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A Single Gene Encodes a Selective Toxin Causal to the Development of Tan Spot of Wheat

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The identification and characterization of pathogenicity factors are essential to an understanding of the molecular events that regulate the interaction of plant-pathogenic microbes with their hosts. We have isolated the gene that encodes a host-selective toxic protein produced by the fungus *Pyrenophora tritici-repentis* and confirmed that this gene functions in the plant as the primary determinant of pathogenicity in the *Pyrenophora*–wheat interaction. These results demonstrate that a single gene encodes the production of a host-selective toxin and that transformation of this gene into a non-toxin-producing isolate of *P. tritici-repentis* leads to both toxin production and pathogenicity.

INTRODUCTION

The identification and characterization of factors that account for the ability of microorganisms to cause plant disease (pathogenicity factors) are essential to our understanding of the molecular events that determine the consequences of host–pathogen interactions. Fungal plant pathogens that produce host-selective toxins (HSTs) are ideal organisms to address this objective because HSTs are readily identifiable pathogenicity factors. HSTs, produced by at least 16 species of fungi, are unique metabolites inferred to be involved in pathogenesis because they are toxic only to hosts susceptible to the fungus but not to resistant plants or nonhosts and because toxin production is strictly correlated with pathogenicity. Based on observations of the precise specificity of these compounds and conventional genetic analyses of toxin production and pathogenicity, these HSTs are believed to play a causal role in plant pathogenesis (Yoder, 1980).

The identification and cloning of genes responsible for toxin production would provide the technology for the direct assessment of the role of HSTs in disease development. However, based on analyses of their chemical structures (Macko, 1983), it appears that, with one exception, the majority of these compounds are very likely the products of multifunctional enzymes or complex enzymatic pathways rather than direct gene products. Consequently, a molecular genetic analysis of their biosynthesis is complicated. The complexity of toxin production is evident from the molecular genetic analysis of the biosynthesis of HC-toxin (Panaccione et al., 1992; Ahn and Walton, 1996; Walton, 1996), the cyclic peptide toxin (Gross et al., 1982; Walton et al., 1982; Pope et al., 1983) produced by *Cochliobolus carbonum*, and HmT toxin (Bronson, 1991; Tzeng et al., 1992; Yang et al., 1994; Yoder et al., 1994), the polyketide toxin (Kono and Daly, 1979; Kono et al., 1981) produced by *C. heterostrophus*. However, the HST produced by the wheat pathogen *Pyrenophora tritici-repentis* is unique among all known HSTs because it is a protein (Ballance et al., 1989; Tomas et al., 1990; Tuori et al., 1995) and thus very likely the direct product of a single gene. These characteristics represent significant experimental advantages for molecular cloning of the gene responsible for toxin biosynthesis in this pathogen. Consequently, this HST provides a unique opportunity for transformation of a tox^- isolate to the tox^+ phenotype and subsequently for a functional analysis of the role of this HST in disease development.

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 to 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 an 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, 1989a) and is correlated with susceptibility to the pathogen (Tomas and Bockus, 1987; Lamari and Bernier, 1989a, 1989b), indicating a causal role of the toxin in pathogenesis. In this study, our approach to investigating the *P. tritici-repentis*–wheat interaction was to isolate and clone the gene(s) responsible for pathogenesis and determine how expression of this gene(s) impacts host function such that a disease interaction results. Our objective in cloning the gene for toxin production was to analyze directly the role of the toxin as a determinant of pathogenicity. Furthermore, cloning the gene for this protein toxin should provide insight into the origin of

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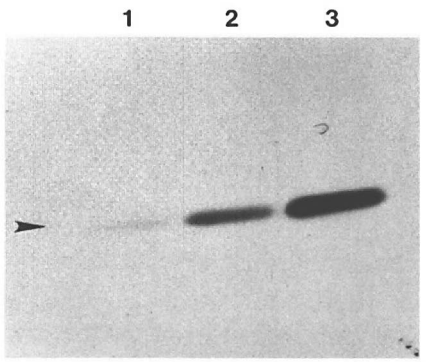


Figure 1. Protein Gel Blot Analysis of Crude Culture Filtrates from the Time Course of Toxin Production.

Total protein precipitated from 1-mL aliquots of culture filtrate at day 5 (lane 1), day 6 (lane 2), and day 7 (lane 3) was analyzed by SDS-PAGE. Protein gel blot analysis with polyclonal antibodies specific for ToxA indicates the accumulation of ToxA in culture filtrates (arrowhead).

pathogenicity and toxin production in this and possibly other fungal pathogens.

RESULTS

To facilitate the genetic analysis of pathogenicity in the *Pyrenophora*–wheat interaction, we previously purified a protein with host-selective toxicity, designated ToxA (Tuori et al., 1995), from culture filtrates of the fungus. Mass spectral analysis of purified ToxA indicated a mass of 13,208 D. Polyclonal antibodies raised against ToxA were shown by protein gel blot analysis and indirect immunoprecipitation to be specific for this protein (Tuori et al., 1995).

