A ToxA-like protein from *Cochliobolus heterostrophus* induces light-dependent leaf necrosis and acts as a virulence factor with host selectivity on maize

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ABSTRACT

ToxA, the first discovered fungal proteinaceous host-selective toxin (HST), was originally identified in 1989 from the tan spot fungus *Pyrenophora tritici-repentis* (*Ptr*). About 25 years later, a homolog was identified in the leaf/glume blotch fungus *Stagonospora nodorum* (*Parastagonospora nodorum*), also a pathogen of wheat. Here we report the identification and function of a ToxA-like protein from the maize pathogen *Cochliobolus heterostrophus* (*Ch*) that possesses necrosis-inducing activity specifically against maize. *ChToxA* is encoded by a 535-bp open reading frame featuring a *ToxA*-specific intron with unusual splicing sites (5'-ATAAGT...TAC-3') at conserved positions relative to *PtrToxA*. The protein shows 64% similarity to *PtrToxA* and is predicted to adopt a similar three-dimensional structure, although lacking the arginyl-glycyl-aspartic acid (RGD) motif reported to be required for internalization into sensitive wheat mesophyll cells. Reverse-transcriptase PCR revealed that the *ChTOXA* gene expression is up-regulated *in planta*, relative to axenic culture. Plant assays indicated that the recombinant *ChToxA* protein induces light-dependent leaf necrosis in a host-selective manner on maize inbred lines. Gene deletion experiments confirmed that *Chtox*A mutants are reduced in virulence on specific ToxA-sensitive maize lines, relative to virulence caused by wild-type strains. Database searches identified potential *ChToxA* homologues in other plant-pathogenic ascomycetes. Sequence and phylogenetic analyses revealed that the corresponding ToxA-like proteins include one member recently shown to be associated with formation of penetration hypha. These results provide the first evidence that *C. heterostrophus* is capable of producing proteinaceous HSTs as virulence factors in addition to well-known secondary metabolite-type toxins produced biosynthetically by

polyketide synthase megaenzymes. Further studies on *ChToxA* may provide new insights into effector evolution in host-pathogen interactions.

1. Introduction

The maize pathogen *Cochliobolus heterostrophus* and closely related species in the genus are best known for producing host-selective toxins (HSTs) associated with ability of the fungus to cause severe diseases on host cereal crops (Yoder, 1980; Turgeon and Lu, 2000; Condon et al., 2013). Examples include T-toxin, a polyketide toxin produced by *C. heterostrophus* race T that is essential for high virulence on maize lines with Texas male-sterile cytoplasm (Yang et al., 1996), HC-toxin, a cyclic tetrapeptide produced by *Cochliobolus carbonum* race 1, that conditions pathogenicity on maize lines homozygous recessive at the *Hm1/Hm2* loci encoding carbonyl reductases that detoxify the toxin (Panaccione et al., 1992), and victorin, a chlorinated cyclic pentapeptide produced by *Cochliobolus victoriae* that accounts for Victoria blight on oats carrying the dominant *Pc-2* allele introduced for resistance to crown rust caused by *Puccinia coronata* (Macko et al., 1985). Collectively, these well-characterized HSTs are small molecule secondary metabolites biosynthesized independently of ribosomes, by large multidomain megaenzymes such as polyketide synthases or nonribosomal peptide synthetases.

In addition to secondary metabolite HSTs, some HSTs are produced ribosomally. The first reported fungal proteinaceous HST (ToxA) was isolated from the tan spot fungus *Pyrenophora tritici-repentis* (*Ptr*) about 25 years ago (Balance et al., 1989; Tomas et al., 1990). The toxin is important for induction of necrosis (Ciuffetti et al., 1997) on wheat carrying the *Tsn1* gene (Faris et al., 1996), which encodes a disease resistance-like protein but, in contrast, is required for *sensitivity* to the toxin (Faris et al., 2010). Structurally, ToxA is a small secreted protein encoded by a gene interrupted by a 50-bp

1 intron with unusual splicing sites (5'-ATAAGT...TAC-3'). ToxA is processed to a 13.2-
2 kD mature protein of 118 amino acids (aa), which adopts a unique three-dimensional
3 structure consisting of a β -sandwich fold with two antiparallel β -sheets (Sarma et al.,
4 2005) and a solvent-exposed arginyl-glycyl-aspartic acid (RGD) motif located at the C-
5 terminal region that is essential for ToxA activity (reviewed by Ciuffetti et al., 2010).
6 ToxA had long been considered to be a unique effector HST lacking counterparts in other
7 fungal pathogens until the identification of an ortholog (Friesen et al., 2006) in the
8 leaf/glume blotch fungus *Parastagonospora nodorum* (previously known as
9 *Stagonospora nodorum* [Sn], and used hereafter for convenience) (Friesen et al., 2006).
10 Subsequently an ortholog was also discovered in *S. avenaria tritici* 1, a sister species of
11 *S. nodorum* (McDonald et al., 2013). It has been proposed that ToxA was acquired by *P.*
12 *tritici-repentis* from *S. nodorum* through a lateral/horizontal gene transfer (Friesen et al.,
13 2006). To date, no ToxA-like proteins have been characterized from other plant-
14 pathogenic ascomycetes.

15 We recently reported the cloning of a ToxA-like gene [*ChTOXA* (ToxA in *Ptr* and
16 *Sn*)] from *C. heterostrophus* and showed that it encodes a protein with both sequence and
17 structure similarities to *PtrToxA* (Lu and Edwards, 2014). In this manuscript, we
18 describe *ChTOXA* and *ChTOXA*-like genes from *C. heterostrophus* and closely-related
19 species and functional characterization of *ChToxA* by recombinant protein and gene
20 deletion approaches. We demonstrate that the recombinant *ChToxA* protein induces
21 light-dependent leaf necrosis in a host-selective manner and that the native *ChTOXA* gene
22 product acts as a virulence factor on specific *ChToxA*-sensitive maize lines. Our study
23 provides the first evidence that *C. heterostrophus* produces both proteinaceous and

secondary metabolite-derived HSTs as virulence factors. We also present evidence that potential ToxA homologues exist in other plant pathogenic fungi including both Dothideomycete and Sordariomycete species, and these ToxA-like proteins include one from *Colletotrichum higginsianum* that has been previously characterized as a developmentally regulated extracellular protein (EC13) associated with penetration hypha formation (Kleemann et al., 2012), suggesting that ToxA may be related evolutionarily to certain factors potentially playing a general role in fungal plant pathogenesis.

2. Materials and methods

2.1. Strains, media and crosses

C. heterostrophus inbred lab strains C5 and C4, and field isolates Hm338, Hm540, and PR1x412 (Turgeon lab, Cornell University) were recovered from glycerol stocks and grown on agar plates containing complete medium with xylose (CMX, Tzeng et al., 1992) and incubated under standard conditions (Turgeon et al., 1993). For liquid cultures, fungal conidia were collected from CMX plates and inoculated into 50-100 ml of complete (CM) or minimal medium (MM) containing glucose as a carbon source (Leach et al., 1982a). Crosses between *C. heterostrophus* strains of opposite mating type were performed using procedures described previously (Leach et al., 1982b). Ascospore progeny were isolated from pseudothecia 21-28 days after setting the cross. Both random spores and complete tetrads (containing four sets of identical twins in a single ascus) were isolated.

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2 *2.2. Nucleic acid preparations*

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4 Fungal mycelia were scraped from agar plates or collected from liquid cultures by
5 centrifugation. Maize leaves collected from inoculated plants were cut into small
6 segments (≈ 1.0 cm), tissue samples were transferred into 2-ml Lysing Matrix D tubes
7 containing 1.4-mm ceramic spheres (QBiogene, Morgan Irvine, CA), and frozen in liquid
8 nitrogen for 2 to 5 min immediately before homogenization using a Mini-Bead-beater-16
9 Cell Disrupter (BioSpec Products, Bartlesville, OK). Genomic DNA was extracted from
10 the homogenized tissues in cetyl trimethyl ammonium bromide solution (Teknova Inc,
11 Hollister, CA) with chloroform extraction and ethanol precipitation, then finally
12 resuspended in TE buffer (pH 8.0) with the concentration adjusted to 10 to 100 ng/ μ l.
13 mRNA was extracted from the homogenized tissues using the Dynabeads mRNA Direct
14 Kit (Invitrogen, CA) following the manufacturer's instructions and finally eluted in
15 Tris-HCl buffer (pH 8.0). cDNA synthesis was done using the Superscript III first strand
16 synthesis system (Invitrogen, CA).

