Theor Appl Genet (2009) 120:117–126
DOI 10.1007/s00122-009-1163-6

ORIGINAL PAPER

Identification and characterization of a novel host–toxin interaction in the wheat–*Stagonospora nodorum* pathosystem

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Received: 27 May 2009 / Accepted: 11 September 2009 / Published online: 9 October 2009

Abstract *Stagonospora nodorum*, casual agent of Stagonospora nodorum blotch (SNB) of wheat, produces a number of host-selective toxins (HSTs) known to be important in disease. To date, four HSTs and corresponding host sensitivity genes have been reported, and all four host–toxin interactions are significant factors in the development of disease. Here, we describe the identification and partial characterization of a fifth *S. nodorum* produced HST designated SnTox4. The toxin, estimated to be 10–30 kDa in size, was found to be proteinaceous in nature. Sensitivity to SnTox4 is governed by a single dominant gene, designated *Snn4*, which mapped to the short arm of wheat chromosome 1A in a recombinant inbred (RI) population. The compatible *Snn4*–SnTox4 interaction is light dependent and results in a mottled necrotic reaction, which is different from the severe necrosis that results from other host–toxin interactions in the wheat–*S. nodorum* pathosystem. QTL analysis in a population of 200 RI lines derived from the Swiss winter wheat varieties Arina and Forno revealed a major QTL for SNB susceptibility that coincided with the *Snn4* locus. This QTL, designated *QSnb.fcu-1A*, explained 41.0% of the variation in disease on leaves of seedlings indicating that a compatible *Snn4*–SnTox4 interaction plays a major role in the development of SNB in this population. Additional minor QTL detected on the short arms of chromosomes 2A and 3A accounted for 5.4 and 6.0% of the variation, respectively. The effects of the three QTL were largely additive, and together they explained 50% of the total phenotypic variation. These results provide further evidence that host–toxin interactions in the wheat–*S. nodorum* pathosystem follow an inverse gene-for-gene model.

Communicated by M. Sorrells.

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Introduction

Stagonospora nodorum (Berk.) E. Castell. and Germano [telomorph *Phaeosphaeria nodorum* (E. Mull.) Hedjar.], is a necrotrophic filamentous ascomycete fungus that belongs to the Dothideomycete class of the ascomycota. It has high genetic diversity and a heterothallic mating system (McDonald and Linde 2002). *S. nodorum* causes one of the most economically important and destructive foliar diseases of wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD genomes) and related cereals, affecting both the leaves and the glumes. One of the most effective methods of controlling Stagonospora nodorum blotch (SNB) is the use of host resistance. Inheritance of SNB resistance is complex and most often governed by multiple genes (Fried and Meister 1987; Bostwick et al. 1993; Du et al. 1999), but monogenic inheritance has also been found in some wheat materials (reviewed in Xu et al. 2004 and Friesen et al. 2008a).

Host-selective toxins (HSTs) are essential determinants of pathogenicity or virulence and determine host specificity (Wolpert et al. 2002). Most HSTs are characterized as small secondary metabolites and their production in fungi are under the control of complex enzymatic pathways

(Panaccione et al. 1992). However, several proteinaceous HSTs have also been reported (Strelkov et al. 1998; Tomas et al. 1990; Barthe et al. 2007; Parada et al. 2008; Sarpeleh et al. 2007).

In *S. nodorum*, four proteinaceous HSTs have been identified and in each case sensitivity to the HST was conferred by a single dominant host gene. Using segregating wheat populations, the *Tsn1*–ToxA, *Snn1*–SnTox1, *Snn2*–SnTox2, and the *Snn3*–SnTox3 interactions were shown to account for as much as 95, 58, 47 and 17% of the variation in SNB development on leaves, respectively (Liu et al. 2004b, 2006; Friesen et al. 2006, 2007, 2008b; Faris and Friesen 2009). Therefore, each of the four host–toxin interactions characterized in the wheat–*S. nodorum* pathosystem plays a significant role in the development of SNB. Studies have shown that the effects of compatible host–toxin interactions are largely additive (Friesen et al. 2007, 2008b, 2009), and some have been shown to be epistatic to others (Friesen et al. 2008b). In addition, disease expression is usually influenced by apparent non-HST factors or QTLs with minor effects. Therefore, the level of disease expression ultimately appears to be quantitatively controlled in most cases, especially when multiple host–toxin interactions are operating in the same system.

