New Insights into the Roles of Host Gene-Necrotrophic Effector Interactions in Governing Susceptibility of Durum Wheat to Tan Spot and Septoria nodorum Blotch

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ABSTRACT Tan spot and Septoria nodorum blotch (SNB) are important diseases of wheat caused by the necrotrophic fungi Pyrenophora tritici-repentis and Parastagonospora nodorum, respectively. The P. tritici-repentis necrotrophic effector (NE) Ptr ToxB causes tan spot when recognized by the Tsc2 gene. The NE ToxA is produced by both pathogens and has been associated with the development of both tan spot and SNB when recognized by the wheat Tsn1 gene. Most work to study these interactions has been conducted in common wheat, but little has been done in durum wheat. Here, quantitative trait loci (QTL) analysis of a segregating biparental population indicated that the Tsc2-Ptr ToxB interaction plays a prominent role in the development of tan spot in durum. However, analysis of two biparental populations indicated that the Tsn1-ToxA interaction was not associated with the development of tan spot, but was strongly associated with the development of SNB. Pa. nodorum expressed ToxA at high levels in infected Tsn1 plants, whereas ToxA expression in P. tritici-repentis was barely detectable, suggesting that the differences in disease levels associated with the Tsn1-ToxA interaction were due to differences in pathogen expression of ToxA. These and previous results together indicate that: (1) the effects of Tsn1-ToxA on tan spot in common wheat can range from nonsignificant to highly significant depending on the host genetic background; (2) Tsn1-ToxA is not a significant factor for tan spot development in durum wheat; and (3) Tsn1-ToxA plays a major role in SNB development in both common and durum wheat. Durum and common wheat breeders alike should strive to remove both Tsc2 and Tsn1 from their materials to achieve disease resistance.

Durum wheat [Triticum turgidum ssp. durum (Desf.) Husnot.], also known as pasta or macaroni wheat, is an allotetraploid (2n = 4x = 28, AABB genomes) of worldwide economic importance because it is used to make pasta and other semolina-based products. Durum wheat production is affected by numerous diseases. Among some of the most severe are the foliar diseases tan spot and SNB caused by the necrotrophic fungal pathogens P. tritici-repentis and Pa. nodorum, respectively. Both pathogens are members of the Pleosporales order of fungi and are known to produce NEs (Oliver et al. 2012; Faris et al. 2013 for reviews). When a specific NE is recognized by the corresponding host gene, a host “defense response” ensues, which leads to programmed cell death allowing these necrotrophs to penetrate, feed, and sporulate. The lack of NE recognition by the host leads to resistance. Therefore, these host–pathogen interactions operate in an inverse gene-for-gene manner (Wolpert et al. 2002; Friesen and Faris 2010; Oliver et al. 2012; Faris et al. 2013), and the dominant alleles of the host NE recognition genes are considered susceptibility genes.
The Tsn1-Ptr ToxA, Tsc2-Ptr ToxB, and Tsc1-Ptr ToxC interactions have all been shown to play significant roles in the development of tan spot in common (hexaploid) wheat (*T. aestivum* L. ssp. *aestivum*, 2n = 6x = 42, AABBDG genomes) (Faris et al. 2013; Kariyawasam et al. 2016). These experiments were conducted by inoculating leaves of wheat lines from segregating populations with cultures containing crude, partially purified, or purified cultures of the NEs and inoculating the same wheat lines with conidia produced by the fungus to evaluate the development of tan spot. Many studies showed statistically significant relationships between sensitivity to the NEs and susceptibility to *P. tritici-repentis*. For example, early work with Ptr ToxA showed strong correlations between sensitivity to culture filtrates containing Ptr ToxA and susceptibility to Ptr ToxA-producing isolates (Tomas and Bockus 1987; Lamari and Bernier 1989). Similarly, studies using Ptr ToxB and Ptr ToxC showed that these NEs were also strongly associated with tan spot caused by isolates that produced them (Effertz et al. 2002; Friesen and Faris 2004; Abeysekara et al. 2010). Therefore, the NEs were considered virulence factors, and it was assumed that sensitivity to an NE would lead to disease susceptibility (Anderson et al. 1999). This led to the notion that lines could be screened with NE-containing cultures to more or less predict their reaction to tan spot. However, more recent studies, particularly involving the Tsn1-Ptr ToxA interaction, indicated that NE sensitivity did not always define tan spot susceptibility, and the involvement of the Tsn1-Ptr ToxA interaction in the development of disease was dependent on the genetic background of the host (Friesen et al. 2003; Faris and Friesen 2005; Chu et al. 2008a; Faris et al. 2012; Kariyawasam et al. 2016).