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18 *2.3. Genomic/cDNA cloning and PCR conditions*

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20 Genomic clones of *ChTOXA* and other *TOXA*-like genes were obtained by PCR
21 amplification from fungal genomic DNA using primers (Table S1) designed based on the
22 sequences identified in the databases of the DOE Joint Genome Institute (JGI)
23 (<http://jgi.doe.gov>). PCR was performed in a 25- μ l volume of 1 x GoTaq Green Master

Mix (Promega, Madison, WI) that contained 1 to 10 ng of fungal genomic DNA and 0.5 μ M each primer. PCR was started with an initial preheat for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 55 or 59°C for 15 s, and extension at 72°C for 1-3 min, with a final extension at 72°C for 10 min. For amplification of large genomic DNA fragments, the GoTaq Green Master Mix was replaced by LongAmp Taq Master Mix (New England Biolabs, Ipswich, MA). PCR was started with an initial preheat for 2 min at 94°C, followed by 32 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 30 s, and extension at 65°C for 5 min, with a final extension at 65°C for 10 min. cDNA clones were obtained by reverse-transcriptase (RT)-PCR using primers designed to the genomic sequence (Table S1). The 5' and 3' untranslated regions (UTR) of the *ChTOXA* mRNA were determined using the 5' RACE and 3' RACE System for Rapid Amplification of cDNA Ends kits (Invitrogen, Carlsbad, CA), respectively. PCR products were separated on a 1 % agarose/ethidium bromide gel in TAE buffer. Targeted PCR products were cloned into a pCR2.1 vector using the TOPO TA Cloning Kit with TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared using the Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI). DNA sequencing was done at the Cornell University Biotechnology Resource Center using gene or vector-specific primers.

2.4. Gene expression profiling

Leaf tissues were collected at 8 h, 24 h, 48 h, 72 h, and 96 h post inoculation. mRNA isolation and cDNA synthesis were done as described above. Concentrations of

different cDNA samples were adjusted to be comparable by serial dilutions against the expression level of the *C. heterostrophus* actin gene (Table S1) which was as an internal control. To validate the specificity of the selected *ChACT1* primers, mRNA samples isolated from uninoculated plants were included to check for possible cross-amplification of the maize actin gene (*ZmACT1*, GenBank Acc# XM_008646341, 80% identity to *ChACT1*). No cross-amplification of the maize transcripts was expected for the *ChTOXA*-specific primers because *ChTOXA* has no similarity to the fully sequenced maize genome. PCR profiling was performed using gene-specific primers (Table S1) and the GoTaq Green Master Mix (Promega, Madison, WI) as described above except that the genomic DNA template was replaced by 1 µl of cDNA solution. The identity of the targeted transcripts was confirmed by DNA sequencing of cDNA clones or PCR products purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). All tests were repeated at least twice to verify the observed gene expression patterns.

2.5. Expression of the recombinant *ChToxA* protein

The coding region corresponding to the 12.1-kD mature *ChToxA* was PCR-amplified from the *ChTOXA* cDNA clone using gene-specific primers with suitable restriction sites incorporated at the 5' end (Table S1) and sub-cloned into a pPink α -HC vector (Invitrogen, Carlsbad, CA). The expression construct was transformed into a protease-deficient strain of *Pichia pastoris* following the manufacturer's protocols. Protein expression and isolation were done as described previously (Lu et al., 2013). Protein abundance and molecular weight were determined by SDS-PAGE analysis done

1 following standard protocols (Green and Sambrook, 2012). The concentration of the
2 target protein was estimated by comparison with a series of dilutions of bovine serum
3 albumin (BSA) (New England Biolabs, Ipswich, MA, USA) included in the same SDS-
4 PAGE gel. After SDS-PAGE gel separation, the coomassie blue-stained band
5 corresponding to the 12.1-kD *ChToxA* protein was excised from the gel and subjected to
6 MALDI-TOF/TOF analysis performed at the Cornell University Biotechnology Resource
7 Center as described previously (Lu et al., 2013).

8

9 *2.6. Plant materials and bioassays*

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11 Maize inbred lines were obtained through the North Central Regional Plant
12 Introduction Station (NCRPIS) (Table 1). Plants were grown in 6-inch pots (10-16 plants
13 /per pot) containing SB100 professional growing mix (Sungrow Horticulture, Bellevue,
14 WA, USA.) in a growth chamber at an average temperature of 21°C with a 16-h
15 photoperiod for 2-3 weeks. For *ChToxA* activity assays, the third leaf of each plant was
16 infiltrated with ~20 µl of cell-free culture supernatant (diluted with water) containing the
17 recombinant *ChToxA* protein at the desired concentrations using a 1-ml syringe without
18 the needle (Delasco, Council Bluffs, IA, USA). Control plants were infiltrated with the
19 culture supernatant from a yeast strain transformed with the expression vector only.
20 Treated plants were kept in the growth chamber and symptoms were examined 2-5 days
21 after infiltration. Each treatment included six plants and was repeated at least twice. For
22 virulence assays, fungal conidia were collected from 10-15 days-old cultures grown on
23 CMX plates and suspended in 0.05% Tween 80 solutions with the concentrations

adjusted to 1×10^6 /ml. Plants were inoculated by spraying the conidial suspensions using a sprayer replacement power unit (Preval, Coal City, IL). Inoculated plants were kept in a mist chamber for 16-20 h and then returned to the growth chamber. Symptoms were recorded 3-4 days post inoculation. Each treatment included five plants and was repeated at least twice.

2.7. Targeted gene deletion and marker segregation analysis

The split-marker PCR strategy (Catlett et al., 2003, Turgeon et al., 2010) was used to delete the *ChTOXA* gene from the genome using specific PCR primers (Table S1). Genomic sequences 5' upstream or 3' downstream of the *ChTOXA* open reading frame were PCR-amplified from a genomic DNA clone and then fused with respective sequences of the hygromycin B resistance gene (*hygB*) cassette, which was PCR-amplified from the plasmid pUCATPH (Lu et al., 1994). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequence identity was confirmed by DNA sequencing. Transformation of *C. heterostrophus* (strain C5) was performed using procedures described previously (Turgeon et al., 1987), except that Novozyme 234 was replaced by lysing enzymes from *Trichoderma harzianum* (Sigma, Saint Louis, MO) at 10 mg/per 100 ml. Transformants were purified by isolation of single conidia from fungal cultures grown on CMX agar plates. The deletion of the *ChTOXA* gene from the genome was confirmed by PCR screening using gene/locus-specific primers (Table S1).

For marker segregation analysis, *ChtoxA* mutants, in the strain C5 genetic background, validated by PCR assays were crossed to strain C4 (*hygB^S*; *ChToxA⁺*; *ToxI⁺*; *MAT1-2*), which is the opposite mating type (Leach et al., 1982b). Progeny were isolated from cross #1401 (between C4 and TxC5-6-2) because this cross gave the best fertility. One of the progeny (1401-R30, genotype = *hygB^R*; *ChToxA⁻*; *ToxI⁻*; *MAT1-2*) from cross #1401 was then back-crossed (cross #1402) to the parental strain C5 (genotype = *hygB^S*; *ChToxA⁺*; *ToxI⁻*; *MAT1-1*). For marker segregation analysis, genomic DNA was extracted from CMX cultures of individual progeny and the genotypes were determined by PCR screening using primers designed to *hygB* (Lu et al., 1994), *ChTOXA*, *MAT1-1* or *MAT1-2* (Turgeon et al., 1993), and *PKSI* gene (Yang et al., 1996), respectively (Table S1). Virulence of individual progeny from cross #1402 was determined by inoculation assays on differential maize lines as mentioned above.

2.8. Sequence, structural and phylogenetic analyses

Amino acid sequences of ToxA-like proteins from other fungal species were retrieved from GenBank databases available online from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), the MycoCosm databases available online from the DOE Joint Genome Institute (JGI) (<http://jgi.doe.gov>) and the Fungal Genome Initiative databases available online from the Broad Institute (www.broadinstitute.org). The N-terminal signal peptides of the ToxA-like proteins were determined using the SignalP 4.1 Server available online from the Center for Biological Sequence Analysis, Technical University of Denmark DTU. Nucleotide and amino acid

sequence alignments were generated using the MegAlign programs from Lasergene 8.1 software (DNASTAR Inc. Madison, WI, USA). Structural data of *PtrToxA* (Sarma et al., 2005) were retrieved from the Protein Database Bank available online from the European Bioinformatics Institute. The molecular modeling of the three-dimensional structure of the mature *ChToxA* protein was done through the PHYRE2 Protein Fold Recognition Server available online. The retrieved PDB files of *ChToxA* and *PtrToxA* were examined using the Jmol (Hanson, 2010) and Ribbon (Carson, 1997) programs. For phylogenetic analysis, the amino acids corresponding to the predicted mature ToxA-like proteins were aligned using the CLUSTALX program (Thompson et al., 1997). Phylogenetic analysis was performed using the neighbor-joining method with the PHYLIP 3.61 package (<http://evolution.genetics.washington.edu/phylip.html>) (Felsenstein, 1989) and the unrooted consensus tree (from 1,000 bootstrap replicates) was constructed using the neighbor joining method and drawn using TreeView software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

3. Results

3.1. Characterization of the *ChTOXA* gene

ChToxA was originally annotated in the genome of *C. heterostrophus* (strain C5) by the DOE Joint Genome Institute (JGI) (<http://jgi.doe.gov>) as a hypothetical protein (JGI protein 1019653) encoded by an open reading frame (ORF) located on scaffold_2 (positions 2,349,946-2,350,765) and found as an EST in liquid culture

(fgenes1_kg.2_#_479_#_18_1). Although it has an apparent signal peptide (SP) sequence (see below), this protein was not included in the suite of 180 predicted small secreted proteins (SSPs) predicted in the *C. heterostrophus* genome (Condon et al., 2013). To validate *ToxA*-like identity, we cloned and then sequenced a 2784-bp genomic DNA from strain C5 (GenBank Acc# KJ664923) and confirmed that this genomic clone carried the *ChTOXA* ORF. Analysis of the corresponding cDNA clones, obtained by RT-PCR coupled with 5' and 3' RACE cloning, revealed that the full-length *ChTOXA* transcript consists of 841 base pairs (bp), including 486-bp coding sequences flanked by 74-bp 5' and 281-bp 3' untranslated regions (UTR) (GenBank Acc# KJ664924). The mature *ChTOXA* mRNA is apparently processed from pre-mRNA by removing a 49-bp intron at unusual, *ToxA*-specific splicing sites (5'-ATAAGT...TAC-3') (Fig. 1A).

Further analysis indicated that the genomic regions flanking the *ChTOXA* gene contain highly repetitive DNA, with genes predicted to encode retrotransposon-like proteins (RTP), some of which (e.g., *RTPAI*) are associated with both *ChToxA* and *PtrToxA*, with the copy number and the orientation differing between the two *ToxA* loci (Fig. 1A). The repeats on the *C. heterostrophus* flanks are extensive, as shown in Fig. S1 for positions of 2,300,000 to 2,350,500 on the left flank and 2,350,700 to 2,365,000 on the right. Analysis of ~100 kb genomic DNA sequences carrying *ChTOXA* and *PtrToxA* revealed short “syntenies” (2-5 kb) at the both 5' and 3' flanking regions that contain the repetitive sequences encoding RTP-like proteins (Fig. S2). No significant similarities were detected when the genomic regions surrounding *ChTOXA* and *SnToxA* were compared in the same way, and the homologues of *RTPAI* appear to be absent in the genome of *S. nodorum* (data not shown).

The deduced *ChToxA* protein is 161 aa with moderate (64%) overall similarity to *PtrToxA* (Ciuffetti et al., 1997) and has a ToxA-like three-domain organization that includes the N-terminal SP, the pro-region (N-domain), and the mature toxin (C-domain) (Fig. 1B, top). Sequence alignment indicated that both *ChToxA* and *PtrToxA* contain the asparagine-rich motif known to be essential for ToxA activity (Lu et al., 2014), but the C-terminal Arg-Gly-Asp (RGD) motif in *PtrToxA* is Ser-Gly-Asn (SGN) in *ChToxA* (Fig. 1B, bottom). Homology-based molecular modeling suggested that the 12.1-kD *ChToxA* mature protein would adopt a *PtrToxA*-like three-dimensional structure (Sarma et al., 2005), which consists of an α helix and eight β strands giving rise to two antiparallel β -sheets but having $\beta 5$ and $\beta 6$ shorter than counterparts that flank the RGD motif in *PtrToxA* (Fig. 1C).

3.2. *ChTOXA* is up-regulated during fungal pathogenesis

To determine the expression profile of *ChTOXA*, transcript abundance was compared in *in vitro* culture vs. *in planta* by RT-PCR. The *ChTOXA* transcripts (554 bp, corresponding to coding mRNA plus part of the UTR sequences) were absent in the early stages (8-48 h) of axenic cultures grown in either MM or CM medium, becoming detectable at low levels at ~ 96 h post inoculation. In contrast, the transcripts accumulated to much higher levels in inoculated maize samples starting at 24 h post inoculation (Fig. 2, top panel). Expression levels of *ChACT1* were nearly equal in all treatments (Fig. 2, bottom panel), and neither *ChTOXA* nor *ChACT1* were apparent in control mRNA samples from uninoculated maize plants (Fig. 2, lane 11). These data confirmed that the

ChTOXA gene is expressed and inducible during fungal infection of the host plant. Interestingly, in the same experiments, we found that a second transcript corresponding to a gene we call *ChTOXA**, which encodes a C-terminal truncated protein with homology to *ChToxA* (see below), was not detectable during infection although a 608-bp *ChTOXA** transcript was detected at 96 h at low levels in axenic cultures grown in MM medium, similar to that of *ChTOXA* (Fig. 2, middle panel).

3.3. *ChToxA* protein induces light-dependent leaf necrosis with host-selectivity on maize

To enable functional analysis, we obtained a recombinant *ChToxA* protein through heterologous expression in *Pichia pastoris*, as described previously (Lu et al., 2012). SDS-PAGE analysis indicated that the mature *ChToxA* protein (Fig. 1B) was expressed successfully as a single major species with expected molecular weight of 12.1 kD (Fig. 3A, left). The identity of the 12.1-kD *ChToxA* protein was further validated by MALDI-TOF/TOF analysis in which *ChToxA*-specific fragments were identified (Fig. 3A, right). We then tested the activity and the specificity of the recombinant *ChToxA* protein using leaf infiltration assays on a total of 33 maize inbred lines.

At 72 h post infiltration, nine maize lines (SD43, GS73CO, W570, FS8A, W64ARpp9, H116, CML247, CML277, and CML52) developed typical necrosis, three maize lines (DE811, TZSTRI110, and NC250) showed no visible symptoms, and the remaining 21 lines reacted with intermediate symptoms, including scattered necrotic spots or chlorosis in the infiltrated areas (Table 1, Fig. S1). The typical necrosis induced by *ChToxA* (approximately 0.1-0.5 μ M) on sensitive inbred lines such as W570 was

1 manifested as tissue collapse across the entire infiltrated area (Fig. 3B, left, leaves 1 and
 2 2). Scattered necrotic spots were observed when *ChToxA* was applied at lower
 3 concentrations (e.g., 0.05 μ M) (Fig. 3B, left, leaf 3). A negative reaction was observed on
 4 insensitive maize lines like DE811 regardless of the concentration of *ChToxA* used under
 5 the same conditions (Fig. 3B, right). It should be noted that the observed necrosis
 6 symptoms shown in Fig. S1 and Fig. 3B may look “restricted” to the area surrounding the
 7 primary vein. This was due to the fact that maize leaves are much broader than those of
 8 wheat; a single shot with the same volume is sufficient to cover the entire breadth of the
 9 leaf on wheat (e.g., Manning et al., 2004, 2008), but not on maize. In fact, necrosis
 10 occurred across the entire infiltrated area.

11 Interestingly, like *PtrToxA* whose necrosis-inducing activity depends on light
 12 (Manning et al., 2005), *ChToxA* did not cause any visible symptoms on sensitive maize
 13 lines when the infiltrated plants were incubated in complete darkness (Fig. 3B, left, leaf
 14 5). No reactions were observed on *ChToxA*-treated plants of *ToxA*-sensitive wheat lines
 15 under the same conditions (data not shown). Taken together, these results demonstrated
 16 that *ChToxA* acts as a proteinaceous HST specifically against certain cultivars of maize
 17 and that its necrosis-inducing activity is light-dependent.

18 19 3.4. *ChToxA* mutants are reduced in virulence on some *ChToxA*-sensitive maize lines

20
 21 To explore the function of *ChToxA* in fungal pathogenesis, we first tested if the
 22 sensitivity to *ChToxA* correlated with the susceptibility to the fungus. Inoculation assays
 23 were performed on all 33 maize inbred lines as mentioned above (Table 1). All maize

1 lines were susceptible, but the disease symptoms fell into two major groups. At 5 days
 2 post inoculation (dpi), 30 *ChToxA*-sensitive maize lines developed necrotic lesions, 2-5
 3 mm in size whereas the three *ChToxA*-insensitive maize lines (DE811, TZSTRI110 and
 4 NC250) developed smaller lesions (most <2 mm) (Fig. S3). These initial observations
 5 raised the possibility that *ChToxA* may act as a virulence factor in the *C. heterostrophus*-
 6 maize interaction.