Of the four host–toxin interactions identified in the wheat–*S. nodorum* system, *Tsn1*–SnToxA is best characterized. ToxA is a 13.2 kDa proteinaceous HST (Ballance et al. 1989; Tomas et al. 1990; Tuori et al. 1995) that has the ability to cause necrosis in wheat lines carrying the *Tsn1* gene. Manning and Ciuffetti (2005) showed that ToxA is internalized within cells of sensitive wheat cultivars and localized to cytoplasmic compartments and to chloroplasts. Work on the host side has included high resolution mapping of the *Tsn1* locus (Haen et al. 2004; Lu and Faris 2006; Lu et al. 2006) followed by BAC-based physical mapping, and has led to the isolation of *Tsn1* (J. Faris, unpublished data), which will allow more detailed characterization of the *Tsn1*–ToxA interaction at the molecular level. Host and pathogen components of the three other wheat–*S. nodorum* host–toxin interactions have yet to be isolated.

In previous studies, the Swiss winter wheat cv. Arina was shown to carry major QTLs for SNB resistance associated with the glume on chromosome arms 3BS (*QSng.sfr-3B*) and 4BL (*QSng.sfr-4B*), whereas the cultivar Forno was shown to be highly susceptible to glume blotch (Schnurbusch et al. 2003). However, the AF population has not been previously evaluated for reaction to leaf blotch at the seedling stage. Here, evaluation of the Arina × Forno (AF) population for reaction to SNB on leaves of seedlings led to the identification and characterization of a novel host–toxin interaction in the wheat–*S. nodorum* pathosystem. This work provides further evidence that the wheat–*S. nodorum* pathosystem follows an inverse gene-for-gene system at the host–toxin interface where multiple effector proteins (HSTs) interact with dominant host sensitivity gene products to cause disease.

Materials and methods

Plant materials

A recombinant inbred (RI) population consisting of 200 single seed descent F_5 lines developed by an intraspecific cross between the Swiss winter wheat cultivars Arina and Forno (Paillard et al. 2003) was evaluated using culture filtrates, partially purified toxin, and by spore inoculations. An F_2 population derived from the cross between Arina and Forno consisting of 50 individuals was infiltrated with the partially purified toxin to determine if the host gene conferring sensitivity in this population was dominant or recessive in nature. The wheat lines W-7984, BG223, BG220, and BG261, which serve as differentials for SnTox1, SnTox2, SnTox3, and SnToxA, respectively, were infiltrated with culture filtrates of the Swiss *S. nodorum* isolate Sn99CH 1A7a (hereafter referred to as 1A7a) to determine if the cultures contained novel toxins.

Disease evaluation and toxin bioassays

Previous work on the AF population for reaction to Stagonospora glume blotch was conducted under natural infestation in Switzerland (Schnurbusch et al. 2003). Therefore, we chose to use a Swiss *S. nodorum* isolate to evaluate reaction of the AF population to Stagonospora leaf blotch. We obtained the 1A7a isolate from Dr. Bruce McDonald (Swiss Federal Institute of Technology, Zurich, Switzerland). Inoculum for disease evaluations were prepared from the isolate 1A7a grown in V8-potato dextrose agar (PDA) for 5–7 days as described by Liu et al. (2004b). Three replicates consisting of the 200 AF lines and parents were planted in a completely randomized design (CRD) and used for spore inoculations. Each replicate consisted of three cones (Stuewe and Sons Inc., Corvallis, OR, USA) per line with three plants per cone placed in racks of 98 (Stuewe and Sons). Hence each experimental unit consisted of nine plants. The susceptible hard red spring wheat cultivar Grandin was planted around the borders of each rack to eliminate any edge effect. Plants were inoculated at the two to three leaf stage with the conidial suspensions, with 1×10^6 spores/ml, until runoff as described in Liu et al. (2004b). Inoculated plants were then subjected to 100% relative humidity at 21°C for 24 h in a mist chamber followed by 6 days of incubation in the growth chamber at 21°C under a 12 h photoperiod. The second leaf of the

inoculated plants was scored on a zero to five lesion type scale (Liu et al. 2004b) 7 days post-inoculation.

Culture filtrates of isolate 1A7a were prepared as described by Liu et al. (2004a) and used for initial evaluation of the AF population for toxin sensitivity. The fully expanded secondary leaf of each plant was infiltrated with approximately 25 µl of the active culture filtrate using a 1-ml syringe with the needle removed and the infiltrated region was marked with a non-toxic felt pen. Infiltrated leaves were evaluated 3 days after infiltration and were scored based on the presence or absence of necrosis. Experiments were repeated at least twice. After analyzing the results obtained from both the infiltrations with the culture filtrate and the conidial inoculations, the RI line AF89 was selected as the differential line for the novel toxin (see "Results"). AF89 possessed the toxin sensitivity allele from Arina and resistance alleles at minor QTLs.

Toxin partial purification

Acetone precipitated 1A7a culture filtrates (concentrated sixfold) were subjected to overnight dialysis against water in 3.5 kDa molecular weight cutoff tubing, filtered with a 0.45 µm filter and loaded on to a 1 ml HiTrap SPXL cation exchange column (GE Healthcare) equilibrated with 20 mM sodium acetate starting buffer (pH 4.3) on an AKTAPrime^{TM}plus system (GE Healthcare). The toxin was eluted using 20 mM sodium acetate + 300 mM NaCl elution buffer, pH 4.3, with a flow rate of 1 ml/min with an elution gradient of 0–300 mM NaCl (pH 4.3) over 20 ml. Fractions were infiltrated onto the differential line AF89 (which showed mottled necrosis in response to the culture filtrate) after adjusting the pH by adding 5.0 µl of 1.5 M sodium hydroxide to each 1 ml fraction, to select the active fractions. Fractions with the highest activities were pooled and used to screen the AF population.

Characterization of SnTox4

Pronase (EMD Biosciences Inc., San Diego, CA, USA), which consists of endo- and exo-proteases, was used to determine whether the toxin is a protein. A 1 ml fraction of the acetone precipitated 1A7a culture filtrate (sixfold concentration) was treated with Pronase (final concentration 1 mg/ml in water). Pronase untreated samples along with Pronase alone were kept as controls. All the samples were incubated at 37°C for 3 h and infiltrated onto the differential line AF89. Pronase-treated and untreated samples were also tested on the set of selected 80 AF lines to further confirm the findings.

Active culture filtrates were subjected to ultrafiltration using 30 and 10 kDa Amicon molecular weight cutoff filters (Millipore) in order to determine the size of the toxin. The same subset of 80 AF lines including the differential line AF89 was used to test the partially purified toxin. This set was screened with the concentrates and filtrates of both the 30 and 10 kDa subjected active culture filtrates to verify the presence of SnTox4. Presence of the toxin in the flow-through of each filtration indicated that the active molecule was smaller in size than the filter cutoff (e.g. activity in the filtrate of the 30 kDa filtration indicated a molecule <30 kDa).

Investigation of light dependency for the *Snn4*–SnTox4 interaction was accomplished by testing the SnTox4 differential line AF89, infiltrated with 1A7a culture filtrates containing SnTox4. Two treatments were evaluated including a 48 h dark period after infiltration (treatment 1) and a 16 h photoperiod after infiltration (treatment 2). Three plants were evaluated per replicate per treatment and all plants were subjected to a 16 h photoperiod in the growth chamber at 24°C with a light intensity of 900 µmol m^{-2} s^{-1} prior to infiltration. All infiltrations were performed immediately after plants had been subjected to an 8 h dark period. At the end of each treatment plants were evaluated for sensitivity to the toxin. The entire experiment was replicated once with identical results.

EST marker development and linkage analysis

We assessed the linkage of the genotypic scores of *Snn4* with markers previously mapped in the AF population (Paillard et al. 2003; Schnurbusch et al. 2003, 2004; Tommasini et al. 2007). After it was determined that *Snn4* resided at the distal end of chromosome arm 1AS, NSF-wheat bin mapped EST sequences were downloaded from bin 1AS3-0.86-1.00 (http://wheat.pw.usda.gov/west/binmaps). EST primers were designed using the computer software Primer3 (Rozen and Skaletsky 2000). Each primer was amplified on the parental DNA, which was isolated from plant tissue as described by Faris et al. (2000). PCR conditions were as described in Lu et al. (2006). Amplified products were analyzed on 6% polyacrylamide gels and the polymorphic markers were selected and tested on the 200 individuals of the AF population. Linkage analysis was performed using the computer program MAPMAKER V2.0 (Lander et al. 1987) for Macintosh with the Kosambi mapping function (Kosambi 1944). A maximum θ value of 0.40 and a minimum LOD threshold of 3.0 were used to identify the linkage groups initially using the "two-point/group" command. The marker order was verified using the "ripple" command with a LOD value of 3.0.

QTL analysis

Previously published linkage maps of the AF population (Paillard et al. 2003; Schnurbusch et al. 2003, 2004;

Tommasini et al. 2007) were used to select a subset of 459 markers that gave the best genome coverage to be used for the detection of QTL. Markers significantly associated ($P < 0.001$) with resistance to SNB were identified by simple linear regression using the computer program MapManager QTXb20 (Manly et al. 2001). Composite interval-regression mapping was used to identify chromosomal regions putatively associated with the disease phenotype. A permutation test with 1,000 permutations was performed to determine the critical LOD threshold of the AF population which was found to be 3.2 at an experimental-wise error (α) level of 0.05. Additive effects of the QTL were obtained using MapManager QTXb20 (Manly et al. 2001) and markers with significant main effects were further tested for possible significant interactions ($P < 0.0001$) with each other.

Statistical analysis

Statistical analysis was conducted using the computer software packages Data desk (Data description Inc. Version 4.1), Graphpad (http://www.graphpad.com/quickcalcs) and SAS (SAS Institute Inc. Version 9.1). The average disease reaction types calculated from the three replicates were used along with the marker data of the significant markers to conduct the multiple regression analysis using Data desk. Chi squared tests were conducted using the program Graphpad. Bartlett's χ^2 test for homogeneity of variances among replicates was conducted using SAS, analysis of variance was conducted using the PROC GLM procedure of SAS, and Fisher's protected least significant difference (LSD) was used at $\alpha = 0.05$ to determine the mean separation for the genotypic means.

Results

Identification of an HST produced by the *S. nodorum* isolate 1A7a

Parental lines Arina and Forno were evaluated with culture filtrate of the Swiss isolate 1A7a. The results indicated that Arina exhibited a mottled necrotic reaction 3 days after infiltration and was therefore sensitive to a toxic component of the culture filtrate (Fig. 1D). Forno showed no reaction to the culture filtrate and was therefore considered insensitive (Fig. 1E). The differential lines for SnToxA (BG261), SnTox1 (W-7984), SnTox2 (BG223) and SnTox3 (BG220) infiltrated with 1A7a culture filtrate resulted in no visible reaction (reactions not shown). This indicated that the 1A7a culture filtrate contained at least one new HST, which we designated SnTox4.

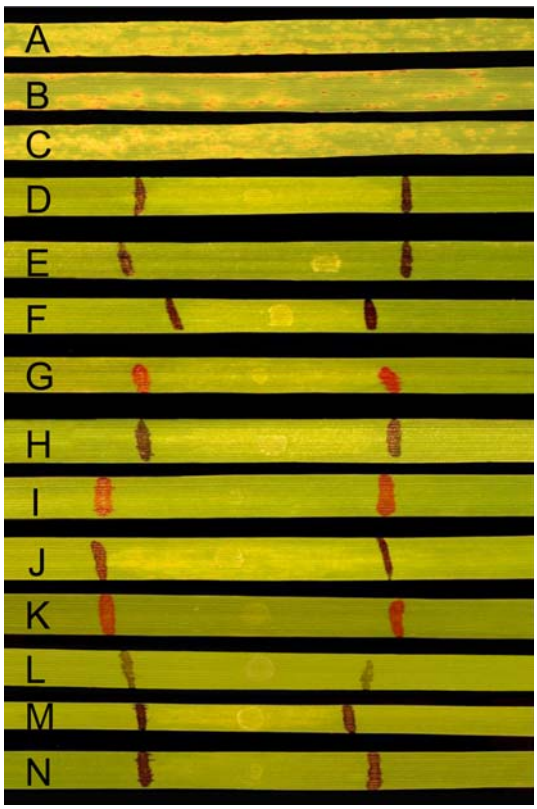

Fig. 1 Leaves inoculated with conidia produced by *Stagonospora nodorum* isolate 1A7a or infiltrated with culture filtrate, partially purified SnTox4, or pronase-treated cultures. Arina (*A*), Forno (*B*), and AF89 (*C*) inoculated with conidia produced by *S. nodorum* isolate 1A7a. Both Arina and Forno were moderately susceptible to SNB with Arina being more susceptible (average disease reaction type 3.2) to the disease than Forno (average disease reaction type 2.8). Reaction of Arina (*D*) and Forno (*E*) to 1A7a culture filtrates. When the differential line AF89 was infiltrated with the concentrate (*F*) and filtrate (*G*) from a 30 kDa filter, and the concentrate of a 10 kDa filter (*H*), all resulted in faint mottled necrosis. No reaction occurred as the result of infiltration of AF89 with filtrate derived from the 10 kDa filter (*I*). Partially purified SnTox4 cultures treated with water alone showed faint mottled necrosis on AF89 (*J*), and when infiltrated with pronase-treated partially purified SnTox4 cultures (*K*) or pronase alone (*L*), AF89 exhibited no reaction. AF89 developed faint mottled necrosis after 48 hours when infiltrated with partially purified SnTox4 and subjected to a normal light/dark regiment (*M*), but no reaction occurred on AF89 when plants were kept in complete darkness after infiltration (*N*)

Identification of the host gene conferring sensitivity to SnTox4

The entire AF population was infiltrated with culture filtrate and partially purified SnTox4 (see below). The population segregated in a ratio of 95 insensitive:105 sensitive in response to both the culture filtrate and the partially purified samples. This fit the expected 1:1 ratio for a single host gene conferring sensitivity ($\chi^2_{df=1} = 0.500$, $P = 0.4795$) in this population.

Fifty F_2 plants derived from the cross between Arina and Forno were infiltrated with the partially purified SnTox4 to determine gene action of sensitivity conferred by the host. The F_2 individuals segregated in a ratio of 38 sensitive:12 insensitive. This fit the expected ratio of 3:1 (sensitive:insensitive) for a single dominant gene conferring sensitivity ($\chi^2_{df=1} = 0.027$, $P = 0.8703$). We propose to designate the gene conferring sensitivity to SnTox4 as *Snn4*.

Chromosomal location of *Snn4*

We assessed the linkage of the genotypic scores of *Snn4* with markers previously mapped in the AF population (Paillard et al. 2003; Schnurbusch et al. 2003, 2004; Tommasini et al. 2007). *Snn4* mapