, 1995), and the blot was stained for 5 min with 0.5% Ponceau-S in 1% acetic acid. The blot was destained in 1% acetic acid until the background was low enough that bands appeared, after which the blot was washed in ddH_2O for 30 min with several washes to remove residual glycine. Ptr ToxA bands were excised from the blot and subjected to microsequencing at The Institute for Molecular Biology, Protein Sequencing Lab, University of Oregon.

Construction of the Full Length cDNA

The preparation and screening of the cDNA library was described previously (Ciuffetti *et al.*, 1997). PCR-amplification of inserts from antibody-positive recombinant λ clones was performed with 10 ng of λ DNA, 50 nM of each primers, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, and with 1-2 units of Taq polymerase and the corresponding reaction buffer obtained from Promega. Lambda clone 17-4-7 was amplified with λ forward and reverse primers and λ clone 22a-4-8 was amplified with internal primer 3 (5'-CCC ACG AGC CTA TAG CAC CAG GTC GT- 3') and λ forward primer. After 2 min at 94° C, the enzyme was added, and the reaction was cycled through 10 cycles of 1 min at 94° C, 1 min at 50° C, and 1.5 min at 72° C. The reactions were then cycled through 35 cycles of 1 min at 94° C, 1 min at 60° C, and 1.5 min at 72° C. Thirty microliters of each PCR reaction were separated by electrophoresis in a 1% agarose gel (1 x TAE, 0.04 M Tris-

acetate, pH 8.0, and 0.001 M EDTA), stained in ethidium bromide, and the bands gel-purified (GeneClean II, BIO101). The fragments were blunt end-cloned into the *Eco* RV site of pSK+ resulting in the separate subcloning of the 5'-end (pCT33) and 3'-end (pCT4) fragments of the Ptr ToxA cDNA. Construction of the chimeric full length cDNA is depicted in Figure 3.3. All ligations and bacterial transformations were carried out according to established protocols (Sambrook *et al.*, 1989).

Detection of the Ptr *ToxA* Gene in Isolates of *P. tritici-repentis* and Other Fungi

DNA preparations for PCR-screening of *P. tritici-repentis* isolates, *P. teres* isolates, and *C. fulvum* were prepared with InstaGene™ Matrix (Bio-Rad) according to the manufacturer's recommendations for DNA preparation from bacteria. Twenty-μL of each DNA preparation were amplified in 100 μL PCR reactions. DNA preparations from *P. tritici-repentis* isolates and *C. fulvum* were amplified with primer-4 and primer-5, with reaction conditions and cycles as described for λ DNA samples. These primers were designed to amplify the entire coding region and the 3'-nontranslated region (see Figure 3.2A). Bases that were changed to facilitate cloning of PCR products are shown in italics, and the resulting restriction sites underlined. Primer-4 (5'-ATC G*CC ATG G*GT TCT ATC CTC GTA CT-3') was designed to create an *Nco* I site flanking the ATG codon. This changed the second codon of the amplified product from R (Arg) to G (Gly). Primer-5 (5'-CGT *GGA TCC* CGT GAT AAA TGT TTC AT-3') was designed to create a *Bam* HI site at the extreme 3'-end of the 3'-nontranslated region, resulting in the loss of the *Nar* I site in the amplified products. DNA preparations from *P. teres* isolates were amplified with primers-4 and -14B (5'-GTC T*GG ATC C*TA ATT TTC TAG CTG CAT TC-3'), designed to amplify only the ORF of the *ToxA* gene (see Figure 3.2A) and to create a *Bam* HI site at

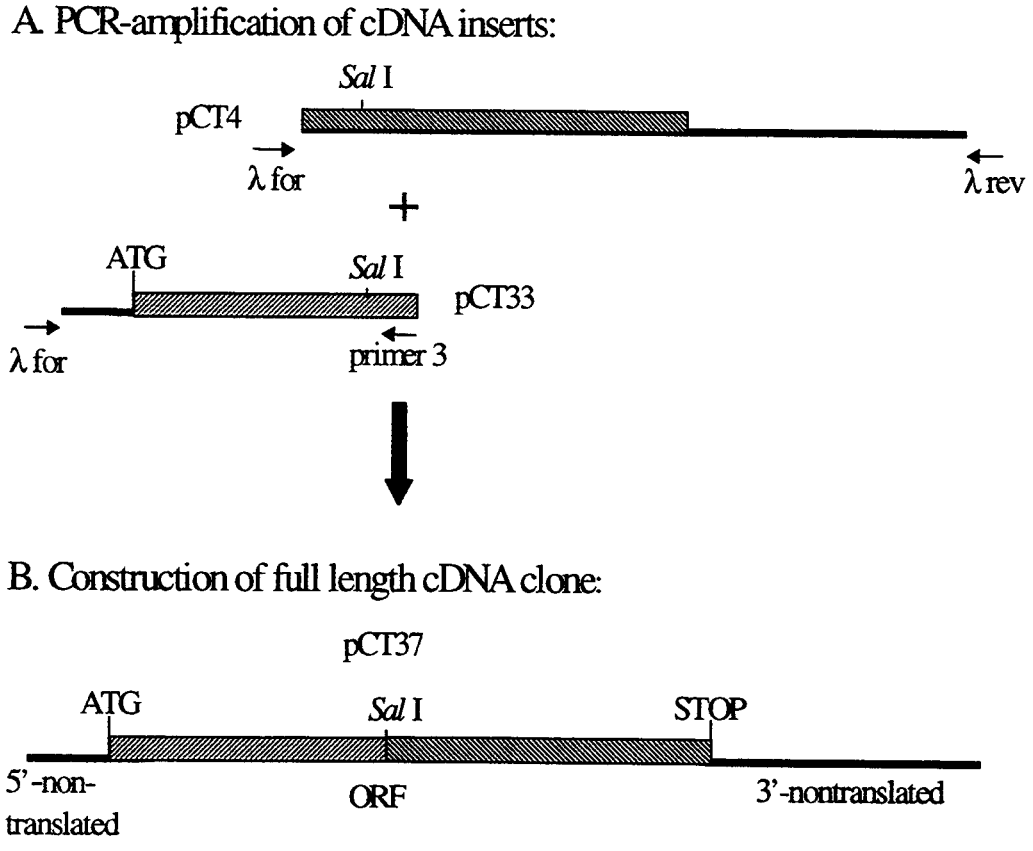


Figure 3.3. PCR-amplification and construction of the full length cDNA clone.

(A) PCR-amplification of cDNA inserts. The insert from λ clone 17-4-7 containing the truncated 3' end of the *ToxA* gene was amplified with λ forward and reverse primers. The 709 bp PCR product was cloned into pSK+ resulting pCT4. The insert from λ clone 22a-4-8 was amplified with λ forward primer and internal primer 3. The 377 bp PCR product was cloned into pSK+ resulting in pCT33.

(B) Construction of full length cDNA clone. A *Sal* I fragment from pCT33 (the internal *Sal* I site and a *Sal* I site 5' to the insert in the plasmid polylinker) was cloned into pCT4 with a *Sal* I fragment removed from it (again, the internal *Sal* I site and a 5' site within the plasmid polylinker) resulting in the chimeric full length cDNA clone, pCT37. The insert of pCT37 is 934 bp, containing an 82 bp 5'-nontranslated region, a 534 bp ORF encoding a 178 amino acid polypeptide of 19,677 D and a 318 bp 3'-nontranslated region.

the extreme 3'-end of the ORF for cloning PCR products. Reaction conditions and cycles were the same as described for λ DNA samples. Bases that were changed to facilitate cloning are shown in italics, and the resulting restriction sites underlined. A 30 μL aliquot of each PCR reaction was separated by electrophoresis in a 1.5 % agarose gel (1 x TAE) and stained in ethidium bromide.

Sequence Analyses and Secondary Structure Analyses

Nucleotide sequence determination was performed at the Central Services Laboratory of the Center for Gene Research and Biotechnology at Oregon State University with dye-primer chemistry on an ABI model 373 automated sequencer (Applied Biosystems Inc, Foster City, CA). Secondary structure predictions were conducted with Wisconsin Package Software Version 9.0, Genetics Computer Group (GCG), Madison, WI.

Results

Generation and Sequencing of Internal Fragments of Ptr ToxA

Attempts to obtain N-terminal amino acid sequence of the purified toxin revealed that the toxin is blocked at the N-terminus. Therefore, we determined the sequence of fragments of the protein generated by partial digestion with trypsin and cleavage with cyanogen bromide. Ten peptide fragments were purified by reversed-phase HPLC and sequenced. The sequence for all of these fragments appeared within the C-domain of the ORF and is shown in Fig. 3.2B. We obtained sequence information for approximately 50% of the Ptr ToxA protein. Comparison of amino acid sequence deduced from

nucleotide sequence with amino acid sequence obtained from the Ptr ToxA peptides provided unequivocal confirmation of the identity of the cloned cDNAs.

Determination of the N-terminus of the Mature Ptr ToxA Protein

In an attempt to determine the amino acid residue at the N-terminus of the mature Ptr ToxA protein, purified Ptr ToxA was treated with the enzyme pyroglutamate amino peptidase to remove a possible pyroglutamate residue from its N-terminus. Sequence information was obtained corresponding to the amino acid sequence of Ptr ToxA immediately following Gln-61. Although attempts were made to repeat this procedure with another source of the enzyme, no amino acid sequence was obtained.

Construction of the Full Length cDNA

The cDNA inserts of five recombinant clones that were picked from the cDNA expression library were PCR-amplified and subcloned into plasmid pBluescript SK+ (pSK+). The nucleotide sequences from all five cDNA inserts were obtained and determined to be truncated cDNAs from the same transcript. Although the largest cDNA insert could not be excised from the λ clone with restriction enzymes or PCR-amplified with λ forward and reverse primers (presumably due to a rearrangement at one of the cloning junctions), the 5'-end of this insert was amplified with λ forward primer and an internal primer and subcloned (Figure 3.3). The construction of a chimeric full length cDNA was accomplished, as shown in Figure 3.3, by fusing the 5'-end of the largest cDNA with the 3'-end of a truncated cDNA.

Detection of the Ptr *ToxA* Gene in Isolates of *P. tritici-repentis* and Other Fungi

Primers designed for PCR-amplification of the *ToxA* gene were used to detect the presence of the gene in various isolates *of P. tritici-repentis* and other fungi. The *ToxA* gene was detected in several isolates of *P. tritici-repentis* (Fig. 3.4A), with apparently identical sized PCR products for all isolates tested. The gene was amplified from all toxin producing isolates tested and was not detected in presumed tox- isolates (Figure 3.4A). These data corroborate the results of previous DNA gel-blot analysis (Ciuffetti *et al.*, 1997), where a truncated cDNA clone (pCT4) comprising only the 3' end of the ORF was used as a probe.

The *ToxA* gene was also detected from DNA of some, but not all, *Pyrenophora teres* isolates (Fig.3.4B), a heterothallic pathogen of barley (Smedegard-Peterson, 1978). A product the same size as for *P. tritici-repentis* was obtained only when primers designed to amplify within the coding region of the gene were used. Primers specific to the promoter or the 3'-nontranslated regions of the *ToxA* gene would not amplify the gene. Nucleotide sequence analysis of the PCR products from two *P. teres* isolates, Pt-MN-85-36E3 and Pt-R3B, revealed that the products had the exact sequence as the *ToxA* gene of *P. tritici-repentis* isolate BFP. The Ptr ToxA protein has not been detected from culture filtrates of any of the selected isolates of this fungus that we tested when cultured under standard conditions for toxin production used with *P. tritici-repentis*. The gene was not detected in the one race of *Cladosporium fulvum* tested (Figure 3.4A), *Helminthosporium carbonum, Helminthosporium victoriae, Alternaria alternata,* or *Graphium* sp.

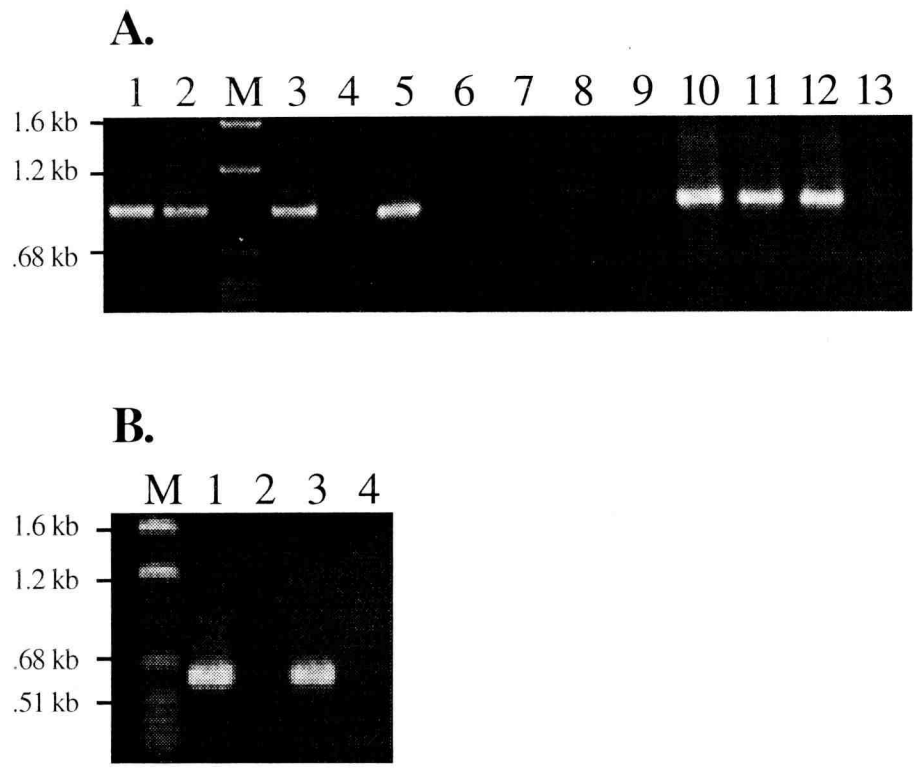


Figure 3.4. PCR-Amplification of *ToxA* from *Pyrenophora tritici-repentis and P. teres*. Ethidium bromide stained 1.5% agarose gels with molecular mass markers of 1.6, 1.2, 0.68, and 0.51 kb shown in lane M of both gels.

(A) PCR screening of *P. tritici-repentis* isolates. Shown are toxin-producing isolates SD-1 (lane 1), SD-2 (lane 2), SD-6 (lane 3), BFP (lane 5), SD-8 (lane 10), SD-16 (lane 11), and SD-19 (lane 12) and nontoxin-producing isolates SD-11 (lane 6), SD-13 (lane 7), SD-20 (lane 8), and MCR-I (lane 13). Also shown is *Cladosporium fulvum* (lane 4). All toxin-producing isolates produce the expected size band (913 bp) for the genomic copy of the *ToxA* gene when amplified with primers 4 and 5 (see Figure 3.8A). No bands are visible in the nontoxin-producing isolates (SD-11, -13, -20 or MCR-I) or *C. fulvum*.

(B) PCR screening of representative *P. teres* isolates. Shown are *P. teres* isolates Pt-R3B (lane 3) and Pt-GR-91-33-31 (lane 4), along with *P. tritici-repentis* isolates BFP (lane 1) and nontoxin-producing isolate SD-20 (lane 2) as controls. All PCR products obtained from *P. teres* isolates with *ToxA* ORF-specific primers are of the expected size (595 bp) for the genomic copy of the gene when amplified with primers 4 and 14B (see Figure 3.2A).

Secondary Structure Analyses of the Ptr ToxA ORF

The secondary structure predictions for the *ToxA* ORF are presented in Table 3.1. The ORF contains two possible N-glycosylation sites at amino acids 36 and 70. The majority of the N-domain, or amino acids 23-61, approximately, is predicted to have alpha helical secondary structure, while the majority of the C-domain, or amino acids 62-178, approximately, is predicted to comprise beta sheets, with a few small regions of turns and a prominent alpha helical hydrophilic tail at the extreme C-terminus.

Discussion

The generation of internal peptide fragments of the Ptr ToxA protein and the sequencing of these fragments resulted in the determination of amino acid sequence for approximately half of the protein (Fig 3.2), and proved invaluable in determining the identity of putative toxin-encoding cDNA clones (Ciuffetti *et al.*, 1997). Amino acid sequencing of Ptr ToxA peptide fragments also provided evidence that the mature toxin is not modified by N-glycosylation. Although two possible sites of N-glycosylation (Bause, 1983) were identified within the *ToxA* ORF (amino acids 36-38 and amino acids 70-72, Table 3.1), the sequence of the second site was detected following Edmun degradation during sequencing of one of the peptide fragments of Ptr ToxA, indicating that this site does not undergo glycosylation. We have no data indicating whether the first site, which is within the N-domain, is glycosylated.

The deduced ORF from the *ToxA* gene encodes a protein with a calculated mass of 19,707 D, which is very close to that predicted from immunoprecipitation of *in vitro* translation products (Ciuffetti *et al.*, 1997). Based on the determined mass of 13,208 D

Table 3.1. *ToxA* Open Reading Frame Secondary Structure Predictions.
Amino acid position (Pos) in the ORF is shown with constituent amino acids (AA) designated by the one letter code. N-Glycosylation sites (GlycoS) are shown with potential sites indicated by 'G'. Also shown is hydrophilicity probability (Hyphil) calculated according to Kyte and Doolittle (1982); surface probability (SurfPr) calculated according to Emini *et al.* (1985); secondary structure predictions (CF-Pred) according to Chou and Fasman (1978) and (GORPred) according to Garnier *et al.* (1978), where 'B' indicates β sheet, 'H' indicates α helix, 'T' indicates turn and no prediction is indicated by a period; and, antigenicity index (AI-Ind) according to Jameson and Wolf (1988).

Pos	AA	GlycoS	HyPHil	SurfPr	CF-Pred	GORPred	AI-Ind
1	M	.	-0.275	0.682	.	B	-0.150
2	R	.	-0.980	0.440	.	B	-0.450
3	S	.	-1.517	0.255	.	B	-0.450
4	I	.	-1.843	0.213	B	B	-0.450
5	L	.	-2.114	0.090	B	B	-0.600
6	V	.	-3.157	0.058	B	B	-0.600
7	L	.	-3.157	0.111	B	B	-0.600
8	L	.	-2.771	0.136	B	H	-0.600
9	F	.	-2.486	0.185	B	H	-0.600
10	S	.	-2.143	0.226	H	H	-0.600
11	A	.	-2.200	0.204	H	H	-0.600
12	A	.	-2.200	0.194	H	H	-0.600
13	A	.	-2.057	0.146	H	H	-0.600
14	V	.	-2.429	0.146	H	H	-0.600
15	L	.	-1.943	0.224	H	H	-0.600
16	A	.	-1.586	0.320	H	H	-0.600
17	A	.	-1.100	0.666	H	.	-0.600
18	P	.	0.000	1.398	T	.	0.200
19	T	.	0.286	1.398	T	.	0.800
20	P	.	1.043	2.311	.	.	0.900
21	E	.	1.529	2.311	.	.	0.900
22	A	.	1.357	1.585	.	.	0.900
23	D	.	1.443	1.606	.	.	0.900
24	P	.	1.714	1.606	T	.	1.300
25	G	.	0.571	1.114	T	H	1.300
26	Y	.	0.229	0.495	.	H	0.300
27	E	.	0.286	0.641	H	H	0.300
28	I	.	-0.486	0.534	H	H	-0.600
29	V	.	-0.943	0.295	H	H	-0.600
30	K	.	-0.629	0.295	H	H	-0.600
31	L	.	-1.386	0.425	H	H	-0.600
32	F	.	-1.000	0.579	H	H	-0.600
33	E	.	0.100	0.465	H	H	0.300
34	A	.	-0.343	0.756	H	H	-0.300
35	A	.	0.314	1.170	H	H	0.450
36	N	G	1.214	1.170	.	H	0.900
37	S	.	0.171	0.955	T	H	0.850
38	S	.	0.929	1.579	T	H	1.300
39	E	.	0.929	0.992	H	H	0.750
40	L	.	1.071	1.450	H	H	0.900
41	D	.	1.014	1.071	H	H	0.900
42	A	.	0.357	0.510	H	H	0.450
43	R	.	-0.029	0.828	H	H	-0.150
44	G	.	-0.029	0.409	.	H	-0.300
45	L	.	-0.029	0.676	H	.	-0.300
46	S	.	0.357	0.363	H	.	0.300
47	L	.	-0.186	0.529	H	.	-0.300
48	D	.	-0.786	0.529	H	T	-0.200

Table 3.1, continued

Pos	AA	GlycoS	HyPHil	SurfPr	CF-Pred	GORPred	AI-Ind
49	W	.	0.314	0.790	H	T	0.700
50	T	.	0.429	1.481	H	.	0.450
51	L	.	1.614	1.738	H	.	0.750
52	K	.	1.171	1.635	H	.	0.900
53	P	.	0.500	0.934	T	.	1.150
54	R	.	-0.143	0.934	T	.	0.250
55	G	.	0.900	0.809	T	.	0.950
56	L	.	0.843	0.906	H	.	0.750
57	L	.	1.257	0.906	H	.	0.750
58	Q	.	1.114	1.586	H	.	0.900
59	E	.	1.114	1.903	H	T	1.300
60	R	.	1.771	3.093	H	T	1.300
61	Q	.	1.957	0.957	H	T	1.150
62	G	.	1.186	0.547	T	T	1.350
63	S	.	0.800	0.374	T	T	1.350
64	C	.	-0.486	0.151	T	T	0.000
65	M	.	-0.886	0.221	B	B	-0.600
66	S	.	-1.586	0.116	B	B	-0.600
67	I	.	-1.200	0.347	B	B	-0.600
68	T	.	-0.614	0.542	B	B	-0.600
69	I	.	-0.229	0.542	B	B	-0.150
70	N	G	0.300	1.514	B	.	0.600
71	P	.	1.171	1.622	T	.	1.300
72	S	.	1.186	3.100	T	.	1.300
73	R	.	1.229	1.431	T	.	1.100
74	P	.	1.229	1.488	T	T	1.500
75	S	.	1.500	1.786	T	T	1.500
76	V	.	0.743	0.639	B	.	0.750
77	N	.	0.157	0.409	B	.	0.450
78	N	.	0.429	0.529	B	.	0.450
79	I	.	-0.286	0.529	B	B	-0.150
80	G	.	0.814	0.549	B	B	0.750
81	Q	.	-0.329	0.239	B	B	-0.150
82	V	.	-0.329	0.570	B	B	-0.300
83	D	.	0.429	0.772	B	B	0.450
84	I	.	-0.229	0.331	B	B	-0.150
85	D	.	-1.371	0.312	.	B	-0.450
86	S	.	-1.314	0.154	.	B	-0.600
87	V	.	-1.757	0.218	B	B	-0.600
88	I	.	-0.471	0.255	B	B	-0.600
89	L	.	-0.743	0.295	B	B	-0.600
90	G	.	-0.800	0.393	B	.	-0.600
91	R	.	-0.457	0.566	B	.	-0.450
92	P	.	-0.457	0.481	T	.	-0.050
93	G	.	0.143	0.481	T	.	0.850
94	A	.	0.200	0.329	H	.	0.300
95	I	.	-0.314	0.224	H	.	-0.300
96	G	.	-0.043	0.392	H	.	-0.300
97	S	.	-0.643	0.320	H	.	-0.600
98	W	.	0.114	0.734	H	.	0.300
99	E	.	1.257	1.193	H	.	0.750
100	L	.	0.800	0.771	H	.	0.600
101	N	.	0.043	0.514	.	T	0.700
102	N	.	0.014	0.428	B	T	0.700
103	F	.	-1.129	0.364	B	B	-0.600
104	I	.	-0.529	0.224	B	B	-0.600
105	T	.	-1.571	0.115	B	B	-0.600
106	I	.	-1.571	0.213	B	B	-0.600
107	G	.	-0.529	0.596	.	B	-0.600
108	L	.	-0.486	0.307	.	.	-0.600