The vast majority of studies involving tan spot have been conducted in common wheat and relatively few have been conducted in durum wheat. P. K. Singh et al. (2008) showed that two linked recessive genes on chromosome arm 3BL conferred resistance to race 3 (produces Ptr ToxC) and race 5 (produces Ptr ToxB) isolates in tetraploid wheat, indicating that the Tsc1-Ptr ToxC and Tsc2-Ptr ToxB interactions were not involved. In another tetraploid wheat mapping study, Chu et al. (2010a) evaluated a durum doubled haploid population that segregated for Ptr ToxA sensitivity with race 1 (produces Ptr ToxA and Ptr ToxC) and race 2 (produces Ptr ToxA) isolates, and found that neither the Tsn1-Ptr ToxA nor the Tsc1-Ptr ToxC interaction was relevant in the development of disease. Therefore, although the three host gene-NE interactions in the wheat-*P. tritici-repentis* system have been shown to play variable roles in disease development in hexaploid wheat, none of them have been shown to be associated with the development of tan spot in durum wheat thus far.

To date, nine interactions involving specific wheat genes and cognate NEs have been reported in the wheat-*Pa. nodorum* system, and all have been shown to play significant roles in the development of SNB (Liu et al. 2004a,b, 2006; Friesen et al. 2006, 2007, 2008, 2009, 2012; Abeysekara et al. 2009, 2012; Chu et al. 2010b; Zhang et al. 2011; Gao et al. 2015; Shi et al. 2015). One of these interactions is the

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**Figure 1** Leaves of Langdon and Altar 84 infiltrated with ToxA and Ptr ToxB. Langdon is sensitive to necrosis caused by ToxA and Altar 84 is insensitive, whereas Langdon is insensitive to the chlorosis caused by Ptr ToxB and Altar 84 is sensitive.

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*Tsn1-SnToxA* (hereafter, both Ptr ToxA and SnToxA will be referred to as ToxA) interaction. Friesen et al. (2006) showed that the ToxA gene was horizontally transferred from *Pa. nodorum* to *P. tritici-repentis* sometime prior to 1940. This event likely played a role in the development of a new pathogen becoming an economically significant disease, and henceforth *Tsn1* operated as a susceptibility gene for both tan spot and SNB. Liu et al. (2006) showed that the ToxA proteins derived from both *P. tritici-repentis* and *Pa. nodorum* functioned in the same way to elicit cell death when the proteins were infiltrated into wheat leaves. Numerous studies have shown that the *Tsn1-ToxA* interaction plays a major role in conferring susceptibility to *Pa. nodorum* in hexaploid wheat (Friesen et al. 2006, 2007, 2008, 2009, 2012; Chu et al. 2010b), and at least two studies have demonstrated the prominence of *Tsn1* in conferring susceptibility in tetraploid wheat (Faris and Friesen 2009; Friesen et al. 2012). Therefore, unlike for tan spot, *Tsn1* has been shown to be an important SNB susceptibility gene in both hexaploid and tetraploid wheat.

The primary objectives of this research were to determine the roles of the *Tsn1-ToxA* and *Tsc2-Ptr ToxB* interactions in a biparental population derived from two durum varieties. We also evaluated the role of the *Tsn1-ToxA* interaction in governing tan spot susceptibility in a second tetraploid wheat population, which was the same population used by Faris and Friesen (2009) to show that the interaction explained 95% of the variation in the development of SNB. Finally, we evaluated ToxA transcription in plants inoculated with *Pa. nodorum* or *P. tritici-repentis* to determine whether ToxA expression levels were correlated with the levels of disease caused by these two pathogens.

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**MATERIALS AND METHODS**

**Plant materials**

A segregating population of 127 recombinant inbred lines (RILs) was developed from a cross between the CYMMIT-bred durum variety “Altar 84” and the North Dakota durum variety “Langdon” (LDN). The RILs were developed by advancing the plants to the F$_2$ generation by single seed descent (SSD). Preliminary experiments indicated that Altar 84 was sensitive to Ptr ToxB and insensitive to ToxA and Langdon was sensitive to ToxA and insensitive to Ptr ToxB, respectively (Figure 1). This population is hereafter referred to as the AL population.

The second population was derived from a cross between LDN and LDN-DIC 5B, which is a genetic stock where a pair of 5B chromosomes derived from the *T. turgidum* ssp. *dicoccoides* accession Israel A was substituted for the native 5B chromosomes in the LDN background (Joppa 1993). This population, hereafter referred to as the LD5B population, consisted of 85 recombinant inbred chromosome lines (RICLs)

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S. K. Virdi et al.
Table 1 Summary of markers mapped in each chromosomegenome in the Altar 84 × Langdon population

| Chromosome | Markers | Phenotype | Total | Length (cM) | Marker Density (cM/Marker) | Markers with Distorted Ratios |
|------------|---------|-----------|-------|-------------|----------------------------|------------------------------|
| 1A         | SSR 4, CAPS 65, SNP - | - | 69 | 144.49 | 2.1 | 0 |
| 1B         | SSR 20, CAPS 53, SNP - | - | 74 | 159.84 | 2.2 | 12 |
| 2A         | SSR 4, CAPS 57, SNP - | - | 61 | 178.68 | 2.9 | 2 |
| 2B         | SSR 9, CAPS 36, SNP 1 | 1 | 46 | 196.73 | 4.2 | 22 |
| 3A         | SSR 6, CAPS 47, SNP - | - | 53 | 159.97 | 3.0 | 0 |
| 3B         | SSR 3, CAPS 31, SNP - | - | 34 | 167.19 | 4.9 | 9 |
| 4A         | SSR 5, CAPS 50, SNP - | - | 55 | 128.57 | 2.3 | 0 |
| 4B         | SSR 2, CAPS 15, SNP - | - | 17 | 107.95 | 6.3 | 8 |
| 5A         | SSR 6, CAPS 52, SNP - | - | 58 | 130.76 | 2.3 | 26 |
| 5B         | SSR 23, CAPS 80, SNP 1 | 1 | 104 | 211.72 | 2.0 | 71 |
| 6A         | SSR 3, CAPS 43, SNP - | - | 46 | 123.34 | 2.7 | 2 |
| 6B         | SSR 7, CAPS 88, SNP - | - | 95 | 111.10 | 1.2 | 52 |
| 7A         | SSR 6, CAPS 66, SNP - | - | 72 | 262.41 | 3.6 | 0 |
| 7B         | SSR 2, CAPS 58, SNP - | - | 60 | 124.40 | 2.1 | 4 |
| A genome   | SSR 34, CAPS 380, SNP - | - | 414 | 1128.22 | 2.7 | 30 |
| B genome   | SSR 66, CAPS 361, SNP 2 | 2 | 430 | 1078.93 | 2.5 | 178 |
| Total      | SSR 100, CAPS 741, SNP 2 | 2 | 844 | 2207.15 | 2.6 | 208 |

SSR, simple sequence repeat; CAPS, cleaved amplified polymorphic sequence; SNP, single nucleotide polymorphism.

and was used by Faris and Friesen (2009) to evaluate the role of the Tsn1-ToxA interaction in conferring SNB susceptibility. Here, it was used to evaluate the role of the Tsn1-ToxA interaction in conferring susceptibility to tan spot caused by the race 2 (ToxA producing) isolate 86–124.

All plants were grown in cones containing SB100 (Sun Gro Sunshine; Sun Gro Horticulture, Vancouver, BC) soil mix with 10–20 granules of Osmocote fertilizer (Scotts Company LLC, Marysville, OH) added to each cone. For disease evaluations and NE infiltrations, all plants were grown in the greenhouse at an average temperature of 21°C with a 16 hr photoperiod.

**NE production and infiltration assays**

ToxA and Ptr ToxB were expressed in the yeast strain *Pichia pastoris* X33 and cultured as described for the NE SnTox3 in Liu et al. (2009). Harvested cultures were used to directly infiltrate the second leaf of wheat plants at the two-leaf stage. Infiltrations were conducted using a 1 ml needleless syringe on the secondary leaf until 2–3 cm of leaf was infiltrated. The boundaries of the infiltrated region were marked using a nontoxic permanent marker. The infiltrated plants were then placed in a growth chamber at 21°C for 6 d of incubation at 21°C/5°C night. Plants were evaluated for 4 d after infiltration and scored as sensitive or insensitive based on the presence or absence of chlorosis for Ptr ToxB or necrosis for ToxA. The entire AL population and parents were infiltrated twice with ToxA and three times with Ptr ToxB. The infiltration scores, i.e., insensitive vs. sensitive, were converted to genotypic scores for placement of the Tsn1 and Tsc2 loci on the genetic linkage maps relative to the molecular markers. For the LD5B population, the reaction of each line to ToxA and the placement of the Tsn1 locus on the genetic linkage map were previously determined (Faris and Friesen 2009).

**Disease evaluations**

For *P. triticirepentis* disease evaluation, the AL population and parents were screened with race 2 isolates 86–124 and L13–35, which produce ToxA, and the race 5 isolate DW5 which produces Ptr ToxB. The LD5B population was screened with 86–124. Isolates were grown on V8-potato dextrose agar (Difco PDA; Becton, Dickinson and Company, Sparks, MD) plates for 5–7 d in the dark, and inoculum was prepared as described in Lamari and Bernier (1989) and Ali et al. (2010). Parents and the RIL population were planted in a completely randomized design consisting of three replicates separated by time for conidial inoculations. Each replicate consisted of a single cone per line with three plants per cone placed in racks of 98 cones. The tan spot-susceptible wheat variety “Jerry” was planted in the borders of each rack to reduce edge effects. Plants were inoculated until runoff at the two- to three-leaf stage with 3000 spores per ml and two drops of Tween 20 (polyoxyethylene sorbitan monolaurate; J.T. Baker Chemical Co., Phillipsburg, NJ) per 100 ml of inoculum. Inoculated plants were placed in a mist chamber with 100% relative humidity at 21°C for 24 hr, and then moved to a growth chamber for 6 d of incubation at 21°C under a 12 hr photoperiod. Inoculated plants were rated using a 1–5 lesion type scale (Lamari and Bernier 1989) at 7 d postinoculation, where one is resistant and five is susceptible.

For *P. nodorum* disease evaluations, the AL population was screened with *P. nodorum* isolate Sn2000, which is known to produce ToxA (Friesen et al. 2006). Inoculum production and inoculation procedures were done as described in Liu et al. (2004a). After inoculation, plants were placed in a mist chamber with 100% relative humidity at 21°C for 24 hr, and then moved to a growth chamber at 21°C with a 12 hr photoperiod. Disease evaluation was carried out at 7 d after inoculation by scoring lesions on the second leaf using the 0–5 scale described by Liu et al. (2004).

**Marker genotyping**

The AL population and parents were genotyped using the iSelect array containing 9000 wheat single nucleotide polymorphism (SNP) markers (Cavanagh et al. 2013) as described in Faris et al. (2014). Simple sequence repeat (SSR) markers were also used to genotype the AL population and were selected from the following libraries: MAG (Xue et al. 2008), WMS (gwm) (Röder et al. 1998), WMC (Somers et al. 2004), HBG (Torada et al. 2006), CFD (Sourdille et al. 2004), and BARC (Song et al. 2005). SSR primer sets were used to amplify the parental DNA using polymerase chain reaction (PCR) conditions as outlined in Lu et al. (2009). PCR amplifications were performed in 10 μl reactions consisting of 100 ng of DNA template, 1.5 mM MgCl₂, 0.125 mM dNTPs, 4 pmol of primers, and 1 unit of Taq DNA polymerase. The
PCR conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 30 sec, the appropriate annealing temperature for 30 sec, and 72°C for 1 min. The annealing temperature of each primer was obtained from the Graingenes website (http://wheat.pw.usda.gov/GG2/index.shtml). Fragments were electrophoresed on 6% polyacrylamide gels which were made using 46 ml of H2O, 6 ml 10× TBE (Tris-borate-EDTA), 9 ml of 40% acrylamide/bis-acrylamide, 40 ml tetramethylethylenediamine (TEMED), and 350 ml of 10% ammonium persulfate. Gels were stained with GelRed (Biotium, Inc.) for 10 min and then visualized with a Typhoon 9410 variable mode imager (GE Healthcare, Waukesha, WI). Markers that revealed polymorphisms between the parents were then used to genotype the AL population.

In addition to the SSR and SNP markers, a cleaved amplified polymorphic sequence (CAPS) marker (Xfcp667) based on the genomic sequence of the Snm1 gene (Shi et al. 2016) was used to map the Snm1 locus. A fragment of the Snm1 gene was PCR-amplified as described for the SSR markers above using an annealing temperature of 65°C and the primers FCP667F: 5’-TGCGTCGATAGGAGTGGT-3’ and FCP667R: 5’-ATGCGTAGGAGCAGGGAAGTA-3’. The 898 bp amplicon was then digested with the restriction enzyme HpyCH4IV, which cleaves the Altar 84 fragment, but not the LDN fragment, thus revealing a codominant polymorphism. The digested amplicons were electrophoresed on 2% agarose gels, stained with ethidium bromide, and photographed.

**Linkage, QTL, and statistical analysis**

Linkage analysis was conducted using the computer program MapDisto 1.8.1 (Lorieux 2012) to generate linkage maps. First, marker grouping was done by using the command “find groups” with a logarithm of odds (LOD) > 3.0 and an Rmax value = 30.0. The “order sequence,” “check inversions,” “ripple order,” and “drop locus” commands were then used to determine the best order for each group. The Kosambi mapping function (Kosambi 1944) was used to calculate linkage distances.

In the AL population, multiple interval mapping (MIM) was used to determine the effects of the Tsn1 and Tsc2 loci in causing disease and to identify additional QTL associated with disease using the computer program QGene v.4.3 (Joehanes and Nelson 2008). An LOD of 3.6 was declared as the threshold for QTL significance based on permutation tests of 1000 iterations for each trait. The homogeneity of variances among the three replicates was determined by Bartlett’s χ² test using SAS program version 9.4 (SAS Institute Inc., Cary, NC). Mean separation of the genotypic means were determined by Fisher’s protected LSD.
at an α level of 0.01. For the LD5B population, the critical LOD threshold of 1.8 declared in Faris and Friesen (2009) was used for determining significant associations for tan spot caused by 86–124. Because the LD5B population segregates for only one linkage group (5B), QTL analysis was conducted using the simple interval mapping (SIM) function in QGene v.4.3 (Joehanes and Nelson 2008).

**Gene expression analysis**

Plants of LDN and LDN-DIC 5B were grown and inoculated with water, *P. tritici-repentis* isolate 86–124, or *Pa. nodorum* isolate Sn2000 as described above. The youngest fully expanded leaf at the time of inoculation was harvested for RNA extraction. Samples for each treatment of both genotypes were collected at 0, 6, 24, 48, 72, and 96 hr after inoculation and immediately frozen in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and first-strand cDNA was synthesized from 1 μg of total RNA using Taqman Reverse Transcription Reagents including an oligo d(T)16 primer (Applied Biosystems, Foster City, CA). Relative quantitative (RQ)-PCR was performed to evaluate ToxA gene expression using primers ToxA.RT.F3 (5ʹ-AACGCCAATACGTGCAGT-3ʹ) and ToxA.cod.1R (5ʹ-GCCTGGCTCTCCCATTTTCACG-3ʹ) in all treatments and sampled time points. Expression of the ToxA gene was compared to the expression of the endogenous wheat ubiquitin gene as described in Faris et al. (2010) using primers Ta.Ubiquitin.F: 5ʹ-GACGTGGCCGACTACACATTTCA-3ʹ and Ta.Ubiquitin.R: 5ʹ-GACGCAGGACGAGACTTGTGAAC-3ʹ. All RQ-PCR experiments were conducted using a 7500 Real-Time PCR System (Applied Biosystems). Each experiment was conducted using three biological replicates each consisting of a single inoculated leaf, and at least three technical replicates per biological replicate were performed. The 10 μl PCR reactions contained 1 × SYBR PCR MasterMix (Applied Biosystems), 0.25 μM each primer, and 5 μl of 10-fold diluted cDNA. The thermocycler procedure was as follows: 10 min of preincubation at 95⁰, followed by 40 cycles for 15 sec at 95⁰ and for 1 min at 60⁰. Efficiencies of the different primer combinations were evaluated using serial dilutions of cDNA (1:5, 1:10, 1:20, and 1:40) and only primers with efficiencies higher than 95% were used for the RQ-PCR. The expression level of the Sn2000-inoculated 48 hr sample was set at 1 as a calibration point. Threshold cycles of the ToxA gene and the endogenous ubiquitin gene were used to calculate the relative expression levels using the 2^−ΔΔCT method.

**Data availability**

Parents and mapping populations are available upon request. Mapping data for the LD5B and the AL populations is also available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

**RESULTS**

**Marker analysis and linkage map construction**

The parental lines of the AL population were screened with 250 SSR primer pairs. Of these, 119 (47.6%) revealed polymorphisms between the parents and were used to genotype the AL population. The 9K SNP array yielded a total of 833 polymorphic SNP markers. One CAPS marker (Xfcp667) that was developed based on the Sm1 gene sequence on chromosome arm 1BS (Shi et al. 2016) was added to the marker set along with the two phenotypic markers Tsn1 and Tsc2 (see below). Therefore, the initial marker dataset consisted of a total of 955 markers. After initial linkage analysis, a total of 111 markers, including 19 SSRs and 92 SNPs, were eliminated from the dataset because they were unlinked, leaving a total of 844 markers in the dataset consisting of 100 SSRs, 741 SNPs, one CAPS, and two phenotypic (Tsn1 and Tsc2) markers.

These markers were assembled into 14 linkage groups that corresponded to the 14 durum wheat chromosomes (Supplemental Material, Table S1) and spanned a total genetic distance of 2207.15 cM with an average marker density of one marker per 2.6 cM (Table 1). The
A-genome chromosomes had 414 markers and spanned 1128.22 cM with an average density of one marker per 2.7 cM, whereas the B-genome chromosomes had a total of 430 markers spanning 1078.93 cM for an average marker density of one marker per 2.5 cM (Table 1). Chromosome 7A was the longest linkage group (262.41 cM) and chromosome 4B was the shortest (107.95 cM). The number of markers per chromosome ranged from 17 (4B) to 104 (5B) (Table 1 and Table S1). Chromosome 6B had the highest marker density at one marker per 1.2 cM, whereas chromosome 4B had the lowest with one marker every 6.3 cM (Table 1). Of the 844 markers, 208 (24.6%), had segregation ratios that deviated significantly (P < 0.05) from the expected 1:1 ratio. These distorted markers were located on 10 chromosomes (1A, 2A, 2B, 3B, 4B, 5A, 5B, 6A, 6B, and 7B).

**Genetic analysis of ToxA and Ptr ToxB sensitivity in the AL population**

Altar 84 was insensitive and Langdon was sensitive to ToxA (Figure 1). The AL population segregated in a ratio of 59 insensitive:68 sensitive for reaction to ToxA and fitted the expected 1:1 ratio for a single host gene conferring sensitivity to ToxA (χ² = 0.65, P = 0.42). Conversion of the ToxA reaction scores to genotypic scores allowed us to map the Tsn1 locus, which was located on the long arm of chromosome 5B as expected and flanked by SNP markers Xiwa7024 and Xiwa6915 at distances of 0.4 and 0.8 cM, respectively (Figure 2 and Table S1).

For reaction to Ptr ToxB, Altar 84 was sensitive and Langdon was insensitive (Figure 1). The AL population segregated in a ratio of 72 insensitive:55 sensitive for reaction to Ptr ToxB and fitted the expected 1:1 ratio for a single host gene conferring sensitivity to Ptr ToxB (χ² = 3.28, P = 0.07). The reactions to Ptr ToxB were converted to genotypic scores and analyzed along with the molecular marker data. Linkage analysis showed that the Tsc2 locus mapped to the short arm of chromosome 2B flanked by SSR markers Xhbg216 and Xwmc25 at distances of 6.6 and 11.3 cM, respectively (Figure 2 and Table S1).

**AL population reaction to P. tritici-repentis**

Altar 84, LDN, and the AL population were screened with the ToxA-producing race 2 isolates 86–124 and L13-35, and the race 5 Ptr ToxB-producing isolate DW5. Bartlett’s Chi-squared test for homogeneity indicated that the variances among replicates for each isolate were not significantly different (86–124: χ² = 2.08, P = 0.35; L13-35: χ² = 1.39, P = 0.50; DW5: χ² = 6.01, P = 0.05); therefore, the means from the three replicates for each isolate were used for further analysis.

Altar 84 and LDN were considered resistant and moderately susceptible to 86–124 with average disease reaction types of 1.17 and 3.50, respectively (Figure 3, Figure 4, and Table 2). The average disease reaction type for the AL population was 3.18, and reaction types ranged from 1.16 to 4.80 (Figure 3 and Table 2). The mean reaction types of Ptr ToxB-sensitive and -insensitive AL lines to DW5 were 2.29 and 3.00, respectively, which were significantly different at the 0.01 level of probability (Table 2). This result indicates that the Tsc2-Ptx ToxB interaction played a significant role in the development of tan spot caused by DW5.

**AL population reaction to Pa. nodorum**

As with the P. tritici-repentis isolates, Bartlett’s Chi-squared test for homogeneity of variances among Pa. nodorum isolate Sn2000 replicates was not significant (χ² = 0.78, P = 0.70), and therefore the means of the three reps were used for further analysis. Altar 84 was resistant to Sn2000 and LDN was highly susceptible with average disease reaction types of 1.33 and 4.83, respectively (Figure 3 and Table 2). The average disease reaction type for the AL population was 3.18, and reaction types ranged from 1.16 to 4.80 (Figure 3 and Table 2). The mean reaction types of ToxA-insensitive and -sensitive AL lines were 2.00 and 3.00, respectively (Table 2). These means were significantly different at the 0.01 level of probability (Table 2), indicating that the Tsn1-ToxA interaction played a significant role in the development of SNB caused by Sn2000.

**QTL analysis of the AL population for reaction to P. tritici-repentis and Pa. nodorum**

For the race 2 isolate 86–124, only one QTL was significantly associated with resistance (Figure 2 and Table 3). This QTL was on the long arm of 6B between markers Xiwa5148 and Xiwa9607, which were at positions 84.4 and 103.4 cM, respectively. This QTL, designated QTs.fcu-6B, had an LOD of 6.9 and explained 22% of the phenotypic variation (Table 3). The resistance effects of QTs.fcu-6B were contributed by Altar 84 (Table 3).

Two significant QTL were associated with the other P. tritici-repentis race 2 isolate, L13-35 (Figure 2). QTL on the short arms of chromosomes 4B and 5B, designated QTs.fcu-4B and QTs.fcu-5B, had LOD values of 4.0 and 4.2, and explained 11 and 12% of the phenotypic variation.
Table 2 Average reaction types of Altar 84, Langdon, and the Altar 84 × Langdon population of recombinant inbred lines to tan spot and Septoria nodorum blotch

| Isolate* | Altar 84 | Langdon | AL Population Range | AL Population Average | ToxA Sensitive Lines | ToxA Insensitive Lines | Ptr ToxB Sensitive Lines | Ptr ToxB Insensitive Lines | Difference Between Sensitive and Insensitive Lines |
|----------|----------|----------|---------------------|-----------------------|---------------------|-----------------------|------------------------|--------------------------|--------------------------|
| 86-124 (race 2) | 1.17 | 3.50 | 1.00–4.16 | 2.31 | 2.39 | 2.21 | – | – | 0.18 |
| L13-35 (race 2) | 2.75 | 4.67 | 1.33–4.83 | 3.52 | 3.64 | 3.36 | – | – | 0.28 |
| DWS (race 5) | 3.16 | 3.50 | 1.25–4.50 | 3.02 | – | – | 2.29 | 1.33 | 0.96* |
| Sn2000 | 1.33 | 4.83 | 1.16–4.80 | 3.18 | 3.00 | 2.00 | – | – | 1.00 |

*Reaction types caused by the P. tritic-repentis isolates (86-124, L13-35, and DWS) were scored using the 1–5 scale for tan spot described by Lamari and Bernier (1989), and reaction types caused by the Pa. nodorum isolate (Sn2000) were scored using the 0–5 scale for Septoria nodorum blotch described by Liu et al. (2004).

It had an LOD of 12.0 and explained 26% of the disease variation.

Table 3 Composite interval mapping analysis of QTL associated with resistance to tan spot caused by P. tritic-repentis races 2 and 5 and resistance to SNB caused by Sn2000 in the Altar 84 × Langdon (AL) population

| QTL | Marker Interval | Marker Position | LOD | R² | Add. | LOD | R² | Add. | LOD | R² | Add. |
|-----|-----------------|-----------------|-----|----|-----|-----|----|-----|-----|----|-----|
| QTs.fcu-2B | Tsc2 | 18.7 | – | – | – | – | – | – | 12.00 | 0.26 | 0.41 |
| QTs.fcu-4B | Xiwa2126-Xgwm113 | 7.1–38.9 | – | – | – | 4.00 | 0.11 | –0.37 | 8.60 | 0.12 | –0.56 |
| QTs.fcu-5B | Xgwm234 | 0.0 | – | – | – | 4.20 | 0.12 | –0.31 | – | – | – |
| QTs.fcu-4B | Xiwa5148-Xiwa967 | 84.4–103.4 | 6.90 | 0.22 | –0.51 | – | – | – | 13.00 | 0.38 | –0.59 |
| Qtnc.fcu-5B | Tsn1 | 110.2 | – | – | – | – | – | – | – | – | – |

A negative value for “Add.” indicates resistance effects derived from Altar 84. A dash indicates that the marker was not significantly associated with resistance. QTL, quantitative trait loci; LOD, logarithm of odds; Add., the additive effects of the QTL.
No expression of ToxA in 86–124-inoculated LDN-DIC 5B plants was detected. Small amounts of Toxα transcript were detected at the 72 and 96 hr time points in the Sn2000-inoculated LDN-DIC 5B plants, but the amounts were about a hundredth of the amount of ToxA transcribed in the Sn2000-inoculated LDN plants (Figure 6).

**DISCUSSION**

**Role of Tsc2-Ptr ToxB in tan spot susceptibility in durum wheat**

One objective of this research was to determine the role of the Tsc2-Ptr ToxB interaction in conferring tan spot susceptibility in a tetraploid durum wheat population. Friese and Faris (2004) first mapped the Ptr ToxB sensitivity gene Tsc2 on chromosome arm 2BS using the ITMI population, which was developed from a cross between the synthetic hexaploid wheat W-7984 and the hard red spring wheat variety Opat 85 (PI 591776). W-7984 was synthesized from crossing Altar 84 with the Ae. tauschii accession CI 18 (WPI 219). Because Altar 84 donated the B-genome chromosomes to W-7984, it also donated the Tsc2 locus, thus rendering W-7984 sensitive to Ptr ToxB. QTL analysis revealed a major QTL on 2BS explaining 69% of the variation and corresponding to the Tsc2 locus, thus indicating that the Tsc2-ToxB interaction was significantly associated with development of the disease in the ITMI population. Other studies have also demonstrated that the Tsc2 gene is a major susceptibility factor in hexaploid wheat (Abeysekara et al. 2010; Singh et al. 2010).

Although the durum variety Altar 84 contributed the dominant Tsc2 allele for Ptr ToxB sensitivity to the synthetic wheat W-7984, the evaluation of the effects of the Tsc2-Ptr ToxB interaction were conducted in a hexaploid wheat background (Friese and Faris 2004). Therefore, in this study, we chose to evaluate a tetraploid population derived from Altar 84 and LDN to determine the effects of the Tsc2-Ptr ToxB interaction in a true tetraploid wheat background. The results indicated that the Tsc2 explained up to 26% of the disease variation. This is the first study to demonstrate that the Tsc2-Ptr ToxB interaction plays a significant role in conferring susceptibility in tetraploid wheat, just as it does in hexaploid wheat.

**Role of Tsn1-ToxA in tan spot**

Another objective of this study was to evaluate the role of the Tsn1-ToxA interaction in conferring tan spot susceptibility in tetraploid wheat. Most of the previous tan spot studies pertaining to the Tsn1-ToxA interaction have been conducted in hexaploid wheat (Faris et al. 2013 for review), and those studies indicated that the Tsn1-ToxA interaction could play a major role (Tomas and Bockus 1987; Lamari and Bernier 1989; Cheong et al. 2004; Singh et al. 2010), a minor role (Friese et al. 2003; Chu et al. 2008a; S. Singh et al. 2008; Faris et al. 2012), or have no effect (Faris and Friese 2005), depending on the genetic background. Three studies prior to the current one involved the evaluation of the Tsn1-ToxA interaction in conferring tan spot susceptibility in tetraploid wheat. In one study, Chu et al. (2010a) evaluated a tetraploid wheat doubled haploid population derived from a cross between the durum variety Lebsock and accession PI 94749 of T. turgidum ssp. carthlicum (LP population) for reaction to the race 2 isolate 86-124 and the race 1 isolate Pt2, which both produce ToxA. Although the population segregated for reaction to ToxA infections, sensitivity to ToxA had no effect on disease. Further QTL analysis showed no significance for the Tsn1 locus on 5BL for reaction to either 86-124 or Pt2. In the second tetraploid wheat study, Chu et al. (2008c) evaluated 172 accessions of wild emmer wheat (T. dicoccoides) for reaction to infiltrations of ToxA and inoculations with the P. tritici-repentis race.
Table 4 Average reaction types of Langdon, LDN-DIC 5B, and the Langdon × LDN-DIC 5B (LD5B) population of recombinant inbred chromosome lines to tan spot and Septoria nodorum blotch

| Isolate | Langdon | LDN-DIC 5B | LD5B Population | ToxA Sensitive Lines | ToxA Insensitive Lines | Difference Between Sensitive and Insensitive Lines |
|---------|---------|------------|-----------------|----------------------|------------------------|-----------------------------------------------|
| 86-124  | 3.60    | 3.00       | 2.25–4.00       | 3.16                 | 3.16                   | 0.12                                          |
| Sn2000  | 3.50    | 0.83       | 0.33–3.83       | 2.40                 | 3.24                   | 0.78                                          |

* P < 0.01.

The data and analysis of Sn2000 in the LD5B population was taken from Faris and Friesen (2009).

Table 5 Simple interval mapping analysis of QTL associated with resistance to tan spot caused by P. tritici-repentis race 2 isolate 86–124 and to SNB caused by the Pa. nodorum isolate Sn2000 in the Langdon × LDN-DIC 5B (LD5B) population

| QTL | Marker Interval | Marker Position | LOD | R² | Add. | LOD | R² | Add. |
|-----|-----------------|-----------------|-----|----|------|-----|----|------|
| QTs.fcu-5B | Xbcd873-Xabg705 | 0–13.6 | 3.80 | 0.19 | –0.18 | – | – | – |
| QSnb.fcu-5B | Tsn1 | 48.2 | – | – | – | 54.0 | 0.95 | –1.30 |

A dash indicates that the marker was not significantly associated with resistance. A negative value indicates resistance effects derived from LDN-DIC 5B. QTL, quantitative trait loci; LOD, logarithm of odds; Add., the additive effects of the QTL.
interaction must be due to differences in the biology of the populations, the differences observed in the effects of the isolates of both these studies evaluated disease produced by ToxA-producing isolates of *P. tritici-repentis* and *Pa. nodorum* on the same population, the differences observed in the effects of the *Tsn1*-ToxA interaction must be due to differences in the biology of the pathogens, *i.e.*, *P. tritici-repentis* vs. *Pa. nodorum*. In line with this, our expression studies indicated that *Pa. nodorum* expressed ToxA at much higher levels than *P. tritici-repentis* on *Tsn1*-containing plants, which is a result reminiscent of those of Faris et al. (2011) and Phan et al. (2016) who both showed that levels of NE expression in *Pa. nodorum* were strongly correlated with levels of disease.

The reason for the differential expression is not known, but as mentioned above, the ToxA gene has existed in *Pa. nodorum* for a very long time and was only recently transferred to *P. tritici-repentis* (Friesen et al. 2006). It is possible that ToxA functions more efficiently in *Pa. nodorum* compared to *P. tritici-repentis*, perhaps due to the presence of additional and/or different transcription factors that may enhance ToxA expression. It is perceivable that transcriptional upregulation of ToxA in *Pa. nodorum*-infected plants may occur due to host recognition signals. In other words, pathogen acknowledgment of host recognition, *i.e.*, the presence of *Tsn1*, may lead to upregulation of ToxA expression. The fact that ToxA expression in the resistant line LDN-DIC 5B was undetectable at 24 hr postinoculation, and levels of ToxA expression in the susceptible line LDN were extremely high at the same time point, would suggest that could be the case. Therefore, it seems that *Pa. nodorum* might possess ToxA-regulating factors that *P. tritici-repentis* does not. More research is needed to test this hypothesis.

**Conclusions**

In conclusion, the *Tsc2*-Ptr ToxB interaction played a significant role in the development of tan spot caused by the Ptr ToxB-producing race 5 isolate DW5. This was the first study to demonstrate this for tetraploid wheat. Therefore, durum wheat breeders should determine whether or not their material possesses the *Tsc2* gene and strive to remove it from their lines using marker-assisted selection. Diagnostic markers for *Tsc2* have been developed and proven to be useful for such purposes (Abeysekara et al. 2010). Second, this research showed that the *Tsn1*-ToxA interaction was not associated with the development of tan spot in two tetraploid wheat populations, however, it played a significant role in the development of SNB in both populations. This is the second study to show this result in tetraploid wheat, and validates work in a different tetraploid wheat population (Chu et al. 2010a; Friesen et al. 2012). Although *Tsn1* may not be relevant for susceptibility to tan spot in these two durum wheat populations, this result needs to be confirmed in other tetraploid wheat populations as well. Regardless of whether or not *Tsn1* is important for tan spot susceptibility in any durum wheat genotype, breeders should still strive to remove *Tsn1* from their materials in an effort to eliminate SNB susceptibility loci and render their lines more resistant. The *Tsn1* gene has been cloned, and numerous diagnostic markers are available for this purpose (Zhang et al. 2009; Faris et al. 2010). More research is needed to determine why the relevance of the *Tsn1*-ToxA interaction in the development of tan spot is affected by different host genetic backgrounds, and why the interaction is more significant in the development of SNB than tan spot.

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