Cloning of the *ToxA* cDNA

The time course for toxin production in culture was evaluated to establish the optimal time for mRNA isolation for the production of cDNA libraries to be used for cloning the *ToxA* cDNA. Antibodies raised against the ToxA protein were used to conduct time-course studies by comparing protein production and toxic activity in culture filtrates. Culture filtrate and mycelia were harvested (Tuori et al., 1995) at intervals from 2 to 14 days after inoculation. Total protein was precipitated from samples of culture filtrates and evaluated by protein gel blot analysis to determine the time of maximum toxin production. Toxin activity was assessed by symptom expression after infiltration (Hagborg, 1970) of leaves of sensitive and insensitive wheat cultivars with culture filtrates (Tuori et al., 1995). The toxin was detected by protein gel blot analysis as early as 2 days and reached a maximum level ~12 days after inoculation. Figure 1 shows the results from 5-, 6-, and 7-day-old cultures. Also, the appearance of toxin activity was correlated with the appearance of the proteinaceous toxin.

Poly(A)$^+$ RNA was prepared from mycelia from 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-ToxA antibody. As shown in Figure 2, gel blot analysis of radiolabeled immunoprecipitation products clearly indicated the presence of an ~19,500-D translation product that reacted with anti-ToxA antibody (lanes 3 and 4).

After confirmation of 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-ToxA antibody. Antibody-positive recombinants were identified at a high frequency. The nucleotide sequences were obtained from five selected cDNA inserts. Our attempts to obtain the N-terminal amino acid sequence of the purified toxin revealed that the toxin is blocked at the N terminus (R.P. Tuori and L.M. Ciuffetti, unpublished data). Therefore, we determined the sequence of internal fragments of the purified protein generated by partial digestion with trypsin and cleavage with cyanogen bromide. Peptide fragments were purified by reversed-phase HPLC, and the purified fragments were sequenced. We obtained sequence information for ~50% of the ToxA protein. Comparison of

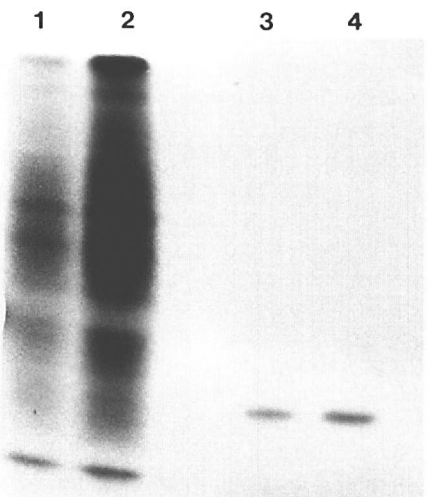


Figure 2. Autoradiography of in Vitro Translation Products.

Shown are products of total poly(A)$^+$ RNA from a combined 6- and 7-day-old culture (lane 1) and from a 10-day-old culture (lane 2). Indirect immunoprecipitation with the anti-ToxA polyclonal antibody of total translation products from days 6 and 7 combined and from day 10 are shown in lanes 3 and 4, respectively. Lanes 1 and 2 were exposed to x-ray film for 3 hr and lanes 3 and 4 for 24 hr.

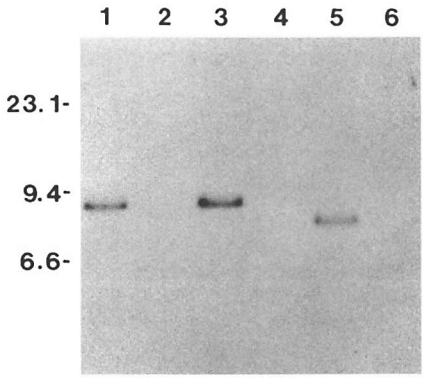


Figure 3. DNA Gel Blot Analysis of tox$^+$ and tox$^-$ Isolates.

Restriction enzyme–digested genomic DNA of tox$^+$ isolates (BFP [lane 1], Ptr-4 [lane 3], and SD-8 [lane 5]) and tox$^-$ isolates (SD-11 [lane 2], EO-3 [lane 4], and SD-20 [lane 6]) was probed with the *ToxA* cDNA insert (plasmid pCT4). Numbers at left indicate molecular length markers in kilobases.

amino acid sequence deduced from nucleotide sequence with amino acid sequence obtained from the protein provided unequivocal confirmation of the identity of the cloned cDNAs.

A Genomic Clone of *ToxA*

The presence or absence of the *ToxA* gene in tox$^+$ (pathogenic) and tox$^-$ (nonpathogenic) isolates of *P. tritici-repentis* was determined by DNA gel blot analysis with a ^{32}P-labeled cDNA insert, encoding ToxA, from the plasmid designated pCT4. Among 13 independent isolates, the *ToxA* gene is present only in tox$^-$ isolates of *P. tritici-repentis.* Results of six isolates probed with the cDNA insert are shown in Figure 3.

A subgenomic library was prepared from a tox$^+$ isolate (BFP), and a genomic clone for the *ToxA* gene was identified by hybridization with the *ToxA* cDNA insert of pCT4. Subsequently, a 1.3-kb subcloned fragment was determined by restriction analysis to contain the *ToxA* gene and its putative endogenous promoter.

A tox$^-$, hygromycin B–sensitive isolate (SD-20) of *P. tritici-repentis* was transformed with a vector containing the genomic copy of the *ToxA* gene and its putative endogenous promoter and the hygromycin B resistance gene for use as a selectable marker. DNA was prepared from the tox$^+$ control isolate (BFP), the tox$^-$ recipient (SD-20), the vector-only control transformant (6-2-2), and tox$^+$ transformants (8-3-4, 9-3-1, and 9-3-2) and analyzed by polymerase chain reaction (PCR). The results are shown in Figure 4A. PCR analysis confirmed that the tox$^+$ transformants contain the *ToxA* gene, whereas the tox$^-$ recipient and the tox$^-$ recipient transformed with the vector only lack the *ToxA* gene. Nucleotide sequence analysis of the PCR products from transformants 8-3-4, 9-3-1, and 9-3-2 confirmed that the products were from the *ToxA* gene.