Table 3.1, continued

Pos	AA	GlycoS	HyPHil	SurfPr	CF-Pred	GORPred	AI-Ind
109	N	.	-0.086	0.703	.	.	-0.300
110	R	.	0.300	0.718	.	.	0.300
111	V	.	0.743	1.454	.	.	0.750
112	N	.	1.386	1.305	.	.	0.900
113	A	.	0.286	0.494	T	B	0.850
114	D	.	0.286	1.305	T	B	1.000
115	T	.	0.286	0.602	B	B	0.450
116	V	.	0.286	0.959	B	B	0.300
117	R	.	-0.100	0.402	B	B	-0.300
118	V	.	0.043	0.546	B	B	0.300
119	N	.	0.443	1.183	B	B	0.450
120	I	.	1.143	0.872	B	B	0.750
121	R	.	0.557	1.162	B	T	1.300
122	N	.	1.800	1.416	B	T	1.300
123	T	.	1.400	2.915	B	T	1.300
124	G	.	2.543	2.393	.	T	1.300
125	R	.	2.543	2.915	.	T	1.300
126	T	.	1.500	1.666	T	.	1.100
127	N	.	0.757	1.180	T	.	1.100
128	R	.	0.057	0.422	B	B	0.450
129	L	.	-0.486	0.422	B	B	-0.600
130	I	.	-0.086	0.455	B	B	-0.300
131	I	.	-0.457	0.244	B	B	-0.600
132	T	.	-0.600	0.494	B	B	-0.600
133	Q	.	0.443	1.134	B	T	0.850
134	W	.	1.186	2.335	B	T	1.300
135	D	.	1.229	1.201	T	.	1.100
136	N	.	1.229	1.001	T	.	1.100
137	T	.	1.371	1.864	.	.	0.900
138	V	.	1.300	1.105	.	.	0.900
139	T	.	1.300	1.147	.	.	0.900
140	R	.	0.200	0.590	T	.	0.650
141	G	.	0.286	1.245	T	.	0.800
142	D	.	1.386	1.494	.	B	0.900
143	V	.	0.743	0.629	B	B	0.600
144	Y	.	-0.300	0.551	B	B	-0.300
145	E	.	-0.300	0.326	B	B	-0.300
146	L	.	-0.300	0.734	B	B	-0.300
147	F	.	0.486	0.734	B	T	0.700
148	G	.	0.043	0.428	.	T	0.700
149	D	.	-1.000	0.428	H	T	-0.200
150	Y	.	-1.100	0.347	H	.	-0.600
151	A	.	-0.200	0.607	H	.	-0.300
152	L	.	-0.200	0.360	H	.	-0.300
153	I	.	-0.057	0.449	H	T	0.100
154	Q	.	-0.186	0.440	H	T	0.250
155	G	.	0.186	0.715	T	T	1.050
156	R	.	0.329	0.884	T	T	1.250
157	G	.	0.614	0.274	T	T	1.550
158	S	.	-0.429	0.228	.	T	-0.050
159	F	.	0.014	0.187	B	B	0.300
160	C	.	-1.271	0.133	B	B	-0.600
161	L	.	-0.686	0.194	B	B	-0.600
162	N	.	-0.686	0.300	B	B	-0.600
163	I	.	0.214	0.934	B	T	0.850
164	R	.	0.671	1.635	B	T	1.300
165	S	.	1.271	1.006	T	T	1.500
166	D	.	1.414	2.811	T	T	1.500
167	T	.	2.557	2.485	T	.	1.300
168	G	.	2.414	2.982	T	.	1.300

Table 3.1, continued

Pos	AA	GlycoS	HyPHil	SurfPr	CF-Pred	GORPred	AI-Ind
169	R	.	2.429	1.878	T	.	1.100
170	E	.	2.571	2.548	T	.	1.100
171	N	.	2.200	2.548	.	.	0.900
172	W	.	2.643	2.253	H	.	0.750
173	R	.	1.457	1.073	H	.	0.750
174	M	.	1.457	1.156	H	.	0.750
175	Q	.	1.457	1.767	H	.	0.900
176	L	.	1.550	1.153	H	.	0.900
177	E	.	0.960	1.490	H	.	0.900
178	N	.	1.675	1.100	.	.	0.900

for the mature toxin (Tuori *et al.*, 1995) and the assumption that the inferred signal sequence of 22 amino acids is proteolytically cleaved following import into the endomembrane system, additional proteolytic processing is apparently involved in the production of the mature Ptr ToxA. Because Ptr ToxA is blocked at the N-terminus and such modifications often result in a mass increase, the two most likely sites for a proteolytic cleavage that would yield mature toxin of 13,208 D are following Arg-60, which would yield a cleaved unmodified peptide of 13,213 D, or after Gln-61, which would yield a cleaved unmodified peptide of 13,085 D. Based on the results of amino acid sequencing of Ptr ToxA following treatment with pyroglutamate amino peptidase, however, the most likely possibility for the site of proteolytic cleavage is following Arg-60. Cleavage at this site would yield a mature protein with a glutamine residue at its N-terminus. The spontaneous or enzymatic cyclization of an N-terminal glutamine yielding a pyroglutamate residue that would be resistant to Edmun degradation is a common post-translational modification of proteins (Harris, 1989). This scenario is particularly attractive because the predicted molecular weight of such a processed Ptr ToxA would be very close to that predicted by mass spectral analysis (~13,197). Although preliminary evidence from N-terminal sequencing of Ptr ToxA following treatment with pyroglutamate amino peptidase indicates that this is highly likely, conclusive data has yet to be obtained.

Although a full length cDNA could not be PCR-amplified from Ptr ToxA-encoding λ clones, we concluded that the extreme N-terminal coding sequences of the Ptr ToxA transcript had been isolated in pCT33 based on several lines of reasoning: 1) the presence of several stop codons in all frames upstream of the putative (and first) ATG codon at base

416 (Fig. 3.2); 2) the strong similarity of the first 22 amino acids of the protein to the signal peptide consensus sequence (von Heijne, 1986) and to other signal peptides (cf. Jamroz *et al.*, 1993; Kersten and Cullen, 1993) which are almost always at the N-terminus of precursor proteins; and 3) the fact that the ORF of the chimeric, full length cDNA directed the production and secretion of active toxin when expressed behind a constitutive fungal promoter in the tox^- isolate of *P. tritici-repentis* (L.M. Ciuffetti, R.P. Tuori and J.M. Gaventa, unpublished data). The construction of a full length cDNA greatly facilitated the over-expression of this gene in *Escherichia coli* (Tuori *et al.*, 1998, in preparation).

The results of both the DNA gel-blot analysis described previously (Ciuffetti *et al.*, 1997) and PCR analysis (Figure 3.4) of tox^+ and tox^- isolates indicate that the *ToxA* gene is present only in tox^+ isolates of *P. tritici-repentis*. This finding is similar to that reported for other HST-producing fungi (Panaccione *et al.*, 1992; Yoder *et al.*, 1994, Ahn and Walton, 1996) and indicates that race differentiation ($path^+/tox^+$ vs $path^-/tox^-$) in this fungus may be due to the acquisition of toxin production rather than a loss of toxin production. An interesting finding from the PCR-screening is that isolates of *P. teres* apparently have a copy of the *ToxA* gene of identical sequence to the *P. tritici-repentis* allele, at least within the ORF. Although *P. teres* can be isolated from wheat (Brown *et al.*, 1993), it is predominantly a barley pathogen. Ptr ToxA was not detected in culture filtrates of selected isolates of *P. teres*, and selected genotypes of barley are not sensitive to this toxin. Therefore, there is currently no evidence implicating Ptr ToxA in the *P. teres*/barley disease interaction. It is interesting to note that the *P. teres* allele can only be amplified with primers annealing within the *ToxA* ORF. No PCR product is obtained when primers either 5' or 3' to the ORF are used for amplification, indicating that the sequence of the *P. teres* allele

diverges from *ToxA* outside the ORF. Confirmation of this finding by Southern blotting and cloning of the *P. teres* gene along with its flanking sequences could provide insight into the regulation and origin of this gene. Other species of *Pyrenophora* as well as other fungi pathogenic to wheat will be assessed for presence of the *ToxA* gene.

Secondary structure predictions of the derived amino acid sequence from the *ToxA* gene indicate that the N-domain is composed mostly of α helices and the C-domain is composed mostly of β sheets with a short α helix region at the extreme C-terminus. This finding provides evidence that these two regions of the ORF comprise functionally distinct structural domains. The secondary structure predictions for the C-terminal domain are very similar to the predictions made by Zhang *et al.* (1996) from the amino acid sequence of purified Ptr ToxA. Several interesting predictions regarding the three-dimensional structure of Ptr ToxA in agreement with empirical biochemical evidence can be drawn from the results shown in Table 3.1: 1, Both cysteine residues of the protein, Cys-64 and Cys-160, are in regions of low hydrophilicity. Surface probability and antigenicity, indicate their probable location on the interior of the protein, as is typical of disulfide bonds. This is consistent with the indication that a disulfide bond is present in the toxin and is required for activity (Tuori *et al.*, 1995); 2, The two regions of the precursor protein surrounding the predicted proteolytic cleavage sites (amino acid 21-24 at the signal peptide-N-domain junction and amino acid 59-62 at the N-domain-C-domain junction) both have high hydophilicity, surface probability and antigenicity values, indicating the likelihood that they are on the exterior of the protein, and therefore, accessible for proteolysis; 3, The region flanking the predicted site of cleavage between the N- and C-domains predicts a turn with an α helix on the N-terminal side and a β sheet on the C-terminal side; 4, Additional regions

of the C-domain can be identified as likely candidates for the active site of Ptr ToxA. These include amino acids 71-75, 122-126, 134-142, and 165-178. All regions have relatively high hydrophilicity, surface probability, and antigenicity index values, and all, interestingly, predicted turn regions in secondary structure. Amino acid region 134-142 also contains the RGD cell attachment motif. Comparisons of hydrophilicity, surface probability, secondary structure and antigenicity index for these and other regions of the ORF may be useful for determining suitable areas of the protein to target for site-directed mutagenesis or specific labeling. Confirmation of these predictions would require the crystal structure determination of Ptr ToxA.

Chapter 4

Heterologous Expression of the Host-Selective Toxin Protein, Ptr ToxA, from *Escherichia coli.*

Robert P. Tuori, Thomas J. Wolpert and Lynda M. Ciuffetti

Submitted to *Molecular Plant-Microbe Interactions*
June, 1998

Abstract

A cultivar-specific, active form of the Ptr ToxA protein, a Host-Selective Toxin (HST) produced by the fungus *Pyrenophora tritici-repentis*, was expressed in *Escherichia coli* as a polyhistidine-tagged, fusion protein consisting of both the N- and C-domains of the *ToxA* open reading frame. This heterologously produced protein was approximately 10-20% as active as native Ptr ToxA. A C-domain fusion protein displayed only trace amounts of activity. These data suggest that: 1) the N-domain is necessary for the correct folding of Ptr ToxA; 2) the N-domain does not function to eliminate activity of the protoxin; and 3) post-translational modifications of Ptr ToxA are not essential for activity. The NC-FP will be useful for receptor binding studies as well as screening wheat cultivars for disease susceptibility.

Introduction

Host-selective toxins (HSTs) are, with one exception, low molecular weight, secondary metabolites that are produced only by selected genera of plant pathogenic fungi. They are inferred to be intimately involved in pathogenesis because: 1, they reproduce the symptoms of disease; 2, they are toxic only to those host plant-cultivars that are susceptible to the toxin-producing fungus; and 3, toxin production is strictly correlated with pathogenicity. Because most HSTs are secondary metabolites, and thus, are very likely the products of multifunctional enzymes or complex biochemical pathways, the genetic analysis of production is extremely difficult and the heterologous expression of these toxins is currently impossible. In contrast, Ptr ToxA, a HST produced by the fungus *Pyrenophora tritici-repentis,* is the protein product of a single gene (Ciuffetti *et al.*, 1997). This protein was purified and shown to be a 13.2 kD, heat-stable polypeptide which induces necrosis when infiltrated into sensitive wheat cultivars (Tuori *et al.*, 1995). The transformation of a nontoxin-producing, nonpathogenic isolate of the fungus to a toxin-producing, pathogenic state was facilitated by the fact that Ptr ToxA is the product of a single gene, and demonstrated, unequivocally, the causal role of a HST in pathogenicity (Ciuffetti *et al.*, 1997).

