ToxA Is Present in the U.S. *Bipolaris sorokiniana* Population and Is a Significant Virulence Factor on Wheat Harboring *Tsn1*

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Abstract

ToxA, a necrotrophic effector originally identified from the tan spot fungus *Pyrenophora tritici-repentis* in 1987, was subsequently identified from *Parastagonospora nodorum* in 2006. More recently, the *ToxA* gene was identified in the spot blotch fungus *Bipolaris sorokiniana* in Australia. Here we show that the *ToxA* gene is also present in the *B. sorokiniana* population in the winter wheat region of southcentral Texas. Leaves from ‘Duster’ wheat showing strong necrotic lesions were collected in Castroville, TX. Fifteen single-spore isolates were collected from separate lesions, and 13 of them harbored the *BsToxA* gene and secreted ToxA in culture based on sensitivity of BG261, the differential line containing the dominant ToxA sensitivity gene, *Tsn1*. Four isolates harboring *BsToxA* and one deficient in *BsToxA* were used to infiltrate two wheat lines harboring *Tsn1* as well as their corresponding *tsn1* mutant lines. Culture filtrates of the isolate lacking

BsToxA did not induce necrosis on any of the lines. Culture filtrates of the four *BsToxA*-containing isolates induced necrosis on the wild type (*Tsn1*) lines but not on the corresponding *tsn1* mutant lines. Sensitivity to these culture filtrates also mapped to the previously identified location for *Tsn1* in the winter wheat mapping population Arina × Forno. Inoculation of one of these ToxA-producing isolates on the same population showed that the *Tsn1* locus accounted for 24.4% of the disease variation. All 13 isolates harbored the same *BsToxA* nucleotide sequence, which was identical to one of the two haplotypes previously identified in Australia. Sensitivity to ToxA is prevalent in popular hard winter wheat cultivars in the central and southcentral winter wheat regions of the United States, showing the potential of a selective advantage for *B. sorokiniana* isolates that harbor the *ToxA* gene.

Helminthosporium leaf blight of wheat is a foliar disease complex made up of the necrotrophic fungal pathogens *Pyrenophora tritici-repentis* and *Bipolaris sorokiniana*, causal agents of tan spot and spot blotch on wheat, respectively. In a 2-year field study located in Rampur, Nepal, Duveiller et al. (2005) used six cultivars to show that grain yield losses ranged from 17 to 55% depending on the cultivar used, with complete crop loss possible in warm high-rainfall years, making Helminthosporium leaf blight the main limiting factor for wheat production in this and surrounding regions. *B. sorokiniana* typically thrives under warm humid conditions and is a major constraint to wheat production in warm humid climates such as South Asia, where *B. sorokiniana* is more damaging than other foliar pathogens such as *P. tritici-repentis* (Duveiller and Dubin 2000). *B. sorokiniana*, however, has been of minimal importance in countries such as the United States, Canada, and Australia, where *P. tritici-repentis* has been more prevalent (Duveiller and Dubin 2000).

The ToxA protein was first discovered by Tomás and Bockus (1987) using culture filtrates of *P. tritici-repentis* infiltrated on wheat lines ranging from resistant to highly susceptible. Culture filtrates induced necrosis in a differential manner that correlated strongly with disease susceptibility. Several groups worked toward the purification of this necrosis-inducing protein, which was ultimately named Ptr ToxA (Ciuffetti et al. 1998). Ptr ToxA is a 13.2-kDa proteinaceous necrotrophic effector, the first protein necrotrophic effector to be discovered (Tuori et al. 1995). Using the protein sequence, two groups eventually cloned the *ToxA* gene (Ballance et al. 1996; Ciuffetti et al. 1997) and showed that it was a single copy gene in the *P. tritici-repentis* genome. Friesen et al. (2006) later identified a nearly identical *ToxA* gene in *Parastagonospora nodorum* (causal agent of septoria nodorum blotch) and showed that *ToxA* had likely been horizontally

transferred from *P. nodorum* to *P. tritici-repentis*. More recently, McDonald et al. (2012) showed that a third fungal species, *Parastagonospora avenaria tritici*, contained the *ToxA* gene and suggested that it was likely obtained through interspecific hybridization.