7 The complete *ChTOXA* ORF was deleted to determine virulence of the mutant
 8 compared to wild type. Five stable hygromycin B-resistant transformants (TxC5-6-2,
 9 TxC5-12-1, TxC5-14-3, TxC5-16-1, and TxC5-19-3), all indistinguishable from their
 10 parental strain in terms of growth rate, morphology, and sporulation, were obtained from
 11 the gene deletion experiment (Fig. 4A), and subjected to further characterization. PCR
 12 amplifications using five pairs of diagnostic primers (Table 1) revealed integration
 13 events. As shown in Fig. 4B, the *hygB*-specific primer pair (pH2501/pH1302) amplified
 14 an 849-bp fragment (internal to *hygB*) from all five hygromycin B-resistant progeny (lane
 15 2-6), but not from the wild type control (lane 1). The *ChTOXA*-specific primer pair
 16 (atgF/tagR) amplified a 535-bp fragment (containing the *ChTOXA* ORF) from the wild
 17 type control (lane 1) and TxC5-16-1 (lane 5), but not from TxC5-6-2, TxC5-12-1, TxC5-
 18 14-3, and TxC5-19-3 (lanes 2, 3, 4 and 6). The third primer pair, which consisted of a
 19 forward primer (5FP1) designed to the genomic region outside the left end (the 5' end) of
 20 the transforming DNA, and a reverse primer (pH1685) designed to *hygB*, amplified a
 21 2,354-bp fragment, while the fourth pair, which consisted of a forward primer (pH375)
 22 designed to *hygB* and a reverse primer (3RP1) designed to the genomic region outside the
 23 right (3') end of the transforming DNA, amplified a 1,138-bp fragment. The third and

fourth primer pairs amplified products from TxC5-6-2, TxC5-12-1, TxC5-14-3, and TxC5-19-3 only (lanes 2, 3, 4 and 6). Targeted gene deletion was validated further by use of the fifth primer pair (5FP1/3RP1), which amplified a 2,799-bp fragment from the wild type control (lane 1) and TxC5-16-1 (lane 5) that was replaced by a 4,801-bp fragment (1,150-bp 5' flank and 1,114-bp 3' flank of native genomic DNA plus the 2,537-bp *hygB* cassette) in TxC5-6-2, TxC5-12-1, TxC5-14-3, and TxC5-19-3 (lanes 2, 3, 4 and 6). DNA sequencing of the purified 4,801-bp PCR fragments confirmed that the replacement of *ChTOXA* by *hygB* occurred precisely without any deletions at the adjacent regions between the native and the transforming DNA (Fig, 4A). These results confirmed that a *Chtox*A mutant was generated in TxC5-6-2, TxC5-12-1, TxC5-14-3, and TxC5-19-3 through a double cross-over-mediated homologous integration at the *ChToxA* locus, and that the native *ChTOXA* gene was intact in TxC5-16-1 in which the transforming DNA integrated in an “ectopic” site in the *C. heterostrophus* genome.

Subsequent plant inoculation assays indicated that the *Chtox*A mutants caused smaller lesions on five *ChToxA*-sensitive maize lines while producing symptoms similar to that of the wild type parental strain on the remaining 25 sensitive lines (Table 1). We focused on W570, one of the five differential maize lines that developed disease symptoms easily distinguishable between the wild type and the *Chtox*A mutants. As shown in Fig. 4C (upper panel), at 5 dpi, W570 plants inoculated with the wild type strain C5 (leaf 1) or the “ectopic” *ChToxA*⁺ transformant TxC5-16-1 (leaf 5) developed large necrotic lesions across most areas of the infected leaves. In contrast, the plants inoculated with the four *Chtox*A mutants all developed weaker symptoms as indicated by the predominance of smaller lesions that retained discernible green areas (leaves 2, 3, 4, and

6). On the other hand, the four *ChtoxA* mutants all caused disease symptoms that were similar to that caused by the wild type when tested on the *ChToxA*-insensitive maize lines such as DE811 under the same conditions (Fig. 4C, lower panel). These results confirmed that the targeted deletion of *ChTOXA* resulted in a mutant that sustains reduced virulence on specific *ChToxA*-sensitive maize lines.

3.5. The host-dependent reduced virulence is genetically linked to the *ChtoxA* null allele

Sixty-three and 48 random progeny ascospores were isolated from cross #1401 and #1402, respectively, and three complete tetrads were isolated from cross #1402 (Table 2). PCR-based marker segregation analysis indicated that all progeny segregated for the parental types only, i.e., all hygromycin B-resistant progeny were *ChToxA*⁻, whereas all hygromycin B-sensitive progeny were *ChToxA*⁺. The observed parental types segregated in a ratio near 1:1 among random spores isolated from both crosses (36:37 and 26:22 for cross #1401 and #1402, respectively) or in a 4:4 pattern for all three complete tetrads isolated from cross #1402 (Table 2). These data confirmed that the deletion of *ChTOXA* in TxC5-6-2 was indeed caused by a single integration event at the *ChToxA* locus that resulted in the replacement of the native *ChTOXA* gene by the *hygB* marker (Fig. 4A).

Plant inoculation assays indicated that the *hygB*-tagged *ChtoxA* null allele co-segregated with the reduced virulence observed on W570. These assays were done for progeny isolated from the backcross (cross #1402) that included all three tetrads (1402-1, 1402-2 and 1402-3) and more than 20 random spore progeny. To estimate the percentage

of reduction in virulence, disease symptoms were recorded at three different time points, i.e., 2 dpi, 3 dpi, and 5 dpi. All hygromycin B-resistant progeny showed reduced virulence just like the *ChToxA*⁻ parent (1401-R30), while all hygromycin B-sensitive progeny caused disease symptoms just like those induced by the wild type parent (strain C5) (Table 2). The disease symptoms observed at 3 dpi on the second leaves of infected plants were found to be the best for comparison of virulence because most lesions were still well-separated, allowing size to be easily measured. For example, in the assay for 1402-1, plants inoculated with the wild type parent (strain C5) and the four hygromycin-B-sensitive progeny developed necrotic lesions with size of ~1.5-2 mm (Fig. 5, leaves #1, and #3-6, top panel), whereas those inoculated with the *ChToxA* mutant parent (1402-R30) and the four hygromycin-B-resistant progeny developed smaller lesions (most ~1 mm) (Fig. 5, leaves #2 and #7-10, top panel). Thus, deletion of *ChTOXA* caused ~ 50% reduction in virulence. No apparent symptomatic differences were observed when the same progeny were tested on the other *ChToxA*-sensitive maize lines, such as SD40 and FS8A, or on the *ChToxA*-insensitive maize line DE811 (data not shown). These results confirmed that the reduced virulence is tightly linked to deletion of *ChTOXA* and that *ChToxA* represents a single genetic locus controlling virulence on specific *ChToxA*-sensitive maize lines.

3.6. *ToxA*-like proteins are found in diverse plant pathogenic ascomycetes

While carrying out BLAST searches with *ChToxA*, we found 13 hypothetical proteins in the fungal genome databases that show homologies to *ToxA*. These proteins

involve species in both the Dothideomycetes and Sordariomycetes. The Dothideomycete species include four in *Cochliobolus*, i.e., *C. carbonum*, *C. victoriae*, and *C. miyabeanus* (a pathogen of rice) and *C. heterostrophus* itself; two in *Pyrenophora*, i.e., *P. teres* f. *teres* (a pathogen of barley) and *P. tritici-repentis* itself, and the other two in the family of *Botryosphaeriaceae*, i.e., *Macrophomina phaseolina* (a pathogen of maize), and *Neofusicoccum parvum* (a pathogen of grapevine). The Sordariomycete species include *Colletotrichum fioriniae* and *C. higginsianum* (the anthracnose fungi), *Fusarium oxysporum* f. sp. *melonis* (a vascular pathogen of melon) and *Claviceps purpurea* (the cereal ergot fungus) (Table 3). Interestingly, we found that the ToxA-like protein identified in *C. higginsianum* was described as a secreted extracellular protein (EC13) associated with fungal penetration hypha formation in a recent study (Kleemann et al., 2012).