Total protein was precipitated from samples of culture filtrate from the control tox$^+$ isolate (BFP), tox$^-$ recipient (SD-20), vector-only control transformant (6-2-2), and tox$^+$ transformants (8-3-4, 9-3-1, and 9-3-2) and evaluated by protein gel blot analysis to determine the presence of the ToxA protein. All tox$^+$ transformants tested produced ToxA in culture at levels similar to the control tox$^+$ isolate, as shown in Figure 4B. As expected, total protein from the tox$^-$ recipient

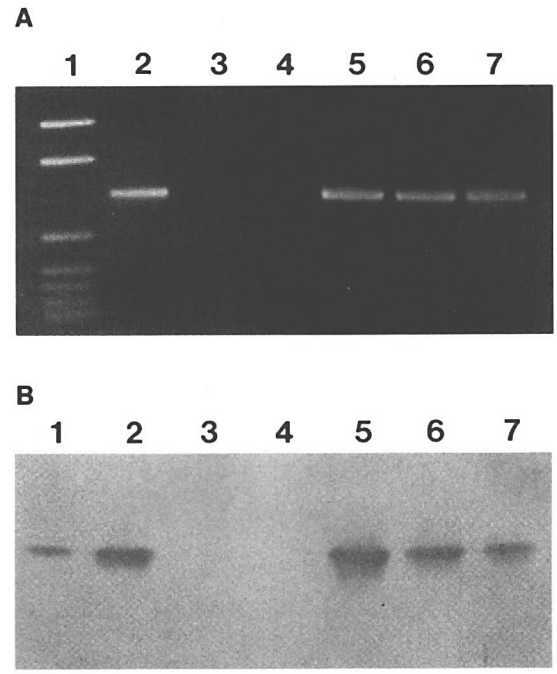


Figure 4. Analyses of tox$^+$ Transformants.

(A) Agarose gel electrophoresis of PCR-amplified genomic DNA. Molecular mass markers (from top to bottom) of 1.6, 1.2, 0.68, and 0.52 kb are shown in lane 1. The tox$^+$ control isolate BFP (lane 2), tox$^-$ recipient SD-20 (lane 3), vector control transformant 6-2-2 (lane 4), and the three tox$^+$ transformants 8-3-4, 9-3-1, and 9-3-2 (lanes 5 to 7) are also shown. Isolate BFP and all tox$^+$ transformants have the expected length band (913 bp) for the genomic copy of the *ToxA* gene when amplified with primers 4 and 5 (see Figure 6A). No bands are visible in either the tox$^-$ recipient (SD-20) or the vector-alone control transformant (6-2-2).

(B) Protein gel blot analysis of ToxA production. Total protein was precipitated from samples of culture filtrate and analyzed on protein gel blots. Shown are purified ToxA (lane 1), the tox$^+$ control isolate BFP (lane 2), the tox$^-$ recipient SD-20 (lane 3), SD-20 transformed with the vector only, 6-2-2 (lane 4), and the three tox$^+$ transformants 8-3-4, 9-3-1, and 9-3-2 (lanes 5 to 7). Isolate BFP and all tox$^+$ transformants show an antibody-positive reaction with the 13.2-kD ToxA protein compared with purified ToxA. No ToxA protein bands are visible in either the tox$^-$ recipient (SD-20) or the vector-only control transformant (6-2-2).

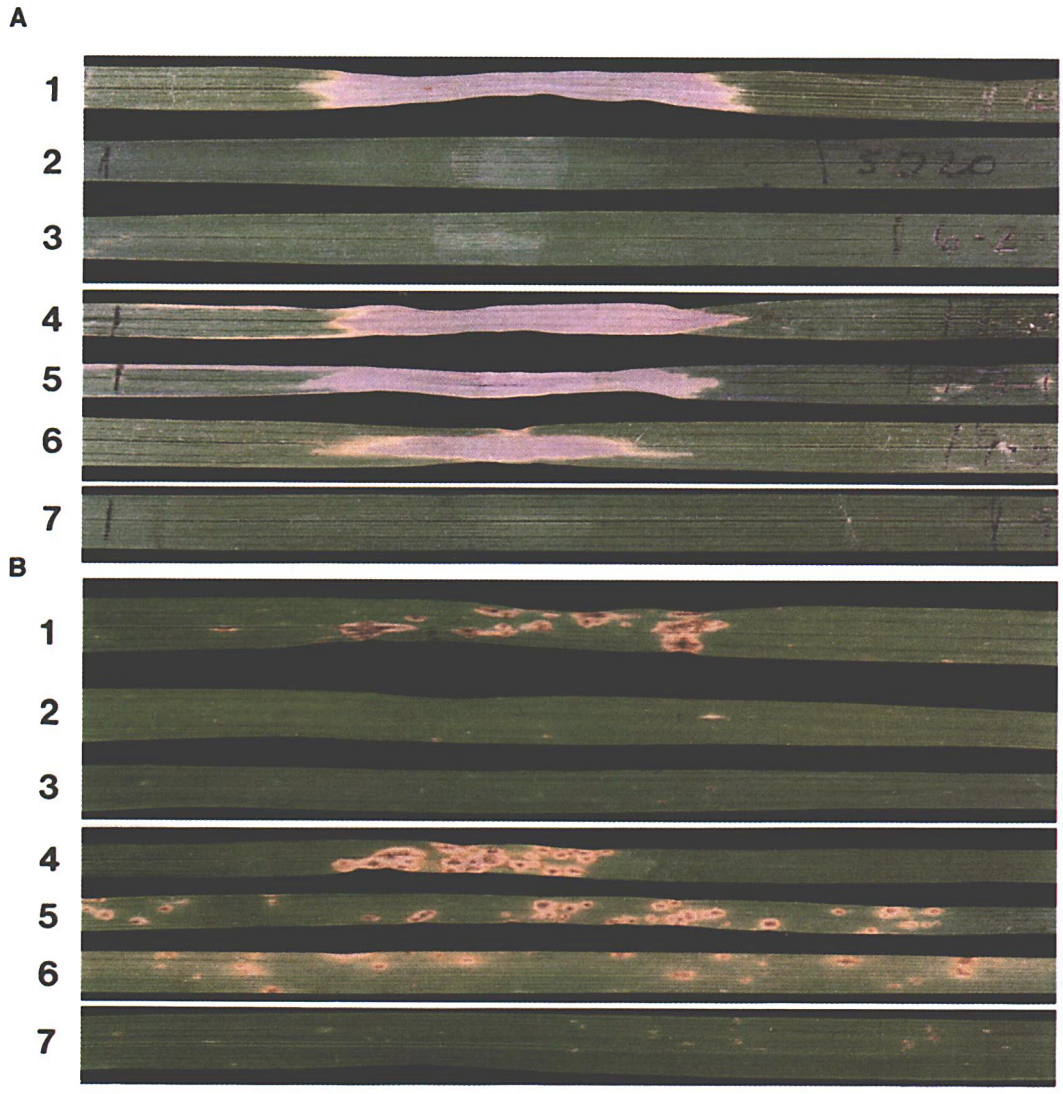


Figure 5. Leaf Infiltration Bioassay of Culture Filtrate and Pathogenicity Assay of tox$^+$ Transformants.

(A) Leaf infiltration bioassay of culture filtrate. Toxin-sensitive wheat (TAM 105) was infiltrated with culture filtrate from 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). Typical toxin-induced tan necrosis is shown on leaves with both the tox$^+$ control isolate and the tox$^+$ transformants. No toxin-induced necrosis developed in sensitive wheat with the tox$^-$ recipient or tox$^-$ recipient transformed with the vector only. In addition, no necrosis developed in the insensitive cultivar with all of the isolates tested. Results of infiltration of culture filtrate with the tox$^+$ transformant 9-3-2 (leaf 7) illustrate a typical reaction of insensitive wheat.

(B) 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) and resistant (Auburn) 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.

and tox⁻ recipient transformed with the vector only did not react with the antibody raised against ToxA (Figure 4B).

Culture filtrates from the tox⁺ control isolate (BFP), the tox⁻ recipient (SD-20), vector-only control transformant (6-2-2), and tox⁺ transformants (8-3-4, 9-3-1, and 9-3-2) were infiltrated into sensitive and insensitive wheat cultivars. The results are shown in Figure 5A. Culture filtrate from all tox⁺ transformants tested caused typical tan spot necrosis in the sensitive wheat cultivar (TAM 105) but not in insensitive wheat (Auburn). The extent of toxin sensitivity was similar to that of preparations from the tox⁺ control isolate. Culture filtrates from the tox⁻ recipient and the tox⁻ recipient transformed with the vector only did not induce necrosis in either the sensitive or insensitive wheat cultivar (Figure 5A).

Determination of the Role of ToxA in Pathogenicity

To determine whether ToxA is responsible for pathogenicity, we inoculated the tox⁺ transformants onto susceptible and resistant cultivars of wheat. The results are shown in Figure 5B. The tox⁺ control isolate (BFP) and the tox⁺ transformants (8-3-4, 9-3-1, and 9-3-2) produced typical tan spot lesions in susceptible wheat (TAM 105) but not in resistant wheat (Auburn). The control vector-only transformant (6-2-2) and the tox⁻ recipient (SD-20) did not develop typical tan spot symptoms in the susceptible or resistant wheat cultivar (Figure 5B).

Sequence Analysis of the *ToxA* Gene

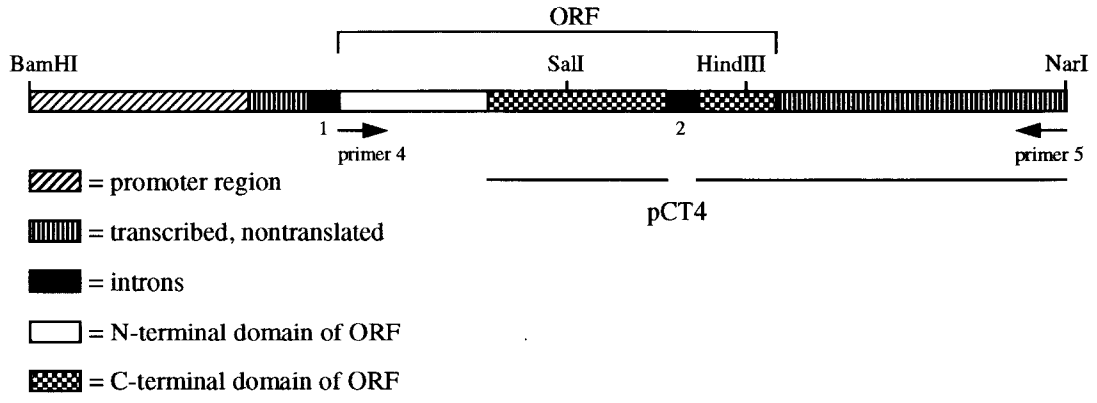
Figure 6A shows the genomic organization of the *ToxA* gene, and Figure 6B shows the nucleotide sequence analysis and predicted amino acid sequence of the genomic copy of the *ToxA* gene. The gene contains two introns. Intron 1 (55 nucleotides) is located in the 5′ leader sequence, and intron 2 (50 nucleotides) is located in the C-terminal domain coding region. Both introns are small, as is typical of fungal genomes (Gurr et al., 1987). Intron 1 has typical eukaryotic splice junctions, 5′(-GT) and 3′(AG-), whereas intron 2, with 5′(-AT) and 3′(AC-), lacks both consensus sequences. The deduced open reading frame (ORF) encodes a protein with a calculated mass of 19,707 D.

DISCUSSION

We concluded that we had isolated a full-length or nearly full-length cDNA for the ToxA protein based on the following: (1) the specificity of the antibody preparations, (2) the detection of cDNA inserts only in libraries prepared from mRNA isolated from mycelia during periods of toxin production, (3) the size of the cDNA inserts, and (4) a comparison of the amino acid sequence from ToxA with the amino acid sequence deduced from the nucleotide sequence. Previous reports (Ballance et al., 1989; Tomas el al., 1990) indicated that maximum toxin activity in culture occurs at 16 to 22 days. Initially, we isolated mRNA from mycelia from this time period and prepared a cDNA library. The library was extensively screened (>10^6 plaques) with both polyclonal and monoclonal antibodies, but we did not detect a single antibody-positive clone (L.M. Ciuffetti and J.M. Gaventa, unpublished data). This is in marked contrast to screens of the library prepared from mycelia at 6 and 7 days of growth, in which antibody-positive clones were detected at a minimum frequency of 1 per 1000 plaques. The inability to detect ToxA clones in the library prepared from 16- to 22-day-old cultures was not anticipated based on the published maxima for toxin activity (Ballance et al., 1989; Tomas et al., 1990) and the high quantities of toxin that we could isolate from cultures during this time period. The time-course studies presented here, which were based on both toxic activity and reaction to the anti-ToxA antibody, indicated that toxin production occurs very early in culture, with the detection of ToxA as early as 2 days. The detection of the toxin very early in growth and the apparent absence of the toxin message at 16 to 22 days suggest both that ToxA is very stable and that toxin production is regulated during growth in culture.

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. Searches in computer data bases for genes homologous to the *ToxA* gene did not reveal the presence of genes with extensive homology. However, based on consensus sequence analysis (von Heijne, 1986) and homology with other signal peptides (cf. Jamroz et al., 1993; Kersten and Cullen, 1993), the first 22 amino acids of the protein comprise the signal peptide necessary for secretion of the toxin. Based on the determined mass of 13,208 D for the mature toxin and the assumption that the inferred signal sequence of 22 amino acids is proteolytically cleaved after import into the endomembrane system, additional proteolytic processing is apparently involved in the production of the mature ToxA. Because the ToxA protein is blocked at the N terminus and such modifications would presumably incur a mass increase, the two most likely sites for a proteolytic cleavage that would yield mature toxin of 13,208 D are after 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. Interestingly, a potential myristoylation site (Towler et al., 1988; Grand, 1989) is found directly after Gln-61 (amino acids 62 to 67). The addition of a myristate to Gly-62 would yield a mature peptide of ~13,290 D. Of the two potential *N*-glycosylation sites (Bause, 1983) found in the ORF (amino acids 36 to 38 and amino acids 70 to 72), the second site is probably not glycosylated because this amino acid sequence was established by Edmun degradation during sequencing of one of the peptide fragments of the ToxA protein. We have no data indicating whether or not the first site is glycosylated. Nine consensus phosphorylation

A



B

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BamHI
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aacttaccTCGACTATCAGCATCCCGTCCTATCTAACAATCGTCCATCGGTATCCAACTCCAACTCTATTCGCAGGGTCCTAGAATCGTA 360
AGTACACGCTTATATCTTGTTGCCAGCGATAGCTGACAATGAATGAATATAGGTCATGCGTTCTATCCTCGTACTTCTTTTCAGCGCCGC 450
                                                    M  R  S  I  L  V  L  L  F  S  A  A

TGCTGTGCTTGCTGCCCCAACGCCTGAAGCCGATCCCGGTTACGAAATCGTTAAACTTTTCGAAGCCGCCAACTCTTCTGAACTCGACGC 540
 A  V  L  A  A  P  T  P  E  A  D  P  G  Y  E  I  V  K  L  F  E  A  A  N  S  S  E  L  D  A
                          ⇑
GCGCGGACTCTCTCTCGACTGGACCCTCAAACCGCGCGGACTCCTACAGGAGCGGCAGGGAAGCTGCATGTCAATCACAATCAACCCTAG 630
 R  G  L  S  L  D  W  T  L  K  P  R  G  L  L  Q  E  R  Q  G  S  C  M  S  I  T  I  N  P  S
                          SalI                    ↑  ↑
TCGTCCGTCTGTCAACAACATCGGCCAAGTCGACATTGACAGTGTTATACTCGGACGACCTGGTGCTATAGGCTCGTGGGAACTGAACAA 720
 R  P  S  V  N  N  I  G  Q  V  D  I  D  S  V  I  L  G  R  P  G  A  I  G  S  W  E  L  N  N

CTTTATTACCATTGGATTGAACCGCGTAAACGCCGATACAGTGCGAGTCAACATCCGAAACACCGGCAGGACTAATCGCCTCATTATTAC 810
 F  I  T  I  G  L  N  R  V  N  A  D  T  V  R  V  N  I  R  N  T  G  R  T  N  R  L  I  I  T

TCAATGGGAATAAGTCTCCCCACCATTTTATTAGATTACTTATTACTAACTTATACTACTAATACTGTCACTCGGGGGGACGTTTATGAG 900
 Q  W  D                                                    N  T  V  T  R  G  D  V  Y  E
                                  HindIII
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 L  F  G  D  Y  A  L  I  Q  G  R  G  S  F  C  L  N  I  R  S  D  T  G  R  E  N  W  R  M  Q

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 L  E  N  *

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                                                    NarI
TCATAGGTGGTAGCTTCCCATAGGTAGCTTTTTGAAATGAAACATTTATCACGGGCGCC                                  1319
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Figure 6. Sequence Analysis of the *ToxA* Gene.

(A) Genomic organization of the *ToxA* locus. Shown is the predicted 534-nucleotide open reading frame (ORF) beginning with the first ATG codon containing the N- and C-terminal domains. The N-terminal 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– to 39–amino acid region with a theoretically determined anionic isoelectric point of 4.55. The C-terminal domain encodes the 117– to 118–amino acid 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-terminal 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-terminal domain and the 3′ nontranslated region minus intron 2.

sites (Kishimoto et al., 1985; Woodgett et al., 1986; Pinna, 1990) have been identified. However, two sites are found in peptide fragments that have been confirmed by direct amino acid sequence. Any of the remaining seven sites could potentially be phosphorylated in the fungus, whereas any of nine sites could potentially be phosphorylated in the host. In addition, a potential cell attachment sequence, a consensus sequence important for interaction with a cell surface receptor (Ruoslahti and Pierschbacher, 1986; d'Souza et al., 1991), has been identified at amino acids 140 to 142. This sequence could be involved in the interaction of toxin with host cells.

As indicated previously (Tuori et al., 1995), it is likely that ToxA and Ptr toxin, a host-selective protein toxin previously described by Tomas et al. (1990), are the same protein. A gift of Ptr toxin from A. Tomas (Pioneer Hi-Bred International, Johnston, IA) comigrated with ToxA on SDS–polyacrylamide gels and reacted with anti-ToxA polyclonal antibodies on protein gel blots. Both ToxA and Ptr toxin are similar in amino acid composition, and both are heat stable (Tuori et al., 1995). However, Ballance et al. (1989) have also reported a host-selective protein toxin called Ptr necrosis toxin. Ptr necrosis toxin has an amino acid content similar to ToxA and Ptr toxin (Tuori et al., 1995) but has been reported as heat labile (Ballance et al., 1989). Also, we previously reported finding a low-molecular-weight anionic protein toxin that appeared to be immunologically unrelated to ToxA (Tuori et al., 1995). Thus, it is possible that multiple host-selective protein toxins or multiple forms of a host-selective protein toxin exist in culture filtrates of the fungus.

Infiltration of culture filtrate from all tox^+ transformants tested showed typical tan spot necrosis in susceptible wheat at levels comparable with culture filtrate obtained from wild-type tox^+ isolates. These data confirm that the *ToxA* gene is responsible for toxin production and consequently that the ToxA protein is a host-selective toxin. These data also suggest that *ToxA* gene expression could account for all of the host-selective toxic activity of culture filtrates on susceptible wheat. The possibility that multiple proteolytic processing is involved in ToxA production is particularly interesting, based on the finding that multiple protein toxins or forms of the toxin have been identified (Tuori et al., 1995). This could account for the formation of proteins with variable N termini and consequently multiple forms of the toxin. As stated previously, our investigation of the toxins produced by this fungus revealed a low-molecular-weight anionic toxin that appeared to be immunologically unrelated to the mature toxin (Tuori et al., 1995). Interestingly, the peptide region between the presumed proteolytic site of the signal peptide and the inferred N terminus of the mature toxin is an anionic peptide with an approximate molecular weight of the anionic toxin previously identified. Future experiments directed toward inactivating the *ToxA* locus in toxin-producing isolates and examining the forms of the toxin produced by tox^+ transformants should help to determine whether any of these possibilities are likely and/or whether other loci exist that encode additional HSTs.

The results of the tox^+ and tox^- isolates probed with the labeled cDNA insert from plasmid pCT4 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 ($path^+/tox^+$ versus $path^-/tox^-$) in this fungus may be due to the acquisition of toxin production rather than to a loss of toxin production.

The most significant finding of this study is that typical tan spot lesions developed on susceptible wheat when inoculated with conidia from each of the three tox^+ transformants. These data clearly demonstrate that toxin production is causal to and apparently sufficient for the pathogenicity of *P. tritici-repentis* on wheat. The sufficiency of expression of the *ToxA* gene for conversion of tox^- isolates to the pathogenic phenotype is noteworthy because it implies that nonpathogenic isolates of this fungus are competent for pathogenesis, with the sole exception of their ability to produce toxin.

In summary, these data confirm that we have isolated the gene for ToxA production and that this gene functions in the plant as the primary determinant of pathogenicity in the *Pyrenophora*–wheat interaction. This host–pathogen interaction has provided a unique opportunity to isolate a single gene for the production of an HST and transformation of a tox^- isolate to the tox^+ phenotype. Transformation has clearly demonstrated that this gene is both necessary and sufficient for pathogenicity of *P. tritici-repentis* on toxin-sensitive wheat genotypes.