Our objectives in this study were four-fold: 1) To facilitate the production of abundant amounts of toxin protein that could be used for disease screening; 2) To produce biologically-active derivatives of Ptr ToxA that could be used for receptor-binding experiments and to assess whether Ptr ToxA undergoes proteolytic processing in *planta*; 3) To help elucidate the functional role of the pro-sequence of the *ToxA* gene; and, 4) To assess the role post-translational processing plays, if any, in the necrosis-

inducing activity of Ptr ToxA. The approach taken to accomplish these goals was the heterologous expression of the *ToxA* gene in *Escherichia coli.*

P. tritici-repentis causes the economically important disease, tan spot of wheat that has been reported worldwide and can lead to significant yield losses (Hosford, 1982). The occurrence of this disease is likely to increase as conventional agriculture shifts to no-till practices (Schuh, 1990; Bailey and Duczek, 1996). Although breeding wheat for resistance to tan spot is currently a major goal of wheat breeders (Cox *et al.*, 1988; Riede *et al.*, 1996), disease screening of wheat in the field for susceptibility to *P. tritici-repentis* is difficult due to environmental fluctuations (Francl, 1995). However, because only those wheat cultivars that are sensitive to the Ptr ToxA protein respond to infection by *P. tritici-repentis* with tan necrosis, screening of wheat for sensitivity to Ptr ToxA provides an easier and more reliable method to identify resistant germplasm than screening by fungal inoculations (Riede *et al.*, 1996). Ptr ToxA can be purified from the cultures of *P. tritici-repentis* yielding an average of approximately 6 mg of purified toxin from a liter of fungal culture filtrate (Tuori *et al.*, 1995). This method, although reliable, is very time-consuming and labor-intensive. It was reasoned that expression of Ptr ToxA in *E. coli*, would provide an easier, less-expensive method for production of toxin that could be used for germplasm screening.

Biologically-active derivatives of Ptr ToxA would provide the tools to initiate a study of the fate of Ptr ToxA in *planta.* Active toxin derivatives that could be radiolabeled, biotinylated and/or tagged as a fusion protein would be advantageous over native Ptr ToxA for receptor-binding experiments because they would allow for several possible ways of detection and affinity-isolation of the protein, and potentially its

receptor. Furthermore, certain derivatives, such as an active fusion protein, could provide the additional advantage that the biologically inactive portion could be detectable even if biologically active portion of the toxin was sterically unavailable for binding due to an interaction in *planta*. In addition, a fusion protein could be useful for assessing whether Ptr ToxA undergoes any detectable processing in *planta*, such as proteolytic cleavage, as both regions of the bipartite fusion protein could be immunologically detected. We hypothesize a very specific interaction occurring between the Ptr ToxA protein and a putative receptor protein, likely on the susceptible wheat cell surface. Alternatively, Ptr ToxA could be proteolytically cleaved, yielding an active peptide that is either taken into the plant cell or interacts with a cell surface receptor.

The heterologous expression of Ptr ToxA was also attempted to help elucidate the functional role of the pro-sequence of the *Ptr ToxA* gene. The *ToxA* open reading frame (ORF) encodes a preproprotein, with an N-terminal signal peptide, followed by a dual domain protoxin (Ciuffetti *et al.,* 1997). The protoxin consists of a ~4.3 kD anionic N-terminal domain, or pro-region, of unknown function and destiny, and a 13.2 kD cationic C-terminal domain that is the mature, secreted Ptr ToxA protein. During the purification of Ptr ToxA protein, we observed an anionic toxic component that was chromatographically, electrophoretically and immunologically distinct from Ptr ToxA (Tuori *et al.*, 1995). Preliminary attempts to characterize the anionic toxic component indicated that it was likely proteinaceous and smaller than Ptr ToxA. The possibility that the N-domain of the protoxin is the observed anionic toxic component was investigated by attempting to express the N-domain of this ORF separately. Pro-regions are common on secreted proteins and they have been hypothesized to: 1, assist in the correct folding of

proproteins; 2, aid in the secretion of small peptides; and 3, prevent formation of active structures prior to proteolytic cleavage and secretion (Eder and Fersht, 1995). The role that the N-domain plays in both the correct folding of Ptr ToxA and/or the suppression of activity prior to final processing was investigated by expressing the protoxin, or N- and C-domains together, in *E. coli.*

Finally, the role that post-translational processing plays, if any, in the necrosis-inducing activity of Ptr ToxA could be assessed by whether an active form of the protein could be produced from *E. coli.* Because it is highly unlikely that correct processing of a eukaryotic protein would occur in a prokaryotic system, it was reasoned that if an active form of the toxin was obtained, any processing that may occur in the native fungus would not be essential for the full activity of Ptr ToxA.

Methods

Plasmid Construction

All expression constructs were produced in the bacterial expression vector pTrc99A (Pharmacia), which directs the expression of N-terminal polyhistidine-tagged (HIS-tagged) fusion proteins in *E coli.* Initial plasmids were constructed to express (1) the N-domain of the *ToxA* ORF (N-FP), (2) the C-domain of the *ToxA* ORF (C-FP) and (3) the N- and C-domains of the *ToxA* ORF together (NC-FP), each as a HIS-tagged fusion protein (Figure 4.1). The C-FP was designed so that the C-domain was expressed with a lysine residue prior to the Ptr ToxA sequences. This would allow for biotinylation of this fusion protein, as no lysine residues are present in Ptr ToxA. The amino-terminal

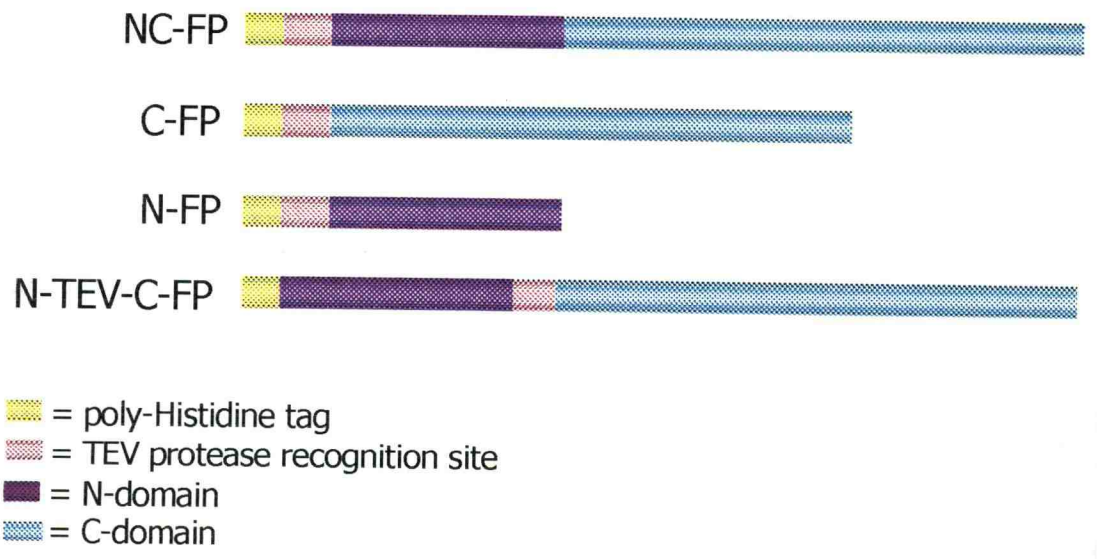


Figure 4.1. Fusion Protein Constructs. An illustration of the four fusion proteins expressed in *E. coli* is shown with the constituent domains of the *ToxA* ORF and the locations of the polyhistidine tag and the TEV protease recognition motif indicated. All constructs were produced in vector pTrc99A.

position of the N-domain, or pro-region of the Ptr ToxA protoxin, was based on signal peptide consensus sequence analysis (von Heijne, 1986) and was predicted to be amino acid residue Asp-23 (Ciuffetti *et al.,* 1997). Based on molecular weight prediction of the C-domain ORF, the amino-terminal position of the C-domain was predicted to be amino acid residue Gln-61 (Ciuffetti *et al.*, 1997). For each construct, primers were designed such that the PCR-amplified portion of the *ToxA* gene was generated with a 5' *Nco* I site and 3' *Bam* HI site. In addition, all 5'-primers for the generation of insert DNA were designed to encode a TEV protease recognition site (Parks *et al.*, 1995) which would separate the HIS-tag and the portion of the *ToxA* ORF. The PCR products were digested with *Nco* I and *Bam* HI, gel-purified with the QIAquick Gel Extraction Kit (Qiagen) and cloned into the *Nco* I and *Bam* HI sites of pTrc99A. Primer 15 (5'-GGG GCC ATG GGG GAG AAT CTT TAT TTT CAG GGC GAT CCC GGT TAC GAA-3') and primer 14B (5'-GCT TGG ATC CTA ATT TTC TAG CTG CAT TCT-3') were used to amplify the insert for the NC-FP expression vector (pCT68). Primer 23 (5'-GGG GCC ATG GGG GAG AAT CTT TAT TTT CAG GGC *AAA* CAG GGA AGC TGC-3') and primer 14B were used to amplify the insert for the C-FP expression vector (pCT70). Primer 23 was designed to encode a lysine codon (shown in italics) immediately following the TEV recognition site. Primer 15 and primer 24 (5'-CCC CGG ATC CTA CCG CTC CTG TAG GAG-3') were used to amplify the insert for the N-FP expression vector (pCT71). Bold letters indicate those bases within the primer which encode the TEV recognition site (amino acid sequence GENLYFQ↑G) and underlined letters indicate the *Nco* I and *Bam* HI restriction sites. Unless otherwise stated all PCR reactions were performed with a standard reaction mix [50 nM each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 1X Taq

reaction buffer, and 1 unit Taq polymerase (Promega)]. Following two minutes at 94 °C, reactions were cycled five times through 30 sec at 94 °C, 30 sec at 50 °C, and 1 min at 72 °C, and then an additional thirty times through 30 sec at 94 °C, 30 sec at 60 °C, and 1 min at 72 °C.

An additional HIS-tagged fusion protein construct was designed that would express the N- and C-domains of the *ToxA* ORF separated by the TEV recognition motif (N-TEV-C-FP, Figure 4.1). The insert for the plasmid (pCT75) that would direct the expression of this fusion protein was generated through initial PCR-amplification of the N- and C-domains separately. The primers for this amplification were designed such that the 3'-end of the coding strand of the N-domain product would be complementary to the 5'-end of the noncoding strand of the C-domain product, with the complementary region being the TEV recognition site. The complementary ends were utilized in a second PCR reaction to prime each other and create a full length product with an internal TEV site. The N-domain was amplified with primer 32 (5'-GGG GCC ATG GAT CCC GGT TAC GAA ATC GTT-3') and primer 30 (5'-TCC CTG **GCC CTG AAA ATA AAG ATT CTC CCC** CCG CTC CTG TAG GAG TCC GCG-3'). The C-domain was amplified with primer 31 (5'-GAG CGG **GGG GAG AAT CTT TAT TTT CAG GGC** CAG GGA AGC TGC ATG TCA ATC-3') and primer 14b. Bold letters, again, indicate those bases within the primer that encode the TEV recognition site and underlined letters indicate the *Nco* I and *Bam* HI restriction sites. The two PCR products were gel-purified as described, and approximately 10 ng of each were then used for a second PCR reaction in standard PCR reaction mix, with the exception that no primers were used in the reaction. Following two minutes at 94 °C, the reaction was cycled two times through 30

sec at 94 °C, 30 sec at 50 °C, and 1 min at 72 °C, and then an additional three cycles through 30 sec at 94 °C, 30 sec at 60 °C, and 1 min at 72 °C. The 50 µL PCR reaction was then supplemented with stocks of primers 32 and 14B, dNTPs, $MgCl_2$, 10X buffer and Taq polymerase such that the final volume of 100 µL was a standard PCR reaction mix. The reaction was cycled thirty times through 30 sec at 94 °C, 30 sec at 60 °C, and 1 min at 72 °C. The full length N-TEV-C PCR product was gel-purified, digested with *Bam* HI and *Nco* I and cloned into the *Nco* I and *Bam* HI sites of pTrc99A resulting in pCT75. TEV protease was obtained from GibcoBRL and used according to the manufacturer's instructions except that 10 mM β-mercaptoethanol instead of DTT was used, and reactions were performed at 16 °C overnight.

All ligations and bacterial transformations were performed as described by Sambrook *et al.* (1989). Plasmids were transformed into *E. coli* cells [BL21(DE3) pLys] carrying the gene for T7 lysozyme. Colonies expressing the NC-FP, C-FP, and N-TEV-C-FP were screened for protein production by conventional colony blot procedures (Sambrook *et al.*, 1989) with anti-Ptr ToxA polyclonal antisera and alkaline phosphatase-conjugated secondary antibody (Promega). Colonies expressing the N-FP were screened with an alkaline phosphatase-nickel conjugate, NiNTA-AP (Qiagen) according to the manufacturer's suggestions.

Purification of Fusion Proteins

E. coli cultures harboring pCT68, pCT70, pCT71, and pCT75 were grown overnight in 5 mL LB (Sambrook *et al.*, 1989) with 100µg/mL ampicillin (LBA) at 37 °C with shaking at 250 RPM. The following morning, 50 mL of fresh LBA were inoculated with 500 µL of the overnight culture and incubated shaking at 37 °C for 1 hr. Fusion

protein expression in cells was induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the cultures were incubated for an additional 3 hr under the same conditions. Cells were harvested by centrifugation at 4000 x g, 4 °C for 20 min and the pellets were resuspended in sonication buffer (50 mM Na_2HPO_4, 5 mM Tris-base, 300 mM NaCl, pH 8.0) with 0.1% Triton-x-100 and 5% 2-mercaptoethanol. Resuspended pellets were sonicated three times, 30 sec each, with 2 min on ice between periods. Insoluble material was then pelleted by centrifugation at 10,000 x g, 4 °C, for 20 min. Pellets were washed three times with sonication buffer with 0.1% Triton-x-100 and 5% 2-mercaptoethanol, and three times with sonication buffer with 5% 2-mercaptoethanol. The final washed pellets, containing inclusion bodies, were resuspended in 1 mL 6 M urea (Sigma) in renaturation buffer (200 mM NaCl, 100 mM Na_2HPO_4, 10 mM Tris-base, 5% glycerol, 0.005 % Tween-20, pH 8.0). All urea used during this study was deionized with AG-501-X8 mixed-bed resin (BioRad), degassed under vacuum and stored at –20 °C. Prior to dialysis, 8M urea stocks were diluted with water and 5X renaturation buffer to give the appropriate urea concentration in 1X renaturation buffer and then the solution was purged of O_2 by bubbling with N_2 for approximately 2 min. Proteins were quantitated with Bradford Assay Reagent (BioRad).

Fusion Protein Refolding

Fusion proteins solubolized in urea were renatured *in vitro* with urea removal accomplished by dialysis. All dialyses were performed in beakers at 4 °C, stirring slowly over the course of four days against 100 mL of renaturation buffer containing decreasing amounts of urea. Prior to refolding, solubolized fusion protein samples were diluted with

8 M urea, 5X renaturation buffer and water, such that the final volume was ½ mL and the final protein concentration was 100 ng/μL in 1X renaturation buffer with 6M urea. The samples were applied into a SpectraPor 3500 MW cut-off membrane and dialyzed overnight against 1X renaturation buffer with 6M urea with one identical buffer change the following morning followed by dialysis for an additional 4 hr to remove residual 2-mercaptoethanol. The buffer was replaced with 1X renaturation buffer with 4M urea and dialyzed for an additional 24 hr. This was repeated again with 1X renaturation buffer with 2M urea, and with 1X renaturation buffer without urea. After the final buffer change, the samples were dialyzed an additional 4 hr against 100mL 1X renaturation buffer with 2 mM EDTA and 100 μg/mL PMSF. Refolding experiments that included reducing and oxidizing reagents contained 2 mM reduced glutathione (Sigma) and 0.02 mM oxidized glutathione (Sigma) throughout the dialyses except for the last dialysis step, where both were omitted. Following the final dialysis, fusion protein samples were assayed for necrosis-inducing activity by infiltrating samples into sensitive (TAM-105) and insensitive (Auburn) wheat cultivars, as described (Tuori *et al.*, 1995). N-TEV-C-FP, following digestion with TEV protease, was also infiltrated into wheat cultivar Norkan, the cultivar used in a previous study to assay for the anionic toxic component (Tuori *et al.*, 1995). SDS-PAGE and western analyses were performed as described (Tuori *et al.*, 1995).

Heat and Reduction Stability

NC-FP and Ptr ToxA at approximately equivalent necrosis-inducing concentrations, 5 μM and 1 μM, respectively, were treated by boiling for 10 min with or without 10 mM 2-mercaptoethanol. The samples were cooled and infiltrated. Both

proteins were also treated in a reducing environment of 10 mM 2-mercaptoethanol overnight at 4 °C and then assayed for activity by infiltration.

Results

Both the NC-FP and the C-FP were produced by *E. coli* in abundance and purified in large quantities, resulting in approximately 2-4 mg per 50 mL bacterial culture (~40-80 mg/L). Following refolding, the NC-FP was approximately 10-20% as active as native Ptr ToxA, while the C-FP displayed only traces of necrosis-inducing activity (Figure 4.2). Removal of the HIS-tag with TEV protease had no effect on the necrosis-inducing activity of either fusion protein. The amount of activity recovered with the NC-FP was independent of the redox state in the dialysis refolding procedure (Figure 4.2). In contrast, what little activity was obtained with the C-FP was absent when redox reagents were present during the refolding procedure (Figure 4.2). Although the HIS-tag on these fusion proteins was initially incorporated for purification with a NiNTA-agarose resin, this was found to be unnecessary due to the accumulation of these proteins as essentially homogeneous aggregates in inclusion bodies. Inclusion bodies isolated from sonicated cells comprised apparently pure fusion protein that was easily solubolized in 6M urea. Purified NC-FP and C-FP appear as the predicted ~19.5 kD and ~15 kD bands in SDS-PAGE, respectively, and both react with antisera raised against purified Ptr ToxA in protein gel blot analysis (Figure 4.3). Separation of *E. coli* lysate into soluble and insoluble fractions demonstrated that both proteins were found almost exclusively in the insoluble fraction whether cells were grown at 30 °C or 37 °C. The refolding procedure utilized in this study resulted in almost complete recovery of soluble protein from the insoluble fraction.

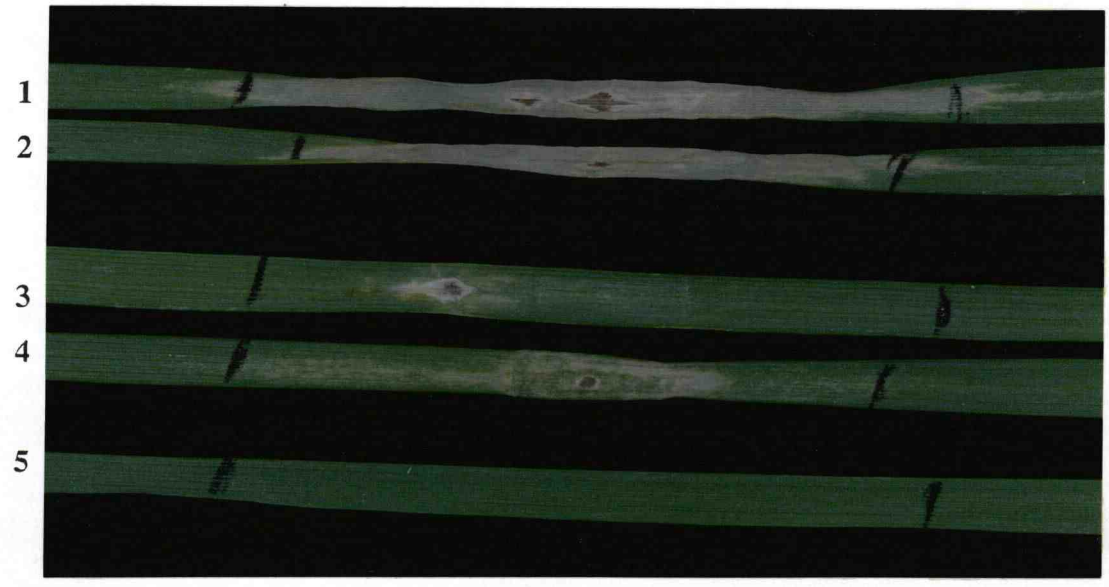


Figure 4.2. Leaf Infiltration Bioassay of Refolded NC-FP and C-FP. Leaves 1 through 4 are the sensitive genotype (TAM-105); leaf 5 is the insensitive genotype of wheat (Auburn). Leaves 1 and 2 were infiltrated with NC-FP at 5 μM, with the sample infiltrated into leaf 1 renatured in the absence of redox reagents and the sample infiltrated into leaf 2 renatured with the presence of redox reagents. A typical necrosis reaction is shown, identical to that induced by ~$1/5^{th}$ to $1/10^{th}$ the concentration of Ptr ToxA. Leaves 3 and 4 were infiltrated with C-FP at 5 μM, with the sample infiltrated into leaf 3 renatured in the presence of redox reagents and the sample infiltrated into leaf 4 renatured with the absence of redox reagents. Leaf 5 was infiltrated with NC-FP at 5 mM, showing a typical insensitive reaction.

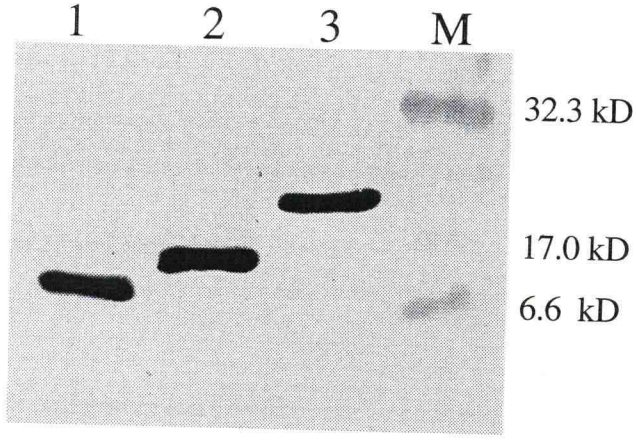


Figure 4.3. Protein Gel Blot Analysis of Refolded Fusion Proteins. Five-hundred ng samples of Ptr ToxA (lane 1), C-FP (lane 2), and NC-FP (lane 3) were detected with anti-Ptr ToxA antibodies, following electrophoresis in a 17% SDS-polyacrylamide gel and electroblotting onto a nitrocellulose membrane. Rainbow Protein Molecular Weight Markers (BioRad) are shown in lane M.

The N-FP was not produced in detectable quantities. Upon induction, cultures harboring pCT71 appeared to stop growing and the appropriate size fusion protein was never detected in protein gel-blot analysis with an alkaline phosphatase-Ni conjugate (Qiagen) for detection of polyhistidines.

The NC-FP displays heat stability and reduction sensitivity essentially similar to native Ptr ToxA (Figure 4.4). Boiling for 10 min or incubation in a reducing environment does not affect the amount of activity of either protein. However, boiling in the presence of a reducing agent eliminates all Ptr ToxA activity and the great majority, although not all, of NC-FP activity.

The N-TEV-C-FP was purified and refolded exactly the same as the NC-FP, yielding equivalent amounts, however, it appeared to be slightly less active than the NC-FP upon infiltration into either TAM-105 or Norkan leaves. Because these studies are currently in progress, no data is shown regarding the N-TEV-C-FP. Proteolytic cleavage at the TEV recognition site between the N- and C-domains of this fusion protein resulted in approximately 10% of the protein being cleaved. Infiltration of partially cleaved N-TEV-C-FP into TAM-105 and Norkan leaves did not result in an increase in the amount of necrosis-inducing activity.

Discussion

This study describes the heterologous production of an active form of the Ptr ToxA protein. The NC-FP can be isolated from *E. coli* cells at approximately ten times the quantities that Ptr ToxA is purified from cultures of *P. tritici-repentis* and with significantly less technical effort. Although results from the bioassay used for toxin

Figure 4.4. Heat and Reduction Sensitivity of Ptr ToxA and NC-FP. Leaves of the sensitive genotype of wheat (TAM-105) are shown following infiltration with samples of NC-FP at 5 μM (leaves 1 through 4) and Ptr ToxA at 1 μM (leaves 5 through 8). Samples 1 and 5 are controls (no treatment), samples 2 and 6 were boiled for 10 min prior to infiltration, samples 3 and 7 were treated with reducing agent prior to infiltration, and samples 4 and 8 were boiled for 10 min in the presence of reducing agent prior to infiltration.

activity, the leaf infiltration assay, are difficult to quantitate, the NC-FP appears to have only 10-20 % the activity of native Ptr ToxA (Fig. 4.2). Alternatively, it could be interpreted that the presence of the N-domain on the NC-FP results in ~80-90% inhibition of the activity of this protein. However, N-TEV-C-FP following digestion with TEV protease, has an equivalent level of activity as the undigested fusion protein (data not shown), indicating that the presence of the N- and C-domains in *cis* does not affect the activity of the C-domain. The level of activity of the NC-FP is sufficiently high that it could be useful for routine screening of wheat cultivars for toxin sensitivity, eliminating the need for both standard toxin purification and/or conidial inoculations to screen for disease susceptibility.