Sequence analysis indicated that these potential ToxA homologues (hereafter ToxA* for convenience) have weak to moderate (38-54%) similarities to *ChToxA* and/or *PtrToxA*. Interestingly, all of the ORFs encoding these ToxA* proteins lack introns (Table 3). Amino acid sequence alignment revealed that all ToxA* proteins have an N-terminal SP, plus at least four motif-like sequences, and two cysteine residues at conserved positions (Fig. 6A). An “LXXR” motif was located at the end of the N-domain in *PtrToxA*, and is likely a proteolytic signature for mature toxin processing. The “LXVXIXN”, “LILTXY” and “WXXQ” motifs located within the C-domain correspond to β 4, β 5, and β 8, respectively in *PtrToxA* shown in Fig. 1B. The two conserved cysteine residues were predicted to form a disulfide bridge in all ToxA* proteins as they do in ToxA. In addition, an asparagine-rich motif was found in five ToxA* proteins with a

consensus of WXXNNXIXVXI (Fig. 6A). Not surprisingly, the N-domain (which is not part of the active mature toxin) was found to be highly variable among ToxA* proteins except for the “LXXR” motif.

Phylogenetic analysis suggested that the ToxA-related proteins fall into three major groups: Group I contains ToxA proteins only, Group II includes ToxA* proteins all from Dothideomycetes that have the “WXXN(S)NXIXVXI” motif, and Group III consists of ToxA* proteins all from Sordariomycetes, except for the two *Pyrenophora* (Fig. 6B). Homology-based modeling did not predict ToxA-like structures for any ToxA* proteins (data not shown).

3.7. The ToxA* protein in *C. heterostrophus* is C-terminally truncated and not expressed during fungal pathogenesis

The *C. heterostrophus* ToxA* protein (*ChToxA**) was identified by BLAST searches with the counterparts in other *Cochliobolus* spp.. This protein is encoded in the genomes of both strain C5 and C4 as identified from the JGI databases. To our surprise, *ChToxA** was found to lack the C-terminal 75 residues present in its counterparts in the other three *Cochliobolus* species. This C-terminal truncation is caused by a single nucleotide mutation in the *ChTOXA** ORF (position 270) that changes the TAC (as in *C. carbonum* and *C. victoriae*) or CTC (as in *C. miyabeanus*) codon to TAA resulting in an early stop (Fig. S4). The stop codon occurs in the ORFs (498 bp) of strain C4 and C5, and also in the *ChTOXA** genes we sequenced from three additional isolates of *C. heterostrophus* (Hm338, Hm540 and PR1x412). RT-PCR indicated that the *ChTOXA**

gene is expressed (Fig. 2, middle panel) and cDNA cloning and DNA sequencing confirmed the identity of the *ChTOXA** mRNA, suggesting that a truncated *ChToxA** protein may be actually produced in fungal cultures, but is dispensable for fungal pathogenesis, as it was not evident in plant inoculated material (Fig. 2).

4. Discussion

The present study extends our research on proteinaceous HSTs into the *C. heterostrophus*-maize interaction, a pathosystem best known for secondary metabolite-derived HSTs (Yoder, 1980; Turgeon and Lu, 2000; Condon et al., 2013). Here we provided solid evidence that *C. heterostrophus* produces a proteinaceous HST (*ChToxA*) that is structurally similar to *PtrToxA*, the first discovered fungal proteinaceous HST (Balance et al., 1989; Tomas et al., 1990). We demonstrated that *ChToxA* acts as a host-specific virulence factor in fungal pathogenesis. The sequence and phylogenetic analyses presented in this work prompt us to examine the distribution of ToxA-like proteins in ascomycete fungi.

ChToxA is a protein ortholog of PtrToxA and SnToxA

ToxA had been known as a unique necrotrophic effector produced by only two ascomycete pathogens, *P. tritici-repentis* (Ciuffetti et al., 1997) and *S. nodorum* (Friesen et al., 2006) until an ortholog was identified recently in the genome of *S. avenaria tritici* 1, a sister species of *S. nodorum* (McDonald et al., 2013). All three pathogens attack the same host plant, wheat. It has been proposed that the gene encoding ToxA, which is

1 nearly-identical (98-99% identity at nucleotide levels) between these three species (Table
 2 3), has been “trafficked” by either horizontal gene transfer between distantly-related
 3 species, i.e., *P. tritici-repentis* and *S. nodorum* (Friesen et al., 2006), or interspecific
 4 hybridization between closely-related species such as the two *Stagonospora* spp.
 5 (McDonald et al., 2013).

6 In this study, we identified a *ToxA*-like gene from *C. heterostrophus*, an
 7 ascomycete pathogen that attacks a different monocotyledonous plant, maize. We
 8 demonstrated *ChTOXA* and *PtrToxA* loci share common features in genomic organization
 9 and that structure of the encoded proteins is highly similar. At the nucleotide level, the
 10 two *ToxA* genes have no significant similarity. However, both genes contain an intron at
 11 the same position that has unique 5’ and 3’ splicing sites (Fig. 1A). At the amino acid
 12 level, the two *ToxA* proteins have moderate (64%) similarity (Table 3); nevertheless, the
 13 deduced mature *ChToxA* protein is predicted to adopt a *PtrToxA*-like three-dimensional
 14 structure (Fig. 1C) with 100% confidence as reported by the Phyre2 server. In addition,
 15 when the *ToxA* proteins are used to query each other’s protein catalogs, they are each
 16 other’s top matches (*Ch* JGI protein ID 1019653 hits *Ptr* JGI protein ID 150574 and *vice*
 17 *versa*). The same is true when *ChToxA* is used to query the *S. nodorum* protein catalog
 18 (best match is JGI protein 10410 which is *SnToxA*). We conclude therefore, that *ChToxA*
 19 is a legitimate designation for the subject of this study.

20 *ChToxA acts as an HST and as a virulence determinant*

22 Our characterization of the recombinant *ChToxA* protein and comparison of HST
 23 and virulence phenotypes of wild type and *Chtox*A mutants provides solid evidence that

1 *ChTOXA* controls the production of a proteinaceous HST, *ChToxA*, which acts as a
 2 virulence factor during fungal pathogenesis to maize. The *ChToxA*-induced necrosis on
 3 maize is light-dependent (Fig. 3B), as is *PtrToxA* activity on wheat (Manning et al.,
 4 2005). The sensitivity to *ChToxA* is predominant among maize inbred lines and differs
 5 phenotypically among sensitive lines treated with the toxin (Table 1). The sensitivity to
 6 *PtrToxA* is also predominant (50-85%) among wheat lines derived from breeding or
 7 artificial selections (Faris et al., 2010; Oliver et al., 2008) and differs in wheat lines
 8 carrying the same sensitivity gene (Tan et al., 2012).

9 *ChtoxA* mutants are reduced in virulence only on particular *ChToxA*-sensitive
 10 maize lines. A simple explanation for this finding is that, like *P. tritici-repentis* (reviewed
 11 by Ciuffetti et al., 2010) and *S. nodorum* (reviewed by Oliver et al., 2012), *C.*
 12 *heterostrophus* may also produce multiple HSTs; thus, eliminating one such HST does
 13 not necessarily result in a discernable reduced virulence phenotype on maize lines
 14 carrying multiple genes for sensitivity to various HSTs. On this issue, there are at least
 15 180 predicted small secreted proteins (SSPs) and a rich reservoir of nonribosomal peptide
 16 synthetases/polyketide synthases encoded in the *C. heterostrophus* genome (Condon et
 17 al., 2013). Some of these may control the production of additional HSTs that induce
 18 disease in certain maize lines in the absence of *ChToxA*.

19 The identification of the maize gene controlling sensitivity to *ChToxA* will be
 20 necessary to unravel common response pathways in the two hosts maize and wheat,
 21 which diverged in the grass family 50-70 million years ago (Kellogg, 2001). Association
 22 mapping of appropriate maize inbred lines (e.g., Flint-Garcia et al., 2005) may be helpful

1 in identifying quantitative trait loci (if any) mediating the phenotypic variations in maize
 2 sensitivity to *ChToxA*.