Figure 6. (continued).

(B) The nucleotide and deduced amino acid sequence of the *ToxA* gene. The promoter region consists of 278 bp (lowercase letters). A potential CAAT motif and TATA box (boldface), thought to be involved in initiation of transcription (Gurr et al., 1987), are located ~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, roman). Introns 1 and 2 are indicated by uppercase italic letters. Amino acid residues 1 to 22 comprise a putative signal peptide, with the probable signal peptide cleavage site shown (open arrow). Two sites of possible proteolytic cleavage that result in the mature ToxA protein are shown (arrows). Approximately 50% of the mature ToxA protein sequence was determined through direct amino acid sequencing, and all of the peptide fragment sequences 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 CNBr; the remainder shown were generated by trypsin digest). Restriction enzyme recognition sites are indicated by underlined nucleic acid sequences. The asterisk represents the stop codon.

METHODS

Fungal Cultures and Plant Material

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). Isolate SD-20 was obtained from G. Buchenau (South Dakota State University, Brookings). Stationary cultures were grown in 500-mL flasks containing 100 mL of modified Fries medium (Tomas et al., 1990), inoculated with a suspension of 3×10^4 conidia per mL, and incubated at 25°C for 7 days under constant fluorescent light.

Wheat plants used for bioassay were grown at 25°C for a 16-hr photoperiod. Plants were grown to the four-leaf stage, and 100 μL of culture filtrate was infiltrated into the third or fourth leaves, as described previously (Tuori et al., 1995). Toxin activity was assayed in the wheat cultivars TAM 105 (sensitive to the toxin and susceptible to the pathogen) and Auburn (insensitive to the toxin and resistant to the pathogen). Necrosis-inducing toxic activity was detected within 1 to 2 days. Infiltrations were repeated at least three times with similar results. Photographs were taken 3 days after leaf infiltration.

Wheat plants were inoculated at the three- to four-leaf stage with a conidial suspension (5×10^3 conidia per mL). Plants were sprayed to drip stage with a deVilbis sprayer connected to an air line. Plants were enclosed in plastic bags to maintain high humidity and kept in dim light during a 16-hr photoperiod for 36 to 48 hr. Plants were returned to 25°C for a 16-hr photoperiod. Photographs were taken 6 days after inoculation. Inoculations were repeated at least three times with similar results. Wheat cultivar Auburn was obtained from G. Shaner (Purdue University, West Lafayette, IN), and TAM 105 was obtained from D. Marshall (Texas A & M University, College Station).

Protein Isolation

Stationary cultures were grown as described above. Total protein was precipitated from 1-mL aliquots of the culture filtrate with a 0.1 volume of 0.15% deoxycholate (kept at room temperature for 15 min) and a 0.1 volume of 72% trichloroacetic acid. The suspension was centrifuged (14,000 rpm for 15 min), the supernatant was decanted, and the pellet was rinsed with 75% EtOH. After centrifugation (14,000 rpm for 5 min), the EtOH was decanted, and the pellet was solubilized in 40 μL of resuspension buffer (2.3% [w/v] SDS, 0.625 M Tris-HCl, pH 6.8) (Ausubel et al., 1996). Protein samples were analyzed by PAGE in 17% polyacrylamide gels, as described previously (Tuori et al., 1995).

Protein Blotting, in Vitro Translation, and Indirect Immunoprecipitations

Polyclonal antibodies were raised against purified ToxA in rabbits at the Monoclonal Antibody Facility of the Center for Gene Research and Biotechnology at Oregon State University. Protein gel blot analysis indicated that the antibodies raised against the toxin react with and are specific to the 13,208-D band associated with toxin activity. All of the protein gel blotting of polyacrylamide gels and immunological detection of proteins were performed as described previously (Tuori et al., 1995).

In vitro translations were performed according to the manufacturer's recommendations (rabbit reticulocyte lysate translation system; Life Technologies, Grand Island, NY). Indirect immunoprecipitation of total translation products was performed as described previously (Tuori et al., 1995), except that rabbit polyclonal antibodies were used.

Preparation of a cDNA Library

Total RNA was prepared (Ausubel et al., 1996) from the combined mycelia of 6- and 7-day-old cultures. Poly(A)$^+$ RNA was prepared by oligo(dT) chromatography (PolyATract mRNA isolation system; Promega). cDNA was prepared according to the manufacturer's instructions (Riboclone cDNA synthesis systems; Promega). EcoRI adapters were ligated to double-stranded cDNA, followed by ligation to EcoRI arms of λgt11. The phage were packaged (Packagene; Promega), and *Escherichia coli* Y1090 cells were infected. Plaques were transferred to Protran nitrocellulose membranes (Schleicher & Schuell) and screened with the anti-ToxA polyclonal antibody as described for protein blotting (Tuori et al., 1995).

Construction of Subgenomic Library

Mycelia were grown overnight from conidial suspension in 0.25 × potato dextrose broth (Difco). To isolate the genomic copy of the *ToxA* gene, genomic DNA from the tox$^+$ isolate (BFP) was digested with a variety of restriction enzymes that did not cut the *ToxA* gene, as indicated by the sequence information obtained from the *ToxA* cDNA inserts. DNA gel blot hybridization was performed with a ^{32}P-labeled cDNA insert of pCT4 (this insert corresponds to nucleotide 594 to 1319 minus intron 2; see Figures 6A and 6B). The pCT4 probe hybridized with a single band of ~9 kb when genomic DNA was digested with SacI. The DNA was isolated from the gel, and the 9-kb inserts were used to construct a subgenomic library in the vector pHA1.3 (Powell and Kistler, 1990). Epicurian *E. coli* XL1-Blue supercompetent cells (Stratagene) were transformed with the library, and the library was screened by colony hybridization, according to established protocols (Sambrook et al., 1989), with the ^{32}P-labeled *ToxA* cDNA insert of plasmid pCT4. Positive colonies were selected and subjected to a second round of purification.

DNA Gel Blot Hybridization

Genomic DNA from tox$^+$ isolates (BFP, Ptr-4, and SD-8) and tox$^-$ isolates (SD-11, EO-3, and SD-20) were digested with EcoRI and separated in an 0.8% agarose gel. Electrophoresis was performed in 1 × TBE buffer (45 mM Tris, 45 mM boric acid, and 1 mM EDTA, pH 7.6). DNA gel blot hybridization was performed with a ^{32}P-labeled cDNA insert of pCT4. High-stringency DNA gel blot hybridization analysis was performed according to established protocols (Sambrook et al., 1989).

Fungal Transformation and Vector Construction

Mycelia were grown by inoculating conidial suspensions into 0.25 × potato dextrose broth. Cultures were grown overnight at 25°C with shaking (125 rpm). Mycelia were harvested by filtration and washed with water. Mycelia (1 to 1.5 g) were added to 8 mL of 1.2 M $MgSO_4$, 10 mM sodium phosphate containing 80 mg of mureinase, 40 mg of driselase, 4 mg of chitinase, and 8500 units of β-glucuronidase and incubated with gentle agitation at 25°C for 2 to 3 hr. The protoplast suspension was filtered through a 20-μm Nitex (Tetko Inc., DePew,

NY) membrane and rinsed with STC (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, and 10 mM $CaCl_2$) (Yelton et al., 1984). The suspension was centrifuged (4500 rpm for 5 min) in an SS34 rotor (Beckman Instruments, Palo Alto, CA). The supernatant was decanted, and protoplasts were counted and diluted to a final concentration of 10^8 protoplasts per mL in STC, polyethylene glycol 4000, and DMSO (80:20:1) and kept on ice or stored in aliquots at −20°C.

Fungal transformation was based on a modification of the procedures of Vollmer and Yanofsky (1986). Spermidine (2 μL of 50 mM), plasmid DNA (3 μg), and 10^7 protoplasts (100 μL of the above given dilution mix) were mixed gently and incubated on ice for 15 min. One milliliter of polyethylene glycol 4000 (50 mM Tris, pH 8.0, and 50 mM CaCl) was added, swirled gently, and incubated at room temperature for 20 min. Nine milliliters of molten regeneration medium (RM) (1.2 M sorbitol, 0.1% yeast extract, 0.1% casein hydrolysate, and 1.5% agar) (Turgeon et al., 1987) was added to the mix and poured onto solidified RM supplemented with 100 μg of hygromycin to give a final concentration of ~50 μg/mL. Protoplasts were plated at 2×10^6 protoplasts per plate. Plates were incubated in the dark at 25°C, and hygromycin-resistant transformants were evident by 3 to 4 days of incubation.

The vectors used for fungal transformation were constructed as follows. A modified hygromycin B phosphotransferase gene (Carroll et al., 1994), obtained from Fungal Genetics Stock Center (Kansas City, KS), in vector pCB1003 was cloned into the SalI site of pBluescript SK−, resulting in pCT48. Transformants generated with pCT48 are referred to as vector-only control transformants. A 1.3-kb BamHI-NarI fragment carrying the *ToxA* locus, determined by restriction analyses, was cut from the 9-kb insert of the subgenomic clone and inserted into the BamHI and ClaI sites of pCT48, resulting in the transformation vector pCT53. Transformants generated with vector pCT53 are referred to as tox^+ transformants.

Polymerase Chain Reaction Amplification of Genomic DNA

Nucleic acids were extracted (Ausubel et al., 1996) from fungal mycelial mats at day 6 or 7 with the following modifications. The volumes were reduced such that all extractions were performed in 12-mL Falcon tubes. The supernatant was saved after the LiCl RNA precipitation step, and DNA was precipitated with ammonium acetate and ethanol. The DNA preparation was treated with RNase A before polymerase chain reaction (PCR) amplification. Primers were designed to amplify the entire coding region and the 3′ nontranslated region (see Figure 6A). Bases that were changed to facilitate cloning of PCR products are shown in italics, and the resulting restriction sites are underlined. Primer 4 (5′-ATCG*CCATGG*GTTCTATCCTCG-TACT-3′) was designed to create an NcoI site flanking the ATG codon. This changed the second codon of the amplified product from R (Arg) to G (Gly). Primer 5 (5′-CGT*GGATCC*CGTGATAAAT-GTTTCAT-3′) was designed to create a BamHI site at the extreme 3′ end of the 3′ nontranslated region, resulting in the loss of the NarI site in the amplified products. PCR was performed with 10 to 50 ng of genomic DNA, 50 nM primers 4 and 5, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 1 to 2 units of Taq polymerase, and the corresponding reaction buffer obtained from Promega. 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 was separated by electrophoresis in a 1% agarose gel (1 × TAE, 0.04 M Tris-acetate, pH 8.0, and 0.001 M EDTA) and stained in ethidium bromide.

Sequence 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, Foster City, CA).

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