The fact that the NC-FP and not the C-FP is active was surprising, however, not without precedent. Several pro-proteins have been expressed as active forms while expression of the mature portion yields only inactive protein (Fukuda *et al.*, 1994; Doyle and Smith, 1996). Indeed, prosequences appear to be particularly important for *in vitro* refolding. The pro-sequence of *Rhizopus niveus* extracellular aspartic proteinase-I was shown to be essential for refolding of the denatured mature enzyme *in vitro* (Fukuda *et al.*, 1994). Similarly, the *Phanerochaete chrysosporium* lignin peroxidase isoenzyme H8 was expressed in *E. coli* as a pro-protein and refolding *in vitro* yielded active enzyme whereas attempts at expression utilizing the mature sequences generated inactive enzyme following *in vitro* refolding experiments (Doyle and Smith, 1996). That NC-FP and not the C-FP is active indicates two important points regarding the function of the N-domain: 1, It likely facilitates the correct folding of the protoxin; and 2, It does not function to eliminate the formation of active toxin prior to proteolytic cleavage. It would, in fact, be

extremely unlikely that Ptr ToxA in any form would be toxic to *P. tritici-repentis*, since, to this date, HSTs (with the exception of HC-toxin produced by *Cochliobolus carbonum*) have been found to be toxic only to the plant hosts susceptible to the toxin-producing fungi (Walton, 1996).

Although, examples of prosequences functioning to modulate activity of the mature protein can be found, such as the *Rhizopus oryzae* extracellular lipase (Beer *et al.*, 1996), where the prosequence prevents mature lipase from damaging the host, the function of the Ptr ToxA prosequence appears similar to the cytotoxic protein restrictocin, produced by *Aspergillus restrictus*. Active pro-restrictocin can be produced both in a heterologous *in vivo* system and in coupled *in vitro* transcription and translations, indicating the pro-region plays no role in inactivating this protein (Yang and Kenaely, 1992). Therefore, the results of over-expression and *in vitro* refolding of the C-FP and NC-FP indicate that the N-domain appears to function not to suppress activity prior to ultimate processing, but to facilitate the correct folding of the protoxin, at least *in vitro* and in *cis*.

The recovery of active NC-FP from *E. coli* also indicates that whatever post-translational or co-translational processing that may occur to this protein in *vivo* prior to secretion is not essential for activity, but could be important for full activity. However, it is most likely that no extensive modifications of Ptr ToxA occur at all. Mass Spectral analysis indicates a molecular weight of approximately 13,208 D for the mature Ptr ToxA (Tuori *et al.*, 1995). Molecular weight calculations for the predicted polypeptide from the *ToxA* ORF indicate that the most likely proteolytic cleavage site within the protoxin would occur after arginine-60, resulting in a mature Ptr ToxA with glutamine-61 at its N-

terminus (Ciuffetti *et al.*, 1997). The cyclization of a glutamyl residue at the N-terminus of a protein, either spontaneously or enzymatically, can result in the formation of a pyroglutamyl residue (Harris, 1989). We have preliminary peptide sequencing data indicating that the N-terminus of Ptr ToxA is a pyroglutamyl residue (data not shown). This would yield a mature polypeptide with a molecular weight extremely close to that predicted by Mass Spectral analysis. Therefore, the Mass Spectral data do not support the possibility of post-translational modifications of the C-domain.

The effect of redox reagents during refolding on activity of the NC-FP and C-FP is somewhat difficult to interpret. Because the *ToxA* ORF predicts two cysteine residues (positions 64 and 160) within the C-domain, and heat in the presence of reducing agents eliminates all toxic activity of Ptr ToxA (Fig 4.4), results strongly suggest that there is a disulfide bond within Ptr ToxA that is essential for activity. Therefore, it was assumed that redox reagents during folding would be necessary for the formation of this bond (Marston and Hartley, 1990), as has been found for the proper folding of other disulfide containing over-expressed proteins (Whitman and Tien, 1996; Beer *et al.*, 1996). This, however, is not the case with the NC-FP. We hypothesize a very compact tertiary structure for Ptr ToxA (or the C-domain) with the disulfide maintaining this conformation from the interior of the protein, and therefore, not accessible to reducing agents in solution. The prosequence (or N-domain) likely allows the protoxin (or the NC-FP) to assume a conformation that brings the two cysteine residues of the C-domain into close proximity to each other, allowing the disulfide to form. Presumably, this conformational change cannot happen in the absence of the N-domain due to insurmountable structural barriers. Because the NC-FP can correctly and efficiently refold on its own, it could be

reasoned that it might be resistant to the effects of redox reagents, either positive or negative. The scant amount of activity seen with the C-FP when redox reagents are omitted from the renaturation procedure could be due to a very small amount of correct folding of the C-FP during renaturation and without the N-domain this protein would be sensitive to reduction by redox reagents. It should be mentioned that the amount of activity observed with the NC-FP after refolding experiments was not consistent when reducing agents were omitted from the inclusion body preparations. This could be due to the oxidation of cysteine residues prior to the reformation of the disulfide bond. Redox reagents, clearly however, are not necessary to promote reformation of the disulfide bond leading to restoration of activity of NC-FP, as was initially expected.

It appears that the NC-FP will be a useful tool for binding studies to identify a potential wheat receptor protein because the NC-FP can be purified in abundance and has similar activity to Ptr ToxA. The N-domain, in itself, can be considered a label. Because it is not involved in the binding of the mature Ptr ToxA polypeptide to its putative receptor, we speculate that this portion of the NC-FP could be labeled with very little, if any, effect on activity of the fusion protein. The N-domain has two lysine residues, which can be specifically biotinylated, and if necessary iodinated. In addition, as an alternative method of detection, antibodies are presently being developed against the NC-FP. This will presumably allow for the immunological detection of the N-domain even if much of the C-domain is sterically unavailable for detection due to an interaction in *planta.*

The inability to detect N-FP from cell lysates of cells harboring pCT71, could be due to this peptide being toxic to *E. coli*, or being particularly unstable. It is interesting to note that attempts to express the N-domain in *E. coli* as an N-terminal biotinylated fusion

protein with the Promega Pinpoint expression vector also resulted in the inability to detect the appropriate size fusion protein in cell lysates (data not shown). Toxicity of certain eukaryotic proteins, however, is not uncommon during prokaryotic expression.

Work with the N-TEV-C-FP is currently in progress and will continue. A large scale digestion of N-TEV-C-FP with TEV protease will be performed, and the resulting C and N-TEV fragments will be separated by either gel-electrophoresis, size-exclusion chromatography or ion-exchange chromatography, and assayed for activity. Although the level of activity of the N-TEV-C-FP did not increase following digestion with TEV protease, indicating both that the N-TEV fragment is apparently not active and that the liberated C-domain fragment is no more active than the undigested N-TEV-C-FP, future work will continue to address the unanswered question of whether the N-domain is the observed anionic toxic component. An N-glycosylation motif is found in the N-domain, and it is possible that this site needs to be modified for necrosis-inducing activity, if any, of the N-domain, and also for the most efficient folding of the protoxin.

In summary, an active form of the Ptr ToxA protein was expressed in *E. coli*. The NC-FP, consisting of both the N- and C-domains of the Ptr ToxA ORF, was ~10-20 % as active as native Ptr ToxA indicating two points: 1, the N-domain appears to function to facilitate the correct folding of the Ptr ToxA protoxin at least *in vitro* and in *cis*; and 2, the N-domain does not function to eliminate activity prior to proteolytic processing. The NC-FP will be useful for both receptor binding studies and for screening wheat cultivars for toxin sensitivity.

Chapter 5

Summary

Conclusions

The identification and characterization of pathogenicity factors are essential to our understanding of the molecular events that determine the consequences of host-pathogen interactions. Fungi that produce host-selective toxins (HSTs) are ideal organisms to address this objective because HSTs are readily identifiable pathogenicity factors and are believed to play a causal role in plant pathogenesis (Yoder, 1980; Walton, 1996). In contrast to other known HSTs, Ptr ToxA, produced by the fungus *Pyrenophora tritici-repentis,* is the protein product of a single gene. This system, therefore, provided a unique opportunity for the genetic analysis of the biosynthesis of this HST and its effect on the development of an economically important crop disease.

This work described the purification, and biochemical and immunological characterization of the major HST, which we designated ToxA, from cultures of *P. tritici-repentis*. Polyclonal antibodies specific for Ptr ToxA were used to immunologically characterize toxic protein fractions of *P. tritici-repentis* culture filtrates, providing evidence that other necrosis-inducing components, both cationic and anionic in nature, are produced by the fungus. Characterization of the Ptr ToxA protein greatly facilitated the molecular genetical analysis of the corresponding toxin gene, which was shown to function as a primary determinant of pathogenicity in the *Pyrenophora*-wheat interaction (Ciuffetti *et al.*, 1997). Also in this study, the development of a heterologous system for the high level expression, purification and renaturation of a cultivar-specific, active form of the Ptr ToxA protein in *E. coli* was reported. These experiments provide evidence

regarding the function of the N-domain and suggest that post-translational modifications of Ptr ToxA are not essential for activity.

Race Differentiation and Toxin Production

Previous reports indicated that several pathotypes (later designated races) of the fungus are distinguishable by the types of toxins they produce (Lamari and Bernier, 1989b; Lamari *et al.,* 1995). Race 1 induces both tan necrosis and extensive chlorosis in susceptible wheat cultivars, race 2 induces only necrosis, race 3 induces only chlorosis, and race 4 is nonpathogenic and elicits neither reaction. A fifth race of the fungus has been identified which also induces chlorosis, however, in different wheat cultivars than race 1 and 3 (Lamari *et al.*, 1995). Interestingly, race 3 and 5 isolates also induce necrosis in some tetraploid wheat cultivars (Lamari and Bernier, 1989b; Lamari *et al.*, 1995).

The major necrosis-inducing toxic protein has been purified by four groups (Ballance *et al.,* 1989; Tomas *et al.,* 1990; Tuori *et al.*, 1995; and Zhang *et al.*, 1996). Both Ballance *et al.* (1989) and Zhang *et al.* (1996) used the same fungal isolate (86-124), designated as race 2, and both groups, therefore, are presumably working with the same protein. Most of our work (Tuori *et al.*, 1995) was done with isolate BFP, a fast growing, high toxin producing, subculture of the isolate used by Tomas *et al.* (1990) for their protein purifications. We, therefore, are presumably working with the same protein as Tomas *et al.* (1990). Prior to the cloning of the *ToxA* gene (Ciuffetti *et al.*, 1997) and the subsequent online report of the cloning of a cDNA encoding the Ptr necrosis toxin (Ballance *et al.*, 1996), there was speculation that the Ciuffetti (Ciuffetti *et al.*, 1997) and Lamari (Ballance *et al.*, 1996) groups may have purified different toxins (Tuori *et al.*, 1995). However, comparison of both the nucleotide sequence and deduced amino acid

sequence from both reports indicate that the same gene had been cloned and, very likely, the same protein had been purified as well. Discrepancies regarding levels of activity and heat stability in earlier reports, therefore, must be due to differences in cultivars used or assay conditions.

We have not independently confirmed the extensive chlorosis reaction described by Lamari and Bernier (1989b, 1991) as being both symptomatically and genetically distinct from the necrosis reaction. To date, we have not observed extensive chlorosis in several wheat lines that have been described as responding specifically with chlorosis (nec^-chl^+), or with necrosis and chlorosis (nec^+chl^+) to race 3 isolates of the fungus (Lamari and Bernier, 1989a) when infiltrated with culture filtrates from several of our isolates. Also, culture filtrate from our standard isolate (BFP) does not induce chlorosis in two wheat lines (Neepawa and Katepwa, nec^+chl^+) that were described as responding differentially with chlorosis to race 5 isolates (Lamari *et al.*, 1995; Orolaza *et al.*, 1995). Therefore, it appears that BFP is, like the majority of *P. tritici-repentis* isolates identified, a race 2 isolate and consequently, does not produce a chlorosis toxin. Alternatively, BFP could be a member of a distinct race not yet characterized.