Host sensitivity to Ptr ToxA was mapped to wheat chromosome arm 5BL with the sensitivity gene being named *Tsn1* (Faris et al. 1996). This gene was a significant factor in both tan spot and septoria nodorum blotch when the infecting pathogens harbored the *ToxA* gene (Friesen et al. 2006; Lamari and Bernier 1989). Faris et al. (2010) cloned *Tsn1* and found that it had similarities to classical resistance-like genes, including nucleotide binding, leucine-rich repeat, and protein kinase domains. This was significant because it demonstrated that a resistance-like gene conferred susceptibility rather than resistance to *P. tritici-repentis* and *P. nodorum* isolates expressing *ToxA* (Friesen et al. 2006).

McDonald et al. (2017) recently discovered a functional *ToxA* gene in several Australian isolates of *B. sorokiniana*. Two nucleotide haplotypes were identified differing by only a single nonsynonymous nucleotide substitution, with one of these haplotypes being identical to the most prevalent *P. tritici-repentis* haplotype (Ciuffetti et al. 1997). This work showed that transfer of virulence genes between species infecting the same host may be more common than previously thought.

In 2016, we collected leaves from winter wheat cultivar ‘Duster’ from winter wheat research plots in Castroville, TX, that were covered with necrotic lesions that contained almost exclusively *B. sorokiniana*. Here we show that *ToxA* was present in the *B. sorokiniana* population of the southcentral winter wheat region of Texas and that, similar to *P. tritici-repentis* and *P. nodorum*, *B. sorokiniana* isolates harboring *ToxA* are effectively using the ToxA-Tsn1 interaction to increase the level of disease on wheat. Given the high prevalence of *Tsn1* in popular hard winter wheat cultivars, this discovery has serious implications for wheat production in the United States, especially in the central and southcentral winter wheat regions of the United States.

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Materials and Methods

Pathogen identification. Leaves of the winter wheat cultivar Duster with distinct necrotic lesions were collected from cooperative research plots operated by Texas A&M University in Castroville,

TX, on 20 April 2016 to identify what pathogen was causing these unique spots. Approximately 10 dry leaves were surface sterilized as described in Liu et al. (2012). Surface-sterilized leaves were placed on water agar under a 12-h photoperiod at room temperature to induce sporulation. Conidia forming out of the necrotic lesions were collected under a stereoscope using a dissecting needle that was flame sterilized and cooled in water agar. Several single spores from each of 15 distinctly separate lesions were transferred to V8 potato dextrose agar (PDA) and allowed to grow. A single colony was randomly chosen from each of the 15 lesions for a second round of single-spore isolation to ensure purity. The fungus was identified based on conidia and conidiophore morphology.

Amplification of *ToxA* from Texas *B. sorokiniana* isolates. Fifteen *B. sorokiniana* isolates collected from leaves as described above were used to investigate the presence of *ToxA*. DNA of each isolate was extracted using the BioSprint 15 DNA Plant Kit (Qiagen). The *ToxA* sequence was polymerase chain reaction (PCR) amplified using *ToxA* primers 226 bp upstream and 161 bp downstream of the start and stop codons, respectively, as described in Friesen et al. (2006). The internal transcribed spacer (ITS) sequence was amplified as a positive control, using primers ITS 1 and ITS 4 (White et al. 1990). A second attempt at *ToxA* amplification was made for any isolate that did not yield *ToxA* amplicons in the first experiment.

Production and infiltrations of SnToxA and *B. sorokiniana* culture filtrates. The 15 *B. sorokiniana* isolates were used to prepare culture filtrates as previously described (Friesen and Faris 2012). Briefly, spores were produced by transferring dried mycelial disks to V8-PDA medium and grown in 24-h light until spores appeared in abundance, typically 5 to 7 days. Approximately 200,000 spores were harvested and inoculated into 60 ml of Fries medium (Liu et al. 2006) and grown with shaking at 27°C for 72 h. Cultures were transferred to a dark growth chamber at 21°C and incubated for an additional week. Culture filtrates were harvested by filtering each flask's contents through a Fisher Scientific Qualitative P5 filter followed by a Millipore Durapore 0.45-µm HV filter. Purified SnToxA was prepared and used as described in Friesen and Faris (2012). Undiluted filtrates as described above were infiltrated using a 1-ml needleless syringe into various wheat lines harboring *Tsn1* or *tsn1* to verify the presence of *BsToxA* in *B. sorokiniana* culture filtrates. Purified SnToxA was used as a positive control as well as to identify the presence of *Tsn1* in uncharacterized wheat lines.

Validation of the presence of *ToxA* in culture filtrates using *Tsn1* and *tsn1* mutant lines. Wheat lines harboring *Tsn1* including Bobwhite and Langdon along with their corresponding *tsn1* mutant lines (Faris et al. 2010) were also used to validate the presence of *BsToxA* in culture filtrates of the Texas *B. sorokiniana* isolates. Infiltrations were done as described above.

Production of spores for inoculation. *B. sorokiniana* spores were harvested after 5 days of growth on V8-PDA as described in Faris and Friesen (2005) and diluted to 3,000 spores/ml with one drop of Tween 20 per 50 ml of inoculum to reduce spore clumping. The spore suspension was inoculated until runoff, at which time plants were placed in chambers at 100% humidity for 24 h followed by 6 days

in a 21°C growth chamber under a 12-h photoperiod. Plants were evaluated on a 1 to 5 scale similar to the tan spot of wheat scale developed by Lamari and Bernier (1989).

Evaluation of plant materials for *ToxA* sensitivity and disease severity. A collection of winter (W) and spring (S) type wheat lines from diverse breeding backgrounds was used to identify the presence of *Tsn1* (*ToxA* sensitivity). These lines included Iba (W), Jamestown (W), Duster (W), BR34 (S), Ruby Lee (W), ND495 (S), Jerry (W), Salamouni (S), and W7984 (synthetic hexaploid) as well as mapping population parental lines Arina (W), and Forno (W) (Table 1). Additionally, this collection of lines was used in inoculation experiments to evaluate their reaction to isolate TexD16Bs1, which was positive for the *ToxA* gene. The *Tsn1* differential line BG261 (Friesen et al. 2006) was used as a *ToxA*-sensitive control.

Twenty-two and 20 of the most popular hard red winter wheat cultivars planted in Texas and Oklahoma, respectively, were chosen based on the Texas Wheat Varieties 2017 and the 2017 Oklahoma Wheat Variety Reports accessed at <https://www.nass.usda.gov>. Lines were evaluated for *ToxA* sensitivity and therefore the prevalence of *Tsn1*. Lines were infiltrated with purified SnToxA at the two- to three-leaf stage as described above.

Mapping sensitivity to culture filtrates and susceptibility to spore inoculations of *B. sorokiniana*. A 120-line F₅ recombinant inbred mapping population developed from a cross of the Swiss winter wheat cultivars Arina and Forno (Paillard et al. 2003) was provided by Beat Keller, University of Zurich, Switzerland, and used to map sensitivity to *B. sorokiniana* culture filtrates and susceptibility to spore inoculations. Culture filtrates of TexD16Bs1 were infiltrated across the Arina × Forno mapping population, which segregates for *ToxA* sensitivity versus insensitivity (*Tsn1/tsn1*) (Abeysekara et al. 2009). Additionally, purified SnToxA was infiltrated side by side as a control. Culture filtrate and SnToxA sensitivity were mapped in the Arina × Forno population as described by Abeysekara et al. (2009).

The Arina × Forno population and parental lines were also grown and subjected to spore inoculation as described by Abeysekara et al. (2009). The *BsToxA*-producing isolate TexD16Bs1 was used to inoculate the Arina × Forno population as described above. A total of three replications were completed for all inoculations, and two replications were completed for all infiltrations. The mean lesion types from the three replications of spore inoculations for the recombinant inbred lines were used to identify significant marker-trait associations using multiple interval mapping in the quantitative trait locus (QTL) analysis program QGene version 4.3 (Joehanes and Nelson 2008). The marker dataset for the Arina × Forno population consisted of 460 markers and was the same as that used by Abeysekara et al. (2009). The critical logarithm of the odds threshold of 3.8 for an experiment-wise significance at the 0.01 level of probability was determined by conducting 1,000 permutations.