3 4 *ChToxA and PtrToxA structural differences*

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 6 We have shown recently that *Ptr* N102 residue located in the asparagine-rich
 7 motif (Fig. 1C) is required for ToxA activity and that the same residue also serves as a
 8 binding site for interacting with wheat pathogenesis-related protein (PR-1-5) that
 9 potentially mediates ToxA-induced necrosis in sensitive wheat (Lu et al., 2014). Our
 10 preliminary experiments indicate that a *ChToxA* recombinant protein having the N85
 11 residue (Fig. 1C) replaced by an alanine fails to induce necrosis on sensitive maize lines.
 12 These results suggest that the asparagine-rich motif may serve as a diagnostic signature
 13 for distinguishing functional ToxA-like proteins, e.g., those lacking this motif (Fig. 6A)
 14 may be less active or nonfunctional. Whether or not the PR-1-5 ortholog exists in maize,
 15 interacts with *ChToxA* and mediates *ChToxA*-induced necrosis on maize remains to be
 16 determined. The entire C-terminal regions where the two ToxA proteins have substantial
 17 dissimilarities, including the absence of the RGD motif in *ChToxA* (Fig. 1B), could
 18 contribute to host specificity. Our preliminary experiments indicated that a recombinant
 19 *ChToxA* protein, in which SGN was replaced with RGD, retains the ability to induce
 20 necrosis on *ChToxA*-sensitive maize (but not on *PtrToxA*-sensitive wheat), like the wild
 21 type *ChToxA*, suggesting that RGD is exchangeable with SGN for *ChToxA* function, but
 22 is not sufficient to alter the host specificity. On the other hand, we were unable to show
 23 this in the opposite way because the attempt to express a recombinant *PtrToxA* protein,

1 in which RGD was replaced with SGN, failed under the same conditions. This was likely
 2 due to the fact that the RGD motif contributes to not only the function, but also the
 3 protein stability in *PtrToxA*. Manning et al (2004) reported that the replacement of the
 4 aspartic acid residue with an alanine in the RGD motif resulted in a rapid degradation of
 5 the recombinant *PtrToxA*-D142A protein. Thus, although it is likely that *ChToxA* and
 6 *PtrToxA* may act in similar ways to induce necrosis in their respective hosts, it remains
 7 to be determined if the SGN motif functions specifically for adhesion to host receptor(s)
 8 as was reported for the RGD motif in *PtrToxA*-wheat interactions (Manning et al., 2004,
 9 2008).

11 *Phylogenetic distribution of ToxA and ToxA**

12 The discovery of *ChTOXA* and *TOXA** genes in other ascomycetes raises the
 13 hypothesis that *ChTOXA* and *PtrToxA* may have originated from a common ancestor,
 14 which might be one of the ancient *TOXA** genes. The essential difference between
 15 *TOXA* and *TOXA** gene is that the latter lacks any introns. There is weaker similarity
 16 between *ToxA* and *ToxA** proteins in the regions (~ 25 aa) between the last two motif
 17 sequences (LILTXY and WXXQ) that begins right after the intron splicing point and
 18 harbors the RGD/SGN motif in *PtrToxA/ChToxA* (Fig. 6A). We note that the *TOXA**
 19 gene is duplicated in *Claviceps purpurea* (Fig. 6B). We speculate that the common
 20 ancestor of *P. tritici-repentis* and *C. heterostrophus* might have had two copies of *ToxA**,
 21 one of which gave rise to *ChTOXA* through a transposon-mediated insertion, which
 22 created the unusual intron. The association of *ChToxA* and *PtrToxA* with blocks of
 23 repeats encoding *RTPAI* (Fig. 1A) offer a clue to how the novel second copy was

relocated in the genome. The facts that repeat elements have been shown to play roles in the evolution of pathogenicity in *P. tritici-repentis* and *C. heterostrophus* (Manning et al., 2013; Santana et al., 2014) support this hypothesis. The percent similarity when all ToxA proteins are compared to *ChToxA* or *PtrToxA* is in the 38-64 range, except for the previously noted remarkably identity between *PtrToxA* and *SnToxA*.

Manning et al (2013) identified a genomic region (~30 kb) in *C. heterostrophus* strain C4 that is syntenic with genomic regions flanking the *PtrToxA* gene. Note that the *ChTOXA* and *ChTOXA** genes are 100% identical between strain C5 and C4. The stretch of syntenic DNA identified by Manning et al is located on scaffold 37 in strain C4 (and on scaffold 5 in strain C5), and is not on the scaffolds harboring *CHTOXA* or *CHTOXA**. In C4, the *CHTOXA* and *CHTOXA** are located on scaffold 114 and scaffold 32, respectively. Thus, this syntenic DNA is not linked to *ChTOXA* in the *C. heterostrophus* genome. However, we cannot exclude the possibility that it is actually part of an “ancient” locus from which *ChTOXA* was mobilized to the current location by *ToxA*-associated transposon activities. It has been proposed that *PtrToxA* is mobile since it can be found on different chromosomes in natural isolates (Manning et al., 2013).

As mentioned and reflected in Table 3, the similarity between *PtrToxA* and *SnToxA* is very high (99%), suggesting that one acquired the gene from the other. Indeed it has been speculated that *P. tritici-repentis* acquired *ToxA* from *S. nodorum* through horizontal gene transfer (HGT) (Friesen et al., 2006). Curiously, unlike *C. heterostrophus* and *P. tritici-repentis*, which have both *TOXA* and *TOXA**, *S. nodorum* does not appear to have *ToxA**-like proteins. In addition, it lacks apparent homologues of *RTPAI* flanking the *ToxA* locus. These facts may support a reverse HGT scenario, i.e., *S.*

1 *nodorum* might have acquired *ToxA* from *P. tritici-repentis*. However, one cannot
 2 exclude the possibility that the two fungi acquired *ToxA* from the same resource
 3 independently. Extensive genome-wide analyses are needed to determine the mechanisms
 4 underlying the evolutionary trajectory.

5 Our database searches revealed that the *ToxA*-like proteins include one member,
 6 i.e., the *ToxA** protein from the anthracnose fungus *Colletorichum higginsianum* (Table
 7 3) that has been previously identified as a potential extracellular effector protein (EC13)
 8 (Kleemann et al., 2012). The *C. higginsianum* *EC13* gene is expressed almost exclusively
 9 in appressoria with penetration hyphae (Kleemann et al., 2012). It will be interesting to
 10 test if EC13 or any of its closely related *ToxA** proteins are essential for fungal virulence
 11 on their respective hosts. Our current experiments indicate that *C. heterostrophus* *ToxA**
 12 may not play a role in fungal pathogenesis since the *ChTOXA** gene is not expressed *in*
 13 *planta* (Fig. 2), and the encoded protein, if expressed, is predicted to be truncated after
 14 the conserved valine residue at the position 89 in comparison with its counterparts in
 15 other *Cochliobolus* spp. (Fig. S4).

16 In conclusion, through exploring fungal genomic and plant germplasm resources
 17 and functional analyses, we have identified the first proteinaceous HST from *C.*
 18 *heterostrophus*. The observed similarity in protein structures and light-dependent
 19 necrosis-inducing activities of *ChToxA* and *PtrToxA* suggests strongly that *C.*
 20 *heterostrophus* and *P. tritici-repentis* may share the same pathogenic strategy in
 21 exploiting plant pathways for necrotrophy on their respective hosts. Thus, further studies
 22 on *ChToxA* and other *ToxA*-related proteins may provide new insights into effector
 23 evolution in host-pathogen interactions.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version.

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Figure Legends

Figure 1. Comparison of the *C. heterostrophus* *ToxA*-like gene (*ChTOXA*) and *P. tritici-repentis* *ToxA*.

(A) Genomic organization of the *ToxA* loci. Open arrows indicate *ChTOXA/PtrToxA* ORFs interrupted by an intron (solid bar, numbers indicating nucleotide positions) with sequences shown below (5' and 3' splicing sites highlighted in red). *RTPAI*, putative gene encoding Retrotransposon-like Protein associated with the *ToxA* locus. Textured boxes indicate the 5' and 3' untranslated regions in the full-length *ToxA* transcripts. Dashed lines indicate genomic regions lacking apparent microsynteny between the two *ToxA* loci.

(B) Domain organization of *ToxA* proteins. Top: Signal peptide (SP), pro-region or N-domain (N) and mature toxin or C-domain (C) are indicated by shaded or open boxes with numbers below indicating the first residue in the full-length proteins. Brackets with dashed lines indicate the predicted disulfide bridges. Bottom: Amino acid sequence alignment. The asparagine-rich and RGD/SGN motifs are highlighted in blue and red, respectively. Triangle indicates the position where the intron sequences are removed in the coding mRNA. The two conserved cysteine residues are indicated by asterisks. Secondary structures are drawn above the sequences following *PtrToxA* (Sarma et al., 2005).

(C) Ribbon diagrams showing protein structures predicted by the Protein Homology/Analog Recognition Engine (Kelley and Sternberg, 2009). Secondary structure elements are highlighted in colors with blue, green and orange for α helices, β

strands and loops, respectively. Relative positions are indicated for the surface-exposed active sites (N102/N85) and the RGD/SGN motifs.

Figure 2. *ChTOXA* and *ChTOXA** are differentially regulated during fungal pathogenesis.