The relation of Ptr ToxA to the other toxic activities (AI and CI) observed in culture filtrates of isolate BFP (Tuori *et al.*, 1995), and to the chlorosis toxin(s) produced by races 1, 3, and 5 remains unknown. A simple explanation would be that this fungus makes several toxins and that different races make different combinations of toxins. There is no reason to assume that there are only five races of the fungus. As culture filtrates and/or conidia are assayed on more wheat cultivars and the reactions closely observed, more races may be identified. Another possibility that has not yet been ruled

out is that multiple toxins could be conditioned by the same gene. Potential alternate proteolytic cleavage of the Ptr ToxA precursor protein, or alternate splicing of the *ToxA* transcript could result in proteins that are significantly different, both chemically and immunologically, yet retain an affinity for the same receptor as Ptr ToxA and, therefore, elicit the same necrotic response.

Proposals for future work

In summary, this work describes the biochemical and molecular genetic analysis of Ptr ToxA, and provides the foundation for the next step of this project, that is, studies on the site and mode of action of Ptr ToxA in susceptible wheat. Procedures for purifying Ptr ToxA have been optimized and a heterologous expression system makes production of the protein straightforward. The importance of Ptr ToxA in pathogenesis has been established and, therefore, the search for its putative receptor becomes more meaningful. An immediate goal for future research is to label either native or heterologously produced Ptr ToxA, and begin receptor binding experiments. Initially, the NC-FP will be biotinylated, and if necessary for detection purposes, radiolabeled. This derivative will be used as a probe with either wheat plasma membrane fractions or protoplasts to assess optimal binding conditions. If this is unsuccessful due to the low specific activity of the fusion protein relative to the native toxin, a mutagenized form of toxin with an added lysine residue (for biotinylation) will be expressed from the tox$^-$ isolate, purified, and labeled. Ligand specificity will be assessed by competition assays using unlabeled toxin or fusion protein. Either form of toxin will be derivatized with a heterobifunctional cross-linker that can be used to photo-affinity label potential interacting wheat proteins. Covalently crosslinked proteins could be affinity

purified and analyzed. This could lead ultimately to the cloning of the receptor gene for Ptr ToxA.

Additional future research should focus on whether multiple toxins or forms of Ptr ToxA are conditioned by the *ToxA* locus. Tox$^-$ isolates transformed with the *ToxA* gene will be screened for production of toxic activities. If necrosis or chlorosis-inducing activities besides Ptr ToxA are observed, this would provide evidence implicating the *ToxA* gene in their production. Truncated forms of Ptr ToxA, generated by either 5'- or 3'- deletions in the ORF, could be expressed from the fungus to determine whether a smaller cationic peptide could be responsible for the CI-fraction activity. Alternatively, the *ToxA* gene from a tox$^+$ isolate could be disrupted and the toxic components produced by the transformed fungus assessed. If necrosis or chlorosis-inducing activities are observed, this would be good indication that other genes are involved in the production of these toxins.

The function and fate of the N-domain of the ToxA ORF will continue to be investigated. Antibodies are being developed against the NC-fusion protein and will be used to assess whether the N-domain peptide is present in culture filtrates of the fungus. If it is detected, the N-domain peptide could then be affinity purified utilizing the antisera developed against it, and its biological activity assayed. This antibody will also be useful for analyzing culture filtrate from a tox$^-$ isolate that has been transformed with a genomic construct of the *ToxA* gene completely missing the C-domain. This construct has been completed, and a fungal transformant is available. However, preliminary studies indicate that no activity is detectable in culture filtrate of the transformant. Immunological analyses with this transformant may provide information regarding the possible secretion

of this peptide fragment. Another approach to studying the N-domain, would be to express an uncleavable form of toxin from the fungus that could be proteolytically cleaved following purification. Expression of an N-TEV-C recombinant protein from the fungus would allow for the production and secretion of N-TEV-C toxin into culture filtrates after potential post-translational processing of the N-domain, such as glycosylation. If cleavage between the N- and C-domains of this recombinant protein does not occur prior to secretion, it could be purified, cleaved with TEV protease and assayed for activity.

Lastly, an area that this work leaves in a particularly unanswered state is the possibility of sequence polymorphisms in the *ToxA* locus from *P. teres*. Southern blotting will be performed to determine, unequivocally, that this gene has been amplified from the *P. teres* genome and not from contaminating DNA. The *P. teres ToxA* allele will be cloned from a subgenomic library and its 5'- and 3'-flanking sequences determined. Presumed sequence polymorphisms in flanking regions could provide insight into the regulation of *ToxA*, and also the evolutionary origin of the *ToxA* gene, as no significant homology to other genes or proteins has yet been obtained from database searches. Although selected barley cultivars do not appear to be sensitive to Ptr ToxA, clues to the function of Ptr ToxA may be gleaned by assessing the regulation of the *P. teres* gene during barley infection and when grown in different culture media. Although no significant homology to other genes has been observed in database searches to

date repeated searches may, in the future, result in the identification of a protein or domain of a protein with notable similarity. This could very well provide insight into the toxin's mode of action, and lead ultimately to the identification of the gene conditioning sensitivity to this toxin.

Bibliography

Ahn, J.H., and Walton, J.D. 1997. A fatty acid synthase gene in *Cochliobolus carbonum* required for production of HC-toxin, cyclo(D-prolyl-L-alanyl-D-alanyl-L-2-amino-9,10-epoxi-8-oxodecanoyl). *Molecular Plant-Microbe Interactions* 10: 207-214.

Ahn, J.H., and Walton, J.D. 1996. Chromosomal organization of *TOX2*, a complex locus required for host-selective toxin biosynthesis in *Cochliobolus carbonum. Plant Cell* 8: 887-897.

Bailey, K.L. and Duczek, L.J. 1996. Managing cereal diseases under reduced tillage. *Canadian Journal of Plant Pathology* 18: 159-167.

Ballance, G. M., Lamari, L., and Bernier, C. C. 1989. Purification of a host-selective necrosis toxin from *Pyrenophora tritici-repentis. Physiological and Molecular Plant Pathology* 35: 203-213.

Ballance GM, Lamari L, Kowatsch R, Bernier CC, 1996. Cloning, expression and occurrence of the gene encoding the Ptr necrosis toxin from *Pyrenophora tritici-repentis. Molecular Plant Pathology On-Line*, http://www.bspp.org.uk/mppol/1996/1209ballance.

Bause, E. 1983. Structural requirements of N-glycosylation of proteins: Studies with proline peptides as conformational probes. *Biochemical Journal* 209: 331-336.

Beer, H.D., Wolhfahrt, G., Schmid, R.D., and McCarthy J.E.G. 1996. The folding and activity of the extracellular lipase of *Rhizopus oryzae* are modulated by a prosequence. *Biochemical Journal* 319: 351-359.

Bronson, C.R. 1988. Ascospores abortion in crosses of *Cochliobolus heterostrophus* for the virulence locus *TOX1. Genome* 30: 12-18.

Bronson, C.R. 1991. The genetics of phytotoxin production by plant pathogenic fungi. *Experientia* 47: 771-776.

Brown, D.A., and Hunger, R.M. 1993. Production of a chlorosis-inducing, host-specific, low molecular weight toxin by isolates of *Pyrenophora tritici-repentis*, cause of tan spot of wheat. *Journal of Phytopathology* (Berl.) 137: 221-232.

Brown, M.P., Steffenson, B.J., and Webster, R.K. 1993. Host range of *Pyrenophora teres f. teres* isolates from California. *Plant Disease* 77: 942-947.

Cantrell, R.G., Elias, E., and Hosford, R.M., Jr. 1985. The inheritance of resistance to tan spot in durum wheat. *Agronomy Abstracts* 77: 50.

Chang, H.R. and Bronson, C.R. 1996. A reciprocal translocation and possible insertion(s) tightly associated with host-specific virulence in *Cochliobolus heterostrophus*. *Genome* 39: 549-557.

Chou, P.Y. and Fasman, G.D. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Advances in Enzymology* 47: 45-147.

Ciuffetti, L.M., Tuori, R.P., and Gaventa, J.M. 1997. A single gene encodes a selective toxin causal to the development of tan spot of wheat. *The Plant Cell* 9: 135-144.

Cox, T.S. Shroyer, J.P. Ben-Hui, L. Sears, R.G., and Martin, T.J. 1988. Genetic improvement in agronomic traits of hard red winter wheat cultivars from 1919 to 1987. *Crop Science* 28: 756-760.

da Luz, W.C., and Hosford, R.M., Jr. 1980. Twelve *Pyrenophora trichostoma* races for virulence to wheat in the Central Plains of North America. *Phytopathology* 70: 1193-1196.

Dewey, R.E., Siedow, J.N., Timothy D.H., and Levings III, C.S. 1988. A 13-kD maize mitochondrial protein in *E. coli* confers sensitivity to *Bipolaris maydis* toxin. *Science* 239: 293-295.

Doyle, W.A. and Smith, T.A. 1996. Expression of lignin peroxidase H8 in *Escherichia coli*: folding and activation of the recombinant enzyme with CA^{2+} and haem. *Biochemical Journal* 315: 15-19.

Dublin, H.J. 1983. Occurrence of *Pyrenophora tritici-repentis* in the Andean countries of South America. *Plant Disease* 67: 1040.

Eder, J. and Fersht, A.R. 1995. Pro-sequence-assisted protein folding. *Molecular Microbiology* 16: 609-614.

Elias, E., Cantrell, R.G., and Hosford, R.M., Jr. 1989. Heritability of resistance to tan spot in durum wheat and its association with other agronomic traits. *Crop Science* 29: 299-304.

Emini, E.A., Hughes, J.V., Perlow, D.S., and Boger, J. 1985. Induction of Hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. *Journal of Virology* 55: 836-839.

Faris, J.D., Anderson, J.A., Francl, L.J., and Jordahl, J.G. 1996. Chromosomal location of a gene conditioning insensitivity in wheat to a necrosis-inducing culture filtrate from *Pyrenophora tritici-repentis*. *Phytopathology* 86: 459-463.

Francl, L.J. 1995. Challenge of bioassay plants in a monitored outdoor environment. *Canadian Journal of Plant Pathology* 17: 138-143.

Fukuda, R., Horiuchi, H., Ohta, A., and Takagi, M. 1994. The prosequence of *Rhizopus niveus* aspartic proteinase-I supports correct refolding and secretion of its mature part in *Saccharomyces cerevisiae. Journal of Biological Chemistry* 269: 9556-9561.

Garnier, J., Osguthorpe, D.J., and Robson, B. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *Journal of Molecular Biology* 120: 97-120.

Gross, M.L., McCrery D., Crow, F., Tomer, K.B., Pope, M.R., Ciuffetti, L.M., Knoche, H.W., Daly, J.M., and Dunkle, L.D. 1982. The structure of HC-toxin. *Tetrahedron Letters* 23: 5381-5384.

Gurr, S.J., Unkles, S.E., and Kinghorn, J.R. 1987. The structure and organization of nuclear genes of filamentous fungi. In *Gene Structure in Eukaryotic Microbes*, J.R. Kinghorn, ed., pp. 93-139. IRL Press: Oxford.

Hagborg, W. A. F. 1970. A device for injecting solutions and suspensions into thin leaves of plants. *Canadian Journal of Botany* 48: 1135-1136.

Hames, B. D. 1981. An introduction to gel electrophoresis. In *Gel Electrophoresis of Proteins: A Practical Approach.* B. D. Hames and D. Rickwood, eds., pp 29-31. IRL Press: Oxford.

Harris, R.B. 1989. Processing of prohormone precursor proteins. *Archives of Biochemistry and Biophysics* 275: 315-333.

Heukshoren, J., and Dernick, R. 1985. Simplified method for silver staining proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6: 103-112.

Hosford, R.M. Jr., 1972. Propagules of *Pyrenophora trichostoma. Phytopathology* 62: 627-629.

Hosford, R.M., Jr. 1982. Tan spot. In *Tan Spot of Wheat and Related Diseases.* R. M. Hosford, Jr., ed., pp. 1-24. North Dakota State University, Fargo.

Hosford, R.M., Jr. and Bush, R.H. 1974. Losses in wheat caused by *Pyrenophora trichostoma* and *Leptoshaeria avenaria* f. sp. *triticea. Phytopathology* 64: 184-187.

Hosford, R.M., Jr. 1971. A form of *Pyrenophora trichostoma* pathogenic to wheat and other grasses. *Phytopathology* 61: 28-32.

Hunger, R. M., and Brown, D. A. 1987. Colony color, growth, sporulation, fungicide sensitivity, and pathogenicity of *Pyrenophora tritici-repentis*. *Plant Disease* 71: 907-910.

Jameson, B.A. and Wolf, H. 1988. The antigenicity index: a novel algorithm for predicting antigenic determinants. CABIOS 4: 181-186.

Jamroz, R.C., Gasdaska, J.R., Bradfield, J.Y., and Law, J.H. 1993. Transferrin in a cockroach: molecular cloning, characterization, and suppression by juvenile hormone. *Proceedings to the National Academy of Sciences, USA* 90: 1320-1324.

Jensen, O. N., Barofsky, D. F., Young, M. C., von Hippel, P. H., Swenson, S., and Seifried, S. E. 1993. Direct observation of UV-crosslinked protein-nucleic acid complexes by matrix-assisted laser desorption ionization mass spectroscopy. *Rapid Communications in Mass Spectrometry* 7: 496-501.

Johal, G.S., and Briggs, S.P. 1992. Reductase activity encoded by the *HM1* disease resistance gene in maize. *Science* 258: 985-987.

Kersten, P.J., and Cullen, D. 1993. Cloning and characterization of a cDNA encoding glyoxal oxidase, a H_2O_2-producing enzyme from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *Proceedings to the National Academy of Sciences, USA* 90: 7411-7413.

Kono, Y., Knoche, H.W., and Daly, J.M. 1981. Structure: Fungal host-specific. In *Toxins in Plant Disease*, R.D. Durbin, eds., pp. 221-257. Academic Press: New York.

Krupinsky, J. M. 1982. Observations on the host range of isolates of *Pyrenophora trichostoma*. *Canadian Journal of Plant Pathology* 4: 42-46.

Krupinsky, J.M. 1992. Grass hosts of *Pyrenophora tritici-repentis*. *Plant Disease* 76: 92-95.

Kyte, J., and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* 157: 105-132.

Laemmli. U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 277: 680-685.

Lamari, L. and Bernier, C.C. 1989a. Evaluation of wheat lines and cultivars to tan spot (*Pyrenophora tritici-repentis*) based on lesion type. *Canadian Journal of Plant Pathology* 11: 49-56.

Lamari, L. and Bernier, C.C. 1989b. Virulence of isolates of *Pyrenophora tritici-repentis* on 11 wheat cultivars and cytology of the differential host reactions. *Canadian Journal of Plant Pathology* 11: 284-290.

Lamari, L., and Bernier, C.C. 1989c. Toxin of *Pyrenophora tritici-repentis*: host specificity, significance in disease, and inheritance of host reaction. *Phytopathology* 79: 740-744.

Lamari, L., and Bernier, C. C. 1991. Genetics of tan necrosis and extensive chlorosis in tan spot of wheat caused by *Pyrenophora tritici-repentis*. *Phytopathology*. 81: 1092-1095.

Lamari, L., Sayoud, R., Boulif, M., and Bernier, C.C. 1995. Identification of a new race of *Pyrenophora tritici-repentis*: Implications for the current pathotype classification system. *Canadian Journal of Plant Pathology* 17: 312-318.

Larez, C.R., Hosford, R.M., Jr., and Freeman, T.P. 1986. Infection of wheat and oats by *Pyrenophora tritici-repentis* and initial characterization of resistance. *Phytopathology* 76: 931-938.

Lim, S.M., and Hooker, A. L. 1971. Southern corn leaf blight: genetic control of pathogenicity and toxin production in race T and race O of *Cochliobolus heterostrophus*. *Genetics* 69: 115-117.

Loughman, R., and Deverell, B.J. 1986. Infection of resistant and susceptible cultivars of wheat by *Pyrenophora tritici-repentis*. *Plant Pathology* 35: 443-450.

Lu, S.W., Lyngholm, L., Yang, G., Bronson, C., Yoder, O.C., and Turgeon, B.G. 1994. Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proceedings to the National Academy of Sciences, USA* 91: 12649-12653.

Marston, F.A.O. and Hartley, D.L. 1990. Solubolization of protein aggregates. *Methods in Enzymology* 182: 264-276.

Morrall, R.A.A. and Howard, R.J. 1975. The epidemiology of leaf spot disease in a native prairie. II. Airborne spore populations of *Pyrenophora tritici-repentis*. *Canadian Journal of Botany* 53: 2345-2353.

Orolaza, N.P., Lamari, L., and Ballance, G. M. 1995. Evidence for a host-specific chlorosis toxin for *Pyrenophora tritici-repentis*, the casual agent of tan spot of wheat. *Phytopathology* 85: 1282-1287.

Panaccione, D.G. Pitkin, J.W. Walton, J.D. Annis, S.L. 1996. Transposon-like sequences at the *TOX2* locus of the plant-pathogenic fungus *Cochliobolus carbonum*. *Gene*. 176: 103-109.

Panaccione, D.G., Scott-Craig, J.S., Pocard, J.-A., and Walton, J.D. 1992. A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. *Proceedings to the National Academy of Sciences, USA* 89: 6590-6594.

Parks, T.D. Howard, E.D. Wolpert, T.J. Arp, D.J., and Dougherty, W.G. 1995. Expression and purification of a recombinant tobacco etch virus NIa proteinase: biochemical analyses of the full-length and a naturally occurring truncated proteinase form. Virology 210: 194-201.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry* 83: 346-356.

Pfender, W. F., Pacey, C.A., and Zhang, W. 1988. Saprophytic growth and pseudothecia production by *Pyrenophora tritici-repentis* in plant tissue held at controlled water potentials. *Phytopathology* 78: 1205-1210.

Pitkin, J.W., Panaccione, D.G., and Walton, J.D. 1996. A putative cyclic peptide efflux pump encoded by the *TOXA* gene of the plant pathogenic fungus *Cochliobolus carbonum. Microbiology* 142: 1557-1565.

Raymond, P.J., Bockus, W.W., and Norman, B.L. 1985. Tan spot of winter wheat: Procedures to determine host response. *Phytopathology* 75: 686-690.

Riede, C.R., Francl, L.J., Anderson, J.A., Jordahl, J.G., and Meinhardt, S.W. 1996. Additional sources of resistance to tan spot of wheat. *Crop Science* 36: 771-777.

Rines, H.W., and Luke, H.H. 1985. Selection and regeneration of toxin-insensitive plants in tissue cultures of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae. Theoretical and Applied Genetics* 17: 16-21.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual, 2nd Edition. Cold Spring Harbor Press: Cold Spring Harbor, NY.

Scheffer, R.P. 1983. Toxins as chemical determinants of plant disease. In *Toxins in Plant Pathogenesis*, J.M. Daly and B.J. Deveral, eds., pp. 1-40. Academic Press: New York.

Scheffer, R.P., Nelson, R.R. and Ullstrup, A.J. 1967. Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae. Phytopathology* 57: 1288-1291.

Schilder, A.M.C., and Bergstrom, G.C. 1995. Seed transmission of *Pyrenophora tritici-repentis*, causal fungus of tan spot of wheat. *European Journal of Plant Pathology* 101: 81-91.

Schuh, W. 1990. The influence of tillage systems on incidence and spatial pattern of tan spot of wheat. *Phytopathology* 80: 804-807.

Scott-Craig, J.S., Panaccione, D.G., Pocard, J.A., and Walton, J.D. 1992. A multifunctional cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7 kb open reading frame. *Journal of Biological Chemistry* 267: 26044-49.

Shabeer, A., Bockus, W.W., and Norman, B.L. 1991. Evidence for cytoplasmic determinants in the reaction of wheat cultivars to tan spot. *Sarhad Journal of Agriculture* 7: 101-104.

Shoemaker, R.A. 1962. Drechslera Ito. *Canadian Journal of Botany* 40:809-836.

Smedegard-Peterson, V. 1978. Genetics of heterothallism in *Pyrenophora graminea* and *P. teres*. *Transactions of the British Mycological Society* 70: 99-102.

Stock, W.S., Brule-Babel, A.L. and G.A., Penner. 1996. A gene for resistance to a necrosis-inducing isolate of *Pyrenophora tritici-repentis* located on 5BL of *Triticum aestivm* cv. Chinese spring. *Genome* 39: 598-604.

Sykes, E.E., and Bernier, C.C. 1991. Qualitative inheritance of tan spot resistance in hexaploid, tetraploid, and diploid wheat. *Canadian Journal of Plant Pathology* 13: 38-44.

Tomas, A., and Bockus, W. W. 1987. Cultivar-specific toxicity of culture filtrates of *Pyrenophora tritici-repentis*. *Phytopathology* 77: 1337-1340.

Tomas, A., Feng, G. H., Reeck, G. R., Bockus, W. W., and Leach, J. E. 1990. Purification of a cultivar-specific toxin from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. *Molecular Plant-Microbe Interactions* 3: 221-224.

Tomas, A., Leach, J. E., and Bockus, W. W. 1988. *In vitro* production and partial purification of a toxin from *Pyrenophora tritici-repentis*. *Phytopathology* 78: 1590 (Abstr.).

Tuori, R.P., Wolpert, T.J., and Ciuffetti, L.M. 1995. Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. *Molecular Plant-Microbe Interactions* 8: 41-48.

Turgeon, B.G., Kodama, M., Yang, G., Rose, M.S., Lu S.W., and Yoder, O.C. 1995. Functional and chromosomal location of the *Cochliobolus heterostrophus TOX1* locus. *Canadian Journal of Botany* 73: S1071-S1076.

Tzeng, T.-W., Lyngholm, L.K., Ford, C.F., and Bronson, C.R. 1992. A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. *Genetics* 130: 81-96.

von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Research* 14: 4683-4690.

Walton, J.D. 1996. Host-selective toxins: Agents of compatibility. *Plant Cell.* 8: 1723-1733.

Walton, J.D., and Panaccione, D.G. 1993. Host-selective toxins and disease specificity: perspectives and progress. *Annual Review of Phytopathology* 31:275-303.

Watkins, J.E. Odvody, G. N., Boosalis, M. G. and Partridge, J. E. 1978. An epidemic of tan spot of wheat in Nebraska. *Plant Disease Reporter* 62: 132-134.

Whitman, R., and Tien, M. 1996. Heterologous expression and reconstitution of fungal Mn Peroxidase. *Archives of Biochemistry and Biophysics* 333: 439-446.

Wolpert, T. J., and Macko, V. 1991. Immunological comparison of the *in vitro* and *in vivo* labeled victorin binding protein from susceptible oats. *Plant Physiology* 95: 917-920.

Wolpert, T.J., Macko, V., Acklin, W., Jaun, B., Seibl, J., Meili, J. Arigoni, D. 1985. Structure of victorin C, the major host-selective toxin from *Cochliobolus victoriae*. *Experientia* 41: 1524-1529.

Yang, G. Rose, M.S. Turgeon, B.G. Yoder, O.C. 1996. A polyketide synthase is required for fungal virulence and production of the polyketide T-toxin. *Plant Cell* 8: 2139-2150.

Yang, R., and Kenealy, W.R. 1992. Effects of amino-terminal extensions and specific mutations on the activity of restrictocin. *Journal of Biological Chemistry* 267: 16801-16805.

Yoder, O.C. 1980. Toxins in pathogenesis. *Annual Review of Phytopathology* 18: 103-129.

Yoder, O.C., Turgeon, B.G., Ciuffetti, L.M., and Schafer, W. 1989. Genetic analysis of toxin production in fungi. In *Phytotoxin and Plant Pathogenesis*, A. Graniti, R.D. Durbin, A. Ballio, eds., pp 43-60. Springer: Berlin.

Yoder, O.C., Yang, G., Rose, M.S., Lu, S.W., and Turgeon, B.G. 1994. Complex genetic control of polyketide toxin production by *Cochliobolus heterostrophus*. In *Advances in Molecular Genetics of Plant-Microbe Interactions*, Vol. 3, M.J. Daniels, J.A. Downie, and A.E. Osbourn, eds., pp. 223-230. Kluwer Academic Publishers: Dordrecht, The Netherlands.

Zhang, H.-F., Francl, L.J., Jordahl, J.G., and Meinhardt, S.W. 1996. Structural and physical properties of a necrosis-inducing toxin from *Pyrenophora tritici-repentis*. *Phytopathology*. 87: 154-160.