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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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 Pcv2 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 (Ballance et al., 1989).
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 intron with unusual splicing sites (5'-ATAAGT...TAC-3'). ToxA is processed to a 13.2-kD mature protein of 118 amino acids (aa), which adopts a unique three-dimensional structure consisting of a S wrench-fold with two antiparallel b-sheets (Sarma et al., 2005) and a solvent-exposed arginyl-glycyl-aspartic acid (RGD) motif located at the C-terminal region that is essential for ToxA activity (reviewed by Ciuffetti et al. (2010)). ToxA had long been considered to be a unique effector HST lacking counterparts in other fungal pathogens until the identification of an ortholog (Friesen et al., 2006). Subsequently an ortholog was also discovered in Stagonospora avernaria tritici 1, a sister species of S. nodorum (McDonald et al., 2013). It has been proposed that ToxA was acquired by P. triticum-repentis from S. nodorum through a lateral/horizontal gene transfer (Friesen et al., 2006). To date, no ToxA-like proteins have been characterized from other plant-pathogenic ascomycetes.

We recently reported the cloning of a ToxA-like gene (ChTOXA [ToxA in Ptr and Sn]) from C. heterostrophus and showed that it encodes a protein with both sequence and structure similarities to PtrToxA (Lu and Edwards, 2014). In this manuscript, we describe ChTOXA and ChTOXA-like genes from C. heterostrophus and closely-related species and functional characterization of ChToxA by recombinant protein and gene deletion approaches. We demonstrate that the recombinant ChToxA protein produces light-dependent leaf necrosis in a host-selective manner and that the native ChTOXA gene product acts as a virulence factor on specific ChToXA-sensitive maize lines. Our study 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., 1997b). Ascospore progeny were isolated from spherotheria 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.

2.2. Nucleic acid preparations

Fungal mycelia were scraped from agar plates or collected from liquid cultures by centrifugation. Maize leaves collected from inoculated plants were cut into small segments (~1.0 cm), tissue samples were transferred into 2-ml Lysing Matrix D tubes containing 1.4-mm ceramic spheres (QiBiogene, Morgan Irvine, CA), and frozen in liquid nitrogen for 2–5 min immediately before homogenization using a Mini-Beadbeater-16 Cell Disruptor (BioSpec Products, Bartlesville, OK). Genomic DNA was extracted from the homogenized tissues using the Dynabeads mRNA Direct Kit (Invitrogen, CA) following the manufacturer’s instructions and finally eluted in Tris·HCl buffer (pH 8.0). cDNA synthesis was done using the Superscript III first strand synthesis system (Invitrogen, CA).

2.3. Genomic/cDNA cloning and PCR conditions

Genomic clones of ChTOXA and other ToXA-like genes were obtained by PCR amplification from fungal genomic DNA using primers (Table S1) designed based on the sequences identified in the databases of the DOE Joint Genome Institute (JGI) (http://www.jgi.doe.gov). PCR was performed in a 25-μl volume of 1 × GoTaq Green Master Mix (Promega, Madison, WI) that contained 1–10 ng of fungal genomic DNA and 0.5 μM 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′ un-translated 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 Escherichia coli cells (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared using the Wizard Plus SV Miniprep 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 Access# 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 GoTag 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 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 following standard protocols (Green and Sambrook, 2012). The concentration of the target protein was estimated by comparison with a series of dilutions of bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA, USA) included in the same SDS–PAGE gel. After SDS–PAGE gel separation, the coomassie blue-stained band corresponding to the 12.1-kD ChToxA protein was excised from the gel and subjected to MALDI-TOF/TOF analysis performed at the Cornell University Biotechnology Resource Center as described previously (Lu et al., 2013).