Ethidium bromide-stained 1% agarose gels were loaded with the amplicons of reverse transcriptase (RT)-PCR products from mRNA isolated from fungal cultures grown in MM, CM or from maize inoculated with *C. heterostrophus* (strain C5) that were collected at different time points as indicated at the top. Lanes 11 were loaded with negative controls (c) in which mRNA isolated from uninoculated maize plants was used for RT-PCR under the same conditions. Numbers on the right indicate size (in base pairs) of the gene-specific amplicons (primer sequences are given in Table S1). *ChACT-1*, *C. heterostrophus* actin gene (internal control). M, 1-kb DNA ladder.

Figure 3. *ChToxA* possesses necrosis-inducing activity with host-selectivity against maize.

(A) Characterization of the recombinant *ChToxA* protein. Left: SDS-PAGE gel was loaded with 25 µl (per lane) of cell-free culture supernatant collected from *P. pastoris* strains expressing the respective recombinant proteins as indicated at top. Negative control (lane 2) was taken from a “mock” strain transformed with the expression vector only. Numbers on the right indicate the predicted molecular masses (in kD). M, protein markers. Right: MALDI-TOF/TOF MS data (through Cornell

1 Biotechnology Resource Center) showing the 18-aa fragment containing residues 94-111
 2 of *ChToxA* (peak = 978.51).

3 (B) Infiltration assays. Maize plants were infiltrated with *ChToxA* at estimated
 4 final concentrations indicated on the right and incubated under light (16 h) – dark (8 h)
 5 photoperiod conditions (leaves 1-3) or complete darkness (leaf 5). Control plants (leaf 4)
 6 were treated with the protein sample of the “mock” strain shown in A (lane 2). Marks
 7 delimit the infiltrated areas. Photographs were taken 72 h post infiltration.

8

9 **Figure 4.** Deletion of the *ChTOXA* gene results in reduced virulence on *ChToxA*-
 10 sensitive maize.

11 (A) Schematic representation of the split mark strategy used to knockout
 12 *ChTOXA*. Open boxes, filled boxes, open arrow, and open boxes with dashed lines
 13 represent PCR fragments (or the corresponding genomic sequences), the hygromycin B
 14 (*hygB*) resistance gene cassette, the *ChTOXA* open reading frame, and the genomic
 15 sequences 5' or 3' outside of the targeted genomic regions, respectively; numbers inside
 16 indicate the size (in base pairs). Thick lines indicate *C. heterostrophus* genome sequence.
 17 Dashed cross lines indicate the expected cross-over between homologous sequences.
 18 Arrows indicate the primers (Table S1) used to validate the integration events and the
 19 expected PCR products with size are indicated by double-arrowed lines.

20 (B) Ethidium bromide-stained gels showing PCR products amplified from DNA
 21 of the wild type parental strain C5 (lane 1) and five transformants, TxC5-6-2, TxC5-12-1,
 22 TxC5-14-3, TxC5-16-1, and TxC5-19-3 (lanes 2-6). Numbers on the right indicate size
 23 (in base pairs) of the expected amplicons shown in (A). M, 1-kb DNA ladder.

(C) Plant assays showing the virulence phenotypes of the wild type strain C5 (leaf 1) and the five transformants (leaves 2-6, in the same order as in B). Plants of the *ChToxA*-sensitive (top panel) and -insensitive (bottom panel) maize lines were inoculated with the same conidial suspensions and incubated under the same conditions. One representative leaf was shown for each treatment. Photographs were taken 5 days post inoculation.

Figure 5. Plant assays showing the virulence phenotypes.

Representative leaves from inoculations with the wild type strain C5 (leaf 1) and the mutant 1401-R30 (leaf 2) parents and eight progeny ascospores (leaves 3-10) isolated from a complete tetrad in cross #1402. An enlargement of the infected areas (boxed) was shown for the leaves infected by the two parental strains (bar inside = 2 mm). One representative leaf was shown for each treatment. Photographs were taken 5 days post inoculation.

Figure 6. Sequence and phylogenetic analyses of potential ToxA homologues from other species of ascomycetes.

(A) Amino acid sequence alignment. The three ToxA proteins (*ChToxA*, *PtrToxA* and *SnToxA*) are shown at the top (lines 1-3); ToxA-like (ToxA*) proteins from species in which the ToxA* protein was identified are shown below. The C-terminal truncated residues (after the point indicated by red asterisk) in the ToxA* protein of *C. heterostrophus* (line 4) are included in the alignment to facilitate motif analysis. Similar residues are highlighted with the same color. Asterisks at bottom of the alignment

1 indicate residues conserved in all proteins with the two cysteines indicated by vertical
 2 arrows. Motif sequences are given at the top. Triangle indicates the position where the
 3 intron sequences are removed in the coding mRNA of *ChToxA* and *PtrToxA* (see Fig.
 4 1B also). Numbers on the right indicate the positions in the full-length proteins. SP,
 5 signal peptide.

6 (B) A phylogenetic tree of the ToxA family. Species of Dothideomycetes are
 7 bolded. The three ToxA proteins are highlighted in red. The two species (*C.*
 8 *heterostrophus* and *P. tritici-repentis*) that have both ToxA and ToxA* proteins are
 9 underlined. Bootstrapping values (>50%, from 1,000 replicates) are indicated on the
 10 branches. Genus abbreviations: *C.*, *Cochliobolus*; *Cl.*, *Claviceps*; *Co.*, *Colletotrichum*; *F.*,
 11 *Fusarium*; *M.*, *Macrophomina*; *N.*, *Neofusicoccum*; *P.*, *Pyrenophora*; Accession numbers
 12 are given in Table 3.

14 Supplemental Figure Legends

16 **Supplemental Figure 1.** The *ChTOXA* gene is surrounded by repetitive DNA sequences.

17 A screenshot of the *ChTOXA* locus ([http://genome.jgi.doe.gov/cgi-](http://genome.jgi.doe.gov/cgi-bin/browserLoad/?db=CocheC5_3&position=scaffold_2:2346666-2354045)
 18 [bin/browserLoad/?db=CocheC5_3&position=scaffold_2:2346666-2354045](http://genome.jgi.doe.gov/cgi-bin/browserLoad/?db=CocheC5_3&position=scaffold_2:2346666-2354045)) is shown.
 19 Red arrow indicates the *ChTOXA* gene (positions 2,349,946-2,350,765). Black bars
 20 indicate various repetitive elements. Brown bars indicate SNPs in different *C.*
 21 *heterostrophus* strains (C4 and C5) or field isolates (Hm338, Hm540 and PR1x412) and
 22 isolates of other *Cochliobolus* spp. (*C. carbonum* 26-R-13, *C. victoriae* F13, *C. lunatis*
 23 m118, and *C. miyabeanus* ATCC-44560).

1

2 **Supplemental Figure 2.** The genomic regions surrounding the *ToxA* loci of *C.*
3 *heterostrophus* and *P. tritici-repentis* contain discontinuous short “syntenies” of
4 repetitive DNA.

5 Dot matrix views generated with the TBLASTX program
6 (<http://blast.ncbi.nlm.nih.gov>) were shown for the alignment between ~100 kb of
7 genomic sequences carrying *ChTOXA* (query) or *PtrToxA* (subject)*. Black dots indicate
8 the regions encoding proteins with significant similarities. Red star indicate the positions
9 corresponding to the ORFs of the aligned *ToxA* genes. Blue circles indicate four scattered
10 short linear regions encoding conserved retrotransposon-like protein (RTP). *The 100-kb
11 genomic DNA of *C. heterostrophus* is part of C5 scaffold_2 (positions 2,300,001-
12 2,400,000) and the 535-bp *ChTOXA* ORF is located at positions 50,030-50,664. The 101-
13 kb genomic DNA of *P. tritici-repentis* is part of supercont1.4 (positions 1,398,479-
14 1,449,979) (<http://www.broadinstitute.org/>) and the 587-bp *PtrToxA* ORF is located at
15 positions 50,416-51,002.

16

17 **Supplemental Figure 3.** Differential reactions of maize inbred lines to *ChToxA* and *C.*
18 *heterostrophus*.

19 Left: Infiltration assays. Two-week old plants were infiltrated with *ToxA* at an
20 estimated final concentration 0.1 μ M. Marks delimit the infiltrated area. Photographs
21 were taken 72 h post infiltration. Right: Fungal infection assays. Three-week old plants
22 were inoculated with conidial suspensions (1×10^6 /ml) of *C. heterostrophus* (strain C5)
23 and incubated under the defined conditions (see main text); symptoms were recorded 96

h post inoculation. Bar = 5 mm (with 1 mm increments). Each treatment (shown by one representative leaf) included 6-10 plants and was repeated at least twice to confirm the observed phenotypes.

Supplemental Figure 4. Amino acid (A) and nucleotide (B) sequence alignments showing the single site mutation in *ChToxA** that causes an early stop in the open reading frame leading to a C-terminal truncated protein.

The red asterisk in A indicates the stop in the actual translated *ChToxA** protein, for which, the truncated residues (75 aa) are still included in the alignment for comparison. The yellow box in B indicates the “C to A” mutation in *ChToxA** at the third position of the codon specifying tyrosine (TAC) in *CcToxA** and *CvToxA** or leucine (CTC) in *CmToxA** proteins. Species abbreviations: *Ch*, *Cochliobolus heterostrophus*; *Cc*, *C. carbonum*; *Cv*, *C. victoriae*; *Cm*, *C. miyabeanus*.

Table 1Maize inbred lines used in this study^a

Line	Origin	Reaction to <i>ChToxA</i> ^b	Reaction to <i>C. het.</i> ^c
W64A	Illinois	++	+++
Creek White	Tennessee	++	+++*
SD43	South Dakota	+++	+++
GS73CO	Mexico	+++	+++
W570	Wisconsin	+++	+++*
SD44	South Dakota	++	+++
SD45	South Dakota	++	+++
FS8A	Florida	+++	+++
ND301	North Dakota	++	+++
ND253	North Dakota	++	+++
ND101	North Dakota	++	+++
DE811	Delaware	-	+
ND257	North Dakota	++	+++
Pa760	Pennsylvania	++	+++
NYRD4058	New York	++	+++
SD40	South Dakota	++	+++
B75	Iowa	++	+++
GEMS-0009	North Carolina	++	+++*
TZSTRI110	West Africa	-	+

P737M20	Michigan	++	+++
W64ARpp9	Wisconsin	+++	+++
R225	Illinois	++	+++
H116	Indiana	+++	+++
FS8B	Florida	++	+++
B37	Iowa	++	+++
NC250	North Carolina	-	+
CML9	Mexico	++	+++
CML247	Mexico	+++	+++
CML264	Mexico	++	+++
CML277	Mexico	+++	+++*
CML52	Mexico	+++	+++*
CML287	Mexico	++	+++
G80	Iowa	++	+++

^a All inbred lines were kindly provided by the North Central Regional Plant Introduction Station (NCRPIS). *ChToxA*-insensitive lines are in bold.

^b The third leaves of two-week-old plants were infiltrated with *ChToxA* and reactions were recorded 72 h post infiltration. "+++", necrosis occurred across entire infiltrated areas; "++", scattered necrotic spots or just chlorosis developed in infiltrated areas; "-", no reactions.

^c Three-week-old plants were inoculated with 1×10^6 /ml conidial suspensions of *C. heterostrophus* (strain C5) and symptoms were recorded 96 h post inoculation. "+++", developed lesions with typical size (2-5 mm); "+", developed smaller lesions (most <2 mm). The five *ChToxA*-sensitive maize lines that developed smaller lesions when infected by *ChToxA* null mutants are indicated by asterisks.

Table 2

Marker segregation in progeny from two crosses between independent *ChToxA* deletion mutants and wild type testers of *C. heterostrophus*

Cross/ ascus# ^a	# spores	<i>ChToxA</i>				Virulence ^b			
		Parental types		Non-parental types		Parental types		Non-parental types	
		<i>hygB^R</i> <i>ToxA⁻</i>	<i>hygB^S</i> <i>ToxA⁺</i>	<i>hygB^R</i> <i>ToxA⁺</i>	<i>hygB^S</i> <i>ToxA⁻</i>	<i>hygB^R</i> <i>Vir⁺</i>	<i>hygB^S</i> <i>Vir⁺⁺⁺</i>	<i>hygB^R</i> <i>Vir⁺⁺⁺</i>	<i>hygB^S</i> <i>Vir⁺</i>
1401	63	36	27	0	0	nd	nd	nd	nd
1402	48	26	22	0	0	13*	13*	0	0
1402-1	8	4	4	0	0	4	4	0	0
1402-2	8	4	4	0	0	4	4	0	0
1402-3	8	4	4	0	0	4	4	0	0

^a Cross #1401 was done between the strain C5-derived transformant Tx C5-6-2 (*ChToxA⁻*; *hygB^R*; *ToxI⁻*; *MAT1-1*) and inbred sister strain C4 (*ChToxA⁺*; *hygB^S*; *ToxI⁺*; *MAT1-2*) of *C. heterostrophus*. Cross #1402 was done between strain 1401-R30 (*ChToxA⁻*; *hygB^R*; *ToxI⁻*; *MAT1-2*), which is a progeny ascospore from cross #1401, and strain C5 (*ChToxA⁺*; *hygB^S*; *ToxI⁻*; *MAT1-1*). 1402-1, -2 and -3 denote three asci isolated from cross #1402 that contained complete tetrads.

^b Virulence was determined by inoculation assays on the *ChToxA*-sensitive maize line W570. *Vir⁺*, weak disease symptoms as indicated by smaller lesions on infected plants; *Vir⁺⁺⁺*, wild type symptoms (Figs. 4 and 5). For cross #1402, a total of 26 (13

hygromycin B-resistant and 13 hygromycin B-sensitive) random spore progeny (asterisks) were selected for plant assays along with the three complete tetrads. nd: not done (except for 1401-R30, which was used as a parent for cross 1402 and tested in subsequent inoculation assays).

Table 3

Sequence characteristics of ToxA and ToxA-like proteins used in this study.

Species ^a	GenBank accession ^b	Length (aa)	SP ^c	Intron	Similarity to <i>ChToxA</i> (%) ^d	Similarity to <i>PtrToxA</i> (%) ^e
Dothideomycetes						
<i>C. heterostrophus</i>	<u>KJ664924</u>	161	Yes	Yes	100 (1-161)	64 (28-178)
<i>C. heterostrophus</i> *	KP698579	165	Yes	No	53 (35-92)	51 (30-117)
<i>C. carbonum</i>	EUC36307	165	Yes	No	48 (36-130)	50 (57-138)
<i>C. victoriae</i>	EUN25607	165	Yes	No	53 (35-92)	48 (57-138)
<i>C. miyabeanus</i>	EUC44184	166	Yes	No	41 (35-160)	43 (51-178)
<i>M. phaseolina</i>	EKG15312	159	Yes	No	52 (20-124)	50 (53-137)
<i>N. parvum</i>	JGI	159	Yes	No	50 (42-130)	48 (57-137)
<i>P. tritici-repentis</i>	<u>AAB61464</u>	178	Yes	Yes	64 (11-161)	100 (1-178)
<i>P. tritici-repentis</i> *	FGI	187	Yes	No	38 (61-150)	39 (53-98)
<i>P. teres</i> f. <i>teres</i>	XP003298007	187	Yes	No	40 (10-160)	54 (26-138)
<i>S. nodorum</i>	<u>ADM80303</u>	178	Yes	Yes	63 (11-161)	99 (1-178)
Sordariomycetes						
<i>Co. fioriniae</i>	EXF72942	175	Yes	No	41 (21-152)	N/A
<i>Co. higginsianum</i>	CCF70887	154	Yes	No	39 (10-160)	N/A
<i>F. oxysporum</i>	EXK24251	167	Yes	No	43 (26-154)	41 (1-178)
f. sp. <i>melonis</i>						

<i>Cl. purpurea</i>	CCE26799	167	Yes	No	N/A	47 (96-143)
<i>Cl. purpurea</i>	CCE26798	170	Yes	No	N/A	45 (62-131)

^a Each species has only one ToxA-like protein except for *C. heterostrophus* and *P. tritici-repentis*, both of which have ToxA and ToxA* (asterisks).

^b There are two proteins identified by translating the corresponding genomic sequences in the following resources: JGI, *Neofusicoccum parvum* UCRNP2 scaffold_240 (the DOE Joint Genome Institute, <http://jgi.doe.gov>); FGI, *P. tritici-repentis* supercont1.6 (the Fungal Genome Initiative, www.broadinstitute.org). Three ToxA proteins included in the phylogenetic analysis (Fig. 6) are underlined. The *SnToxA* protein (ADM80303) used in this study belongs to the H5 (haplotype 5) group, which is predominant among natural populations of *S. nodorum* and is one of the four haplotypes that appear to be more closely related *ChToxA* as indicated by the presence of an aspartic acid residue (D) at position 99 (Fig. 4A), at which a glutamic acid residue (E) is found in other nine haplotypes (Stukenbrock and McDonald, 2007; Tan et al., 2012).

^c SP, signal peptide, predicted by the SignalP Server (<http://www.cbs.dtu.dk>).

^{d,e} Similarity (amino acid) was determined by using the BLAST program. (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Numbers in parentheses indicate the positions of *ChToxA/PtrToxA* residues overlapping those of the aligned homologous proteins. N/A, no significant similarity.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6