Results

Identification of *B. sorokiniana* from wheat lesions. Duster wheat grown in Castroville, TX, contained an abundance of 3 to

Table 1. Diverse set of wheat lines evaluated for *ToxA* sensitivity using purified SnToxA; these lines were also evaluated for susceptibility to a Texas *Bipolaris sorokiniana* isolate harboring the *ToxA* gene (see Figure 2)

Wheat line	Wheat type	Origin	ToxA sensitive
Iba	Hard red winter	Oklahoma State University	+
Jamestown	Soft red winter	Virginia Tech	–
Duster	Hard red winter	Oklahoma State University	+
BR34	Spring	Brazil	–
Ruby Lee	Hard red winter	Oklahoma State University	+
ND495	Hard red spring	North Dakota State University	+
Jerry	Hard red winter	North Dakota State University	+
Salamouni	Soft spring	Landrace	–
W7984	Synthetic hexaploid	CIMMYT	–
Arina	Winter	Swiss	–
Forno	Winter	Swiss	+

5 mm necrotic lesions (Fig. 1A). Spores emerging from surface-sterilized leaves were almost exclusively *B. sorokiniana* and were clearly identified as such based on their typical abundant sporulation (Fig. 1B) and the heavily brown-pigmented oblong, septate conidia (Fig. 1C). Conidia isolated from lesions were reinoculated to show that Duster is highly susceptible to these isolates (Fig. 2). Fifteen *B. sorokiniana* isolates were single-spored to be used in downstream experiments and were named based on their collection location Texas (Tex), cultivar Duster (D), year 2016 (16), and isolates being identified as *B. sorokiniana* (Bs) (i.e., TexD16Bs1 to TexD16Bs15).

Identification of *BsToxA* and production of *BsToxA* in Texas *B. sorokiniana* isolates. Based on the necrotic lesion phenotype found on Duster, which harbors *Tsn1*, and the recent discovery of *ToxA* in *B. sorokiniana* in Australia (McDonald et al. 2017), we used PCR to attempt to amplify *ToxA* from the 15 *B. sorokiniana* isolates. *ToxA* was amplified from 13 of the 15 isolates (Fig. 3). Culture filtrates of the 13 isolates harboring *ToxA* identified by PCR induced

Fig. 1. Isolation of *Bipolaris sorokiniana* from leaf samples of Duster wheat collected in Castroville, TX, in 2016. **A**, Field leaves of the hard red winter wheat line Duster heavily affected by necrotic lesions induced by *B. sorokiniana*. **B**, A lesion after being surface sterilized and incubated on water agar for 5 days showing the production of darkly pigmented spores typical of the spot blotch fungus *B. sorokiniana*. **C**, Close-up view of the *B. sorokiniana* conidiophores and conidia protruding from the infected leaf surface.

necrosis when infiltrated on both Duster wheat and the *Tsn1* differential line BG261; however, the two isolates deficient in *ToxA* showed no necrosis on either line.

***B. sorokiniana* inoculation on a selection of wheat lines.** Several wheat lines from different classes and breeding programs were evaluated for *ToxA* sensitivity (*Tsn1*) and susceptibility to *B. sorokiniana* (Fig. 2). Five of these lines had *Tsn1* and four did not. In this limited set, lines harboring *Tsn1* were visibly more susceptible than lines lacking *Tsn1*. This result led us to further evaluate the Arina × Forno

Fig. 2. Seedling disease reactions of wheat lines with and without *Tsn1* infiltrated or inoculated with culture filtrates or spores of isolate TexD16Bs1, respectively. Top panel, spore inoculations on various winter and spring wheat lines using *Bipolaris sorokiniana* isolate TexD16Bs1. Middle panel, winter wheat lines Arina and Forno inoculated with isolate TexD16Bs1. Bottom panel, infiltration of Bobwhite and Langdon, both of which harbor *Tsn1*, along with four *tsn1* mutants of Bobwhite and two *tsn1* mutants of Langdon using culture filtrates of isolate TexD16Bs1.

Fig. 3. Polymerase chain reaction amplification of *ToxA* from 15 *Bipolaris sorokiniana* isolates (TexD16Bs1 to TexD16Bs15) collected from necrotic lesions on Duster wheat in Castroville, TX. Controls include *Pyrenophora teres* f. *teres* (Ptt0-1) lacking *ToxA*, and *P. tritici-repentis* (Ptr-1C-BFP) and *Parastagonospora nodorum* (LDN03Sn4), both of which harbor *ToxA*.

mapping population to see if *Tsn1* was indeed contributing to spot blotch susceptibility in wheat.

Infiltration of *B. sorokiniana* culture filtrates on *Tsn1* and *tsn1* mutant wheat lines. Five isolates were randomly chosen for additional culture filtrate production and analysis. Culture filtrates of five of the isolates, four of which harbored the *ToxA* gene and one of which did not, were infiltrated on the *Tsn1* lines Bobwhite and Langdon as well as the previously characterized Bobwhite and Langdon *tsn1* ethyl methanesulfonate (ems)-induced mutant lines (Faris et al.

2010). Culture filtrates of TexD16Bs7, which was deficient in *ToxA*, did not induce necrosis on any of the lines; however, culture filtrates of the isolates harboring the *BsToxA* gene (TexD16Bs1, TexD16Bs4, TexD16Bs6, and TexD16Bs9) induced necrosis on Bobwhite and Langdon but not on the *tsn1* mutant lines (LDNems114, LDNems403, BWems123, BWems258, BWems687, and BWems952) (Fig. 2), showing that *BsToxA* was interacting with *Tsn1* alone. This serves as additional evidence that the *BsToxA* protein was interacting with *Tsn1*, resulting in necrosis.

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TexBsToxA      atgcggttctatcctcgacttcttttcagcgccgctgctgtgcttgcgtccccaacgcct
AusBsToxA1     -----tgctgtgcttgcgtccccaacgcct
AusBsToxA2     -----agcgccgctgctgtgcttgcgtccccaacgcct
Pt-1C-BFP_ToxA atgcggttctatcctcgacttcttttcagcgccgctgctgtgcttgcgtccccaacgcct
Sn4_ToxA       atgcggttctatcctcgacttcttttcagcgccgctgctgtgcttgcgtccccaacgcct
                *****

TexBsToxA      gaagccgatcccggttacgaaatcgtaaacttttcgaagccgccaactcttctgaactc
AusBsToxA1     gaagccgatcccggttacgaaatcgtaaacttttcgaagccgccaactcttctgaactc
AusBsToxA2     gaagccgatcccggttacgaaatcgtaaacttttcgaagccgccaactcttctgaactc
Pt-1C-BFP_ToxA gaagccgatcccggttacgaaatcgtaaacttttcgaagccgccaactcttctgaactc
Sn4_ToxA       gaagccgatcccggttacgaaatcgtaaacttttcgaagccgccaactcttctgaactc
                *****

TexBsToxA      gacgcgcgcggactctctctcgactggaccctcaaacgcgcggactcctacaggagcgg
AusBsToxA1     gacgcgcgcggactctctctcgactggaccctcaaacgcgcggactcctacaggagcgg
AusBsToxA2     gacgcgcgcggactctctctcgactggaccctcaaacgcgcggactcctacaggagcgg
Pt-1C-BFP_ToxA gacgcgcgcggactctctctcgactggaccctcaaacgcgcggactcctacaggagcgg
Sn4_ToxA       gacgcgcgcggactctctctcgactggaccctcaaacgcgcggactcctacaggagcgg
                *****

TexBsToxA      cagggaaagctgcatgtcaatcacaatcaaccctagtcgctccgtctgtcaacaacatcggc
AusBsToxA1     cagggaaagctgcatgtcaatcacaatcaaccctagtcgctccgtctgtcaacaacatcggc
AusBsToxA2     cagggaaagctgcatgtcaatcacaatcaaccctagtcgctccgtctgtcaacaacatcggc
Pt-1C-BFP_ToxA cagggaaagctgcatgtcaatcacaatcaaccctagtcgctccgtctgtcaacaacatcggc
Sn4_ToxA       cagggaaagctgcatgtcaatcacaatcaaccctagtcgctccgtctgtcaacaacatcggc
                *****

TexBsToxA      caagtcgacattgacagtgttatactcggacgacctggtgctataggctcgtgggaactg
AusBsToxA1     caagtcgacattgacagtgttatactcggacgacctggtgctataggctcgtgggaactg
AusBsToxA2     caagtcgacattgacagtgttatactcggacgacctggtgctataggctcgtgggaactg
Pt-1C-BFP_ToxA caagtcgacattgacagtgttatactcggacgacctggtgctataggctcgtgggaactg
Sn4_ToxA       caagtcgacattgacagtgttatactcggacgacctggtgctataggctcgtgggaactg
                *****

TexBsToxA      aacaactttattaccattggattgaaccgcgtaaacgcgcgatacagtgcgagtcacatc
AusBsToxA1     aacaactttattaccattggattgaaccgcgtaaacgcgcgatacagtgcgagtcacatc
AusBsToxA2     aacaactttattaccattggattgaaccgcgtaaacgcgcgatacagtgcgagtcacatc
Pt-1C-BFP_ToxA aacaactttattaccattggattgaaccgcgtaaacgcgcgatacagtgcgagtcacatc
Sn4_ToxA       aacaactttattaccattggattgaaccgcgtaaacgcgcgatacagtgcgagtcacatc
                *****

TexBsToxA      cgaacacccggcaggactaatcgccctcattattactcaatgggaataagtctccccacca
AusBsToxA1     cgaacacccggcaggactaatcgccctcattattactcaatgggaataagtctccccacca
AusBsToxA2     cgaacacccggcaggactaatcgccctcattattactcaatgggaataagtctccccacca
Pt-1C-BFP_ToxA cgaacacccggcaggactaatcgccctcattattactcaatgggaataagtctccccacca
Sn4_ToxA       cgaacacccggcaggactaatcgccctcattattactcaatgggaataagtctccccacca
                *****

TexBsToxA      ttttattagattacttattactaacttatactactaataactgtcactcggggggacgttt
AusBsToxA1     ttttattagattacttattactaacttatactactaataactgtcactcggggggacgttt
AusBsToxA2     ttttattagattacttattactaacttatactactaataactgtcactcggggggacgttt
Pt-1C-BFP_ToxA ttttattagattacttattactaacttatactactaataactgtcactcggggggacgttt
Sn4_ToxA       ttttattagattacttattactaacttatactactaataactgtcactcggggggacgttt
                *****

TexBsToxA      atgagctttttggtgattacgctttaattcaaggtagaggaagcttttgcccttaataatac
AusBsToxA1     atgagctttttggtgattacgctttaattcaaggtagaggaagcttttgcccttaataatac
AusBsToxA2     atgagctttttggtgattacgctttaattcaaggtagaggaagcttttgcccttaataatac
Pt-1C-BFP_ToxA atgagctttttggtgattacgctttaattcaaggtagaggaagcttttgcccttaataatac
Sn4_ToxA       atgagctttttggtgattacgctttaattcaaggtagaggaagcttttgcccttaataatac
                *****

TexBsToxA      gatctgatacaggctcgtgaaaattggagaatgcagctagaaaattag
AusBsToxA1     gatctg-----
AusBsToxA2     gatct-----
Pt-1C-BFP_ToxA gatctgatacaggctcgtgaaaattggagaatgcagctagaaaattag
Sn4_ToxA       gatctgattcaggtcgtgaaaattggagaatgcagctagaaaattag
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Fig. 4. Nucleotide sequences of *BsToxA* from Texas (TexBsToxA) and Australia (AusBsToxA1 and AusBsToxA2) aligned to *Parastagonospora nodorum* Sn4 (Sn4_ToxA) and *Pyrenophora tritici-repentis* (Pt-1C-BFP_ToxA) ToxA using Clustal Omega (Sievers et al. 2011). Variation between sequences is highlighted.

Fig. 5. Spot blotch disease association with *Tsn1* (ToxA sensitivity) on chromosome 5B in the Arina × Forno recombinant inbred population using multiple interval mapping analysis. The logarithm of the odds (LOD) significance threshold of 3.8 is shown by a dashed line.

ToxA gene sequencing in the Texas *B. sorokiniana* isolates. *ToxA* was amplified and sequenced from the 13 isolates harboring the *BsToxA* gene. A single haplotype was identified across all 13 Texas isolates (Fig. 4), with this sequence differing by a single nucleotide from the most common *P. tritici-repentis* sequence (Ciuffetti et al. 1997) and being identical to one of the two *BsToxA* haplotypes identified in Australia (McDonald et al. 2017).

Mapping of sensitivity to *BsToxA* and susceptibility to *B. sorokiniana* using the Arina × Forno recombinant inbred line population. Purified SnToxA and culture filtrates of TexD16Bs1 were infiltrated side by side across the Arina × Forno mapping population. Sensitivity to purified SnToxA cosegregated perfectly with sensitivity to culture filtrates of TexD16Bs1 (Fig. 5). Both reactions mapped to wheat chromosome arm 5BL precisely where *Tsn1* had previously been mapped (Abeysekara et al. 2009).