2.6. Plant materials and bioassays

Maize inbred lines were obtained through the North Central Regional Plant Introduction Station (NCRIPS) (Table 1). Plants were grown in 6-in. pots (10–16 plants/per pot) containing SB100 professional growing mix (Sungrow Horticulture, Bellevue, WA, USA) in a growth chamber at an average temperature of 21 °C with a 16-h photoperiod for 2–3 weeks. For ChToXA activity assays, the third leaf of each plant was infiltrated with ~20 μl of cell-free culture supernatant (diluted with water) containing the recombinant ChToxA protein at the desired concentrations using a 1-ml syringe without the needle (Delacso, Council Bluffs, IA, USA). Control plants were infiltrated with the culture supernatant from a yeast strain transformed with the expression vector only. Treated plants were kept in the growth chamber and symptoms were examined 2–5 days after infiltration. Each treatment included six plants and was repeated at least twice. For virulence assays, fungal conidia were collected from 10 to 15 days-old cultures grown on CMX plates and suspended in 0.05% Tween 80 solutions with the concentrations adjusted to 1 x 10⁶/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 3–5 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 ChToxA-sensitive maize lines that developed smaller lesions when infected by ChToxA null mutants are indicated by asterisks.

<table>
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<th>Line</th>
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a All inbred lines were kindly provided by the North Central Regional Plant Introduction Station (NCRIPS). 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 x 10⁷/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.

d For marker segregation analysis, ChToxA mutants, in the strain C5 genetic background, validated by PCR assays were crossed to strain C4 (hygB⁶; ChToXA⁺; ToxA⁻; MATI-2), which is the opposite mating type (Leach et al., 1992b). Progeny were isolated from cross #1401 (between C4 and Tcxs-6-2) because this cross gave the best fertility. One of the progeny (1401-R36, genotype = hygB⁶; ChToXA⁺; ToxA⁻; MATI-2), from cross #1401, was then back-crossed (cross #1402) to the parental strain C5 (genotype = hygB⁶; ChToXA⁺; ToxA⁻; MATI-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, MATI-1 or MATI-2 (Turgeon et al., 1993), and PKS1 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 package (http://evolution.genetics.washington.edu/phylip.html) (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.

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 sβ and ββ shorter than counterparts that flank the RGD motif in PtrToxA (Fig. 1C).

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′-ATAATG…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., RTPA1) 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 2,300,000–2,350,500 on the left flank and 2,350,700–2,365,000 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 RTPA1 appear to be absent in the genome of S. nodorum (data not shown).

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).

3.2. ChTOXA is up-regulated during fungal pathogenesis

To enable functional analysis, we obtained a recombinant ChToxA protein through heterologous expression in P. pastoris, as described previously (Lu et al., 2013). 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, G573CO, 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 μM) on sensitive inbred lines such as W570 was manifested as tissue collapse across the entire infiltrated area (Fig. 3B, left, leaves 1 and 2). Scattered necrotic spots were observed when ChToxA was applied at lower concentrations (e.g., 0.05 μM) (Fig. 3B, left, leaf 3). A negative reaction was observed on insensitive maize lines like DE811 regardless of the concentration of ChToxA used under the same conditions (Fig. 3B, right). It should be noted that the observed necrosis symptoms shown in Figs. S1 and 3B may look “restricted” to the area surrounding the primary vein. This was due to the fact that maize leaves are much broader than those of wheat; a single shot with the same volume is sufficient to cover the entire breadth of the leaf on wheat (e.g., Manning et al., 2004, 2008), but not on maize. In fact, necrosis occurred across the entire infiltrated area.

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

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

To explore the function of ChToxA in fungal pathogenesis, we first tested if the sensitivity to ChToxA correlated with the susceptibility to the fungus. Inoculation assays were performed on all 33 maize inbred lines as mentioned above (Table 1). All maize lines were susceptible, but the disease symptoms fell into two major groups. At 5 days post inoculation (dpi), 30 ChToxA-sensitive maize lines developed necrotic lesions, 2–5 mm in size whereas the three ChToxA-insensitive maize lines (DE811, TZSTRI110 and NC250) developed smaller lesions (most <2 mm) (Fig. S3). These initial observations raised the possibility that ChToxA may act as a virulence factor in the C. heterostrophus–maize interaction.
The complete ChTOXA ORF was deleted to determine virulence of the mutant compared to wild type. Five stable hygromycin B-resistant transformants (TxC5-6-2, TxC5-12-1, TxC5-14-3, TxC5-16-1, and TxC5-19-3), all indistinguishable from their parental strain in terms of growth rate, morphology, and sporulation, were obtained from the gene deletion experiment (Fig. 4A), and subjected to further characterization. PCR amplifications using five pairs of diagnostic primers (Table S1) revealed integration events. As shown in Fig. 4B, the hygB-specific primer pair (pH2501/pH1302) amplified an 849-bp fragment (internal to hygB) from all five hygromycin B-resistant progeny (lanes 2–6), but not from the wild type control (lane 1). The ChTOXA-specific primer pair (atgF/tagR) amplified a 535-bp fragment (containing the ChTOXA ORF) from the wild type control (lane 1) and TxC5-16-1 (lane 5), but not from TxC5-6-2, TxC5-12-1, TxC5-14-3, and TxC5-19-3 (lanes 2, 3, 4 and 6). The third primer pair, which consisted of a forward primer (5FP1) designed to the genomic region outside the left end (5' end) of the transforming DNA, and a reverse primer (pH375) designed to hygB and a reverse primer (3RP1) designed to the genomic region outside the right (3') end of the transforming DNA, amplified a 1138-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 2799-bp fragment from the wild type control (lane 1) and TxC5-16-1 (lane 5) that was replaced by a 4801-bp fragment (1150-bp 5' flank and 1114-bp 3' flank of native genomic DNA plus the 2537-bp hygB cassette).

Fig. 4. Deletion of the ChTOXA gene results in reduced virulence on ChToxA-sensitive maize. (A) Schematic representation of the split mark strategy used to knockout ChTOXA. Open boxes, filled boxes, open arrow, and open boxes with dashed lines represent PCR fragments (or the corresponding genomic sequences), the hygromycin B (hygB) resistance gene cassette, the ChTOXA open reading frame, and the genomic sequences 5' or 3' outside of the targeted genomic regions, respectively; numbers inside indicate the size (in base pairs). Thick lines indicate C. heterostrophus genome sequence. Dashed cross lines indicate the expected cross-over between homologous sequences. Arrows indicate the primers (Table S1) used to validate the integration events and the expected PCR products with size are indicated by double-arrowed lines. (B) Ethidium bromide-stained gels showing PCR products amplified from DNA of the wild type parental strain C5 (lane 1) and five transformants, TxC5-6-2, TxC5-12-1, TxC5-14-3, TxC5-16-1, and TxC5-19-3 (lanes 2–6). Numbers on the right indicate size (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.
indeed caused by a single integration event at the
These data confirmed that the deletion of
wild type and the
developed disease symptoms easily distinguishable between the
ChToxA null allele
Marker segregation in progeny from two crosses between independent
ChtoxA 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 ChtoxA 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 Tcx5-16-1 (leaf
5) developed large necrotic lesions across most areas of the
infected leaves. In contrast, the plants inoculated with the four
ChToxA 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 DBS11 under the same con-
ditions (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 tet-
rads 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<sup>−</sup>, whereas all hygromycin B-sensitive progeny were
ChToxA<sup>+</sup>. 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 tetrad isolated from cross #1402 (Table 2).
These data confirmed that the deletion of ChToxA in Tcx5-6-2 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 back-
cross (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 viru-
ence just like the ChToxA<sup>+</sup> parent (1401-130), while all hygromy-
cin 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), whereas those
inoculated with the ChtoxA mutant parent (1402-130) and the four
hygromycin-B-resistant progeny developed smaller lesions (most
~1 mm) (Fig. 5, leaves #2 and #7–10). Thus, deletion of ChToxA
caused ~50% reduction in virulence. No apparent symptomatic dif-
fferences 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 DBS11 (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. triticiprepentis 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.
### Fig. 5. Plant assays showing the virulence phenotypes. Representative leaves from inoculations with the wild type strain C5 (leaf 1) and the mutant 1403-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 3 days post inoculation.

**Table 3**

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

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<th>Intron</th>
<th>Similarity to ToxA (%)</th>
<th>Similarity to PtrToxA (%)</th>
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* Each species has only one ToxA-like protein except for C. heterostrophus and P. tritic-repentis, both of which have ToxA and ToxA* (asterisks).

* There are two proteins identified by translating the corresponding genomic sequences in the following resources: JGI, Neofuscococcum parvum UCNC22 scaffold; (the DOE Joint Genome Institute, [http://jgi.doe.gov](http://jgi.doe.gov)); FGI, P. tritic-repentis supercont L6; (the Fungal Genome Initiative, [www.broadinstitute.org](http://www.broadinstitute.org)). Three ToxA proteins included in the phylogenetic analysis (Fig. 6) are underlined. The SoToxA protein (ADMB03030) 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 to ChTOXA as indicated by the presence of an aspartic acid residue (D) at position 99 (Fig. 6A), at which a glutamic acid residue (E) is found in other nine haplotypes (Stukenbrock and Mcdonald, 2007; Tan et al., 2012).

* SP, signal peptide, predicted by the SignalP Server ([http://www.cbs.dtu.dk](http://www.cbs.dtu.dk)).

* Similarity (amino acid) was determined by using the BLAST program ([http://blast.ncbi.nlm.nih.gov/BLAST.cgi](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.

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. miyabeaenus**) codon to TAA resulting in an early stop (Fig. 54). 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 (Ballance 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.
4.1. 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 nearly-identical (98–99% identity at nucleotide levels) between these three species, has been “trafficked” by either horizontal gene transfer between distantly-related species, i.e., *P. tritici-repentis* and *S. nodorum* (Friesen et al., 2006), or interspecific hybridization between closely-related species such as the two *Stagonospora* spp. (McDonald et al., 2013).

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

4.2. ChToxA acts as an HST and as a virulence determinant

Our characterization of the recombinant ChToxA protein and comparison of HST and virulence phenotypes of wild type and ChToxA mutants provide solid evidence that ChTOXA controls the production of a proteinaceous HST, ChToxA, which acts as a virulence factor during fungal pathogenesis to maize. The ChToxA-induced necrosis on maize is light-dependent (Fig. 3B), as is PtrToxA activity on wheat (Manning and Ciuffetti, 2005). The sensitivity to ChToxA is predominant among maize inbred lines and differs phenotypically among sensitive lines treated with the toxin (Table 1). The sensitivity to PtrToxA is also predominant (50–85%) among wheat lines derived from breeding or artificial selections (Faris et al., 2010; Oliver et al., 2008) and differs in wheat lines carrying the same sensitivity gene (Tan et al., 2012). ChToxA mutants are reduced in virulence only on particular ChToxA-sensitive maize lines. A simple explanation for this finding is that, like *P. tritici-repentis* (reviewed by Ciuffetti et al. (2010)) and *S. nodorum* (reviewed by Oliver et al. (2012)), *C. heterostrophus* may also produce multiple HSTs; thus, eliminating one such HST does not necessarily result in a discernable reduced virulence phenotype on maize lines carrying multiple genes for sensitivity to various HSTs. On this issue, there are at least 180 predicted small secreted proteins (SSPs) and a rich reservoir of nonribosomal peptide synthetases/polyketide syntheses encoded in the *C. heterostrophus* genome (Condon et al., 2013). Some of these may control the production of additional HSTs that induce disease in certain maize lines in the absence of ChToxA.

The identification of the maize gene controlling sensitivity to ChToxA will be necessary to unravel common response pathways in the two hosts maize and wheat, which diverged in the grass family 50–70 million years ago (Kellogg, 2001). Association mapping of appropriate maize inbred lines (e.g., Flint-Garcia et al., 2005) may be helpful in identifying quantitative trait loci (if any) mediating the phenotypic variations in maize sensitivity to ChToxA.

4.3. ChToxA and PtrToxA structural differences

We have shown recently that *Ptr N102* residue located in the asparagine-rich motif (Fig. 1C) is required for ToxA activity and that the same residue also serves as a binding site for interacting with a wheat pathogenesis-related protein (PR-1-5) that potentially mediates ToxA-induced necrosis in sensitive wheat (Lu et al., 2014). Our preliminary experiments indicate that a ChToxA recombinant protein having the N85 residue (Fig. 1C) replaced by an alanine fails to induce necrosis on sensitive maize lines. These results suggest that the asparagine-rich motif may serve as a diagnostic signature for distinguishing functional ToxA-like proteins, e.g., those lacking this motif (Fig. 6A) may be less active or non-functional. Whether or not the PR-1-5 ortholog exists in maize, interacts with ChToxA and mediates ChToxA-induced necrosis on maize remains to be determined. The entire C-terminal regions where the two ToxA proteins have substantial dissimilarities, including the absence of the RGD motif in ChToxA (Fig. 1B), could contribute to host specificity. Our preliminary experiments indicated that a recombinant ChTOXA protein, in which SGN was replaced with RGD, retains the ability to induce necrosis on ChTOXA-sensitive maize (but not on PtrTOXA-sensitive wheat), like the wild type ChToxA, suggesting that RGD is exchangeable with SGN for ChToxA function, but is not sufficient to alter the host specificity. On the other hand, we were unable to show this in the opposite way because the attempt to express a recombinant PtrToxA protein, in which RGD was replaced with SGN, failed under the same conditions. This was likely due to the fact that the RGD motif contributes to not only the function, but also the protein stability in *PtrToxA*. Manning et al. (2004) reported that the replacement of the aspartic acid residue with an alanine in the RGD motif resulted in a rapid degradation of the recombinant *PtrToxA*-D142A protein. Thus, although it is likely that ChToxA and PtrToxA may act in similar ways to induce necrosis in their respective hosts, it remains to be determined if the SGN motif functions specifically for adhesion to host receptor(s) as was reported for the RGD motif in *PtrToxA*–wheat interactions (Manning et al., 2004, 2008).

4.4. Phylogenetic distribution of ToxA and ToxA’

The discovery of ChTOXA and TOXA’ genes in other ascomycetes raises the hypothesis that ChTOXA and PtrToxA may have originated from a common ancestor, which might be one of the ancient TOXA’ genes. The essential difference between TOXA and TOXA’ gene is that the latter lacks any introns. There is weaker similarity between ToxA and ToxA’ genes in the regions (~25 aa) between the last two motif sequences (LILTX and WXXQ) that begins right after the intron splicing point and harbors the RGD/SGN motif in *PtrToxA*/ChToxA (Fig. 6A). We note that the TOXA’ gene is duplicated in *C. purpurea* (Fig. 6B). We speculate that the common ancestor of *P. tritici-repentis* and *C. heterostrophus* might have had two copies of ToxA’, one of which gave rise to ChTOXA through a transposon-mediated insertion, which created the unusual intron. The association of ChTOXA and PtrToxA with blocks of repeats encoding RTPA1 (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’ genes 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 RPTA1 flanking the ToxA locus. These facts may support a reverse HGT scenario, i.e., S. nodorum might have acquired ToxA from P. tritici-repentis. However, one cannot exclude the possibility that the two fungi acquired ToxA from the same resource independently. Extensive genome-wide analyses are needed to determine the mechanisms underlying the evolutionary trajectory.

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

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

Acknowledgments

We thank Cayley Steen, Mikayla Miller for technical assistance, and the North Central Regional Plant Introduction Station (NCRPIS) for providing maize inbred lines. This study was supported by USDA-ARS CRIS project 54421-21000-037-00D.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.fgb.2015.05.013.

References