Multiple interval mapping of the mean lesion types from spore inoculations indicated that only one genomic region in the Arina × Forno population was significantly associated with disease resistance versus susceptibility, and that region was defined by the *Tsn1* locus on 5BL (Fig. 5). *Tsn1* defined the peak of the regression curve, and it had a logarithm of the odds of 8.0 and explained 24.4% of the disease variation.

ToxA sensitivity in popular Texas and Oklahoma winter wheats. Twenty-two of the 35 most popular hard red winter wheat lines in 2017 reported for Texas and 20 of the 35 most popular hard red winter wheat lines in 2017 reported for Oklahoma were infiltrated with purified SnToxA to identify the prevalence of *Tsn1* in hard red winter wheat (Table 2). Due to overlap between the two sets, 26 lines were evaluated, with 20 of the 26 being sensitive to ToxA (Table 1). Additionally, the top four lines for acreage in Texas (TAM111, TAM112, TAM113, and Gallagher) and the top five lines for acreage in Oklahoma were all sensitive to ToxA (Table 1), showing the prevalence of *Tsn1* in the hard red winter wheat cultivars of the south central United States.

Discussion

In this paper, we report the identification of a functional *ToxA* gene in a Texas population of *B. sorokiniana*. The *ToxA* gene had a high

Table 2. Reaction to ToxA infiltration of popular winter wheat cultivars planted in 2017 in Texas and Oklahoma

TX rank order ^a	OK rank order ^a	Cultivar name	Breeding program origin	ToxA reaction ^b
1	17	TAM111	Texas A&M	+
2	15	TAM112	Texas A&M	+
3	33	TAM113	Texas A&M	+
4	1	Gallagher	Oklahoma State University	+
5	14	WB Cedar	WestBred	–
6		Greer	Syngenta-Agripro	–
9		TAM401	Texas A&M	+
10		TAM105	Texas A&M	+
12	3	Endurance	Oklahoma State University	+
13	34	TAM204	Texas A&M	–
15	18	Jackpot	Syngenta-Agripro	+
18	2	Duster	Oklahoma State University	+
19	7	Jagger	Kansas State University	+
20	19	WB Grainfield	WestBred	+
21		TAM110	Texas A&M	+
22		TAM304	Texas A&M	–
23	9	Winterhawk	Kansas State University	+
26	11	WB 4458	WestBred	–
27	4	Iba	Oklahoma State University	+
29	8	Fuller	Kansas State University	+
31		Hatcher	Colorado State University	+
33	23	Garrison	Oklahoma State University	+
	5	Ruby Lee	Oklahoma State University	+
	13	Everest	Kansas State University	–
	21	Scout 66	University of Nebraska	+
	22	Overley	Kansas State University	+

^a Indicates the rank order of popularity based on percentage of reported acres planted in Texas (https://www.nass.usda.gov/Statistics_by_State/Texas/index.php, accessed 10/24/2017) or Oklahoma (https://www.nass.usda.gov/Statistics_by_State/Oklahoma/Publications/Oklahoma_Wheat_Varieties/index.php, accessed 10/24/2017).

^b Reaction to infiltration of purified SnToxA. + = sensitive, and – = insensitive.

degree of similarity to the *ToxA* gene identified in *P. tritici-repentis* (Ballance et al. 1996; Ciuffetti et al. 1997), *P. nodorum* (Friesen et al. 2006), and *P. avenaria tritici* (McDonald et al. 2012). Our results corroborate a recent report that the *ToxA* gene exists in Australian populations of *B. sorokiniana* (McDonald et al. 2017). The high degree of similarity of *ToxA* sequences among the four species can best be explained by horizontal gene transfer (Friesen et al. 2006) or interspecific hybridization (McDonald et al. 2017), and it is remarkable that this phenomenon has occurred at least three times in the Pleosporales. The exact match of the *ToxA* sequence of our isolates and the sequence of one haplotype described by McDonald et al. (2017) suggests that relatively recent global migration of these strains has occurred.

Spot blotch on wheat has been a major problem in warm, humid regions of the world; however, little is known about how the pathogen is infecting the host. Kumar et al. (2009) used a highly aggressive *B. sorokiniana* isolate collected in India to evaluate the genetics of spot blotch field resistance in a recombinant inbred line population developed from a cross of the resistant Chinese line Yangmai 6 and the Indian cultivar Sonalika. Sonalika was a popular cultivar in the 1970s and 1980s but was highly susceptible to spot blotch. A major-effect QTL on chromosome 5B, the same chromosome that harbors *Tsn1*, was identified. This QTL was isolated in a second population using a progeny recombinant inbred line backcrossed to the susceptible Sonalika (Kumar et al. 2015). Fine mapping in this population showed that this QTL mapped to the long arm of chromosome 5BL and was named *Sb2*. Interestingly, markers in the *Sb2* region placed it in the same region as the *ToxA* sensitivity gene *Tsn1* (Faris et al. 2010), indicating the possibility that the *Sb2* susceptibility allele and *Tsn1* are the same gene. We evaluated three accessions of Sonalika from the national small grains collection (PI 478282, PI 337371, and Cltr 15392) for *ToxA* sensitivity and found that all three accessions were sensitive to purified *ToxA* (data not shown), showing that Sonalika does indeed carry *Tsn1*. This is significant because it shows that *B. sorokiniana* isolates in the regions where spot blotch has major economic impact may already harbor *ToxA*. If this is the case, it would be advisable to eliminate *Tsn1* from all wheat lines planted in areas where spot blotch is a major problem.

Historically, this disease has not been of major concern in the United States or Canada. However, numerous distinct or coalescing necrotic lesions were identified on the popular hard red winter wheat cultivar Duster at a wheat breeding nursery in Castroville, TX. Leaves were collected, surface-sterilized, and incubated to identify the causal pathogen. Lesions contained almost exclusively *B. sorokiniana*, and the majority of the single-spore isolates collected harbored the *BsToxA* gene. The presence of the *BsToxA* gene in *B. sorokiniana* in the southern hard red winter wheat region is significant, especially because the majority of the most popular hard red winter wheat cultivars planted in Texas and Oklahoma harbor *Tsn1* and are therefore potentially more vulnerable to all three of the wheat pathogens that employ *ToxA* as a virulence factor.

Several studies have shown *Tsn1* to be a significant susceptibility factor for tan spot (Faris et al. 2013, review) and septoria nodorum blotch (Friesen et al. 2006; Liu et al. 2006; Viridi et al. 2016), but it has also been shown recently that the importance of this susceptibility factor is background dependent (Chu et al. 2008; Faris and Friesen 2005; Faris et al. 2012; Viridi et al. 2016). Here we show that *Tsn1* is a significant susceptibility factor for spot blotch at the seedling stage in the winter wheat population Arina × Forno. Although the significance of the *ToxA*-*Tsn1* interaction shown here in the Arina × Forno population was performed at the seedling stage, it is likely that this interaction is also significant at the adult plant stage because the distinct necrotic lesions from which the *B. sorokiniana* containing *BsToxA* was isolated were observed at the adult plant stage. Additional work will need to be done to investigate whether *BsToxA* is an effective virulence factor in other backgrounds including spring wheat and durum wheat and at other stages of growth.

It is thought that *P. tritici-repentis*, *P. nodorum*, and *B. sorokiniana* are differentially effective under different environmental conditions. Because *Tsn1* has never been shown to contribute positively to

wheat, it would be advisable to select against *Tsn1* to avoid the impact of this virulence factor present in three major wheat pathogens.

It is also important that these lesions were identified on Duster wheat, which harbors *Tsn1* (*ToxA* sensitive) and is a popular cultivar in both Oklahoma and Texas. Here we also evaluated 26 of the most popular wheat lines planted in Texas and Oklahoma and found that 20 of these lines harbor *Tsn1*. Additionally, 12 of the top 25 Kansas cultivars planted in 2017 are in this list of 26 lines (data not shown), with nine of these 12 lines being sensitive to *ToxA*. This is significant because the presence of *Tsn1* applies selection pressure on at least three pathogens to maintain the *ToxA* gene in the population present in the southcentral hard red winter wheat region of the United States to exploit the *Tsn1* susceptibility target. Maintaining *Tsn1* in popular wheat cultivars not only provides a selective advantage to pathogens that currently harbor *ToxA* but may also provide selection pressure for new or more fit pathogens that acquire *ToxA* through horizontal transfer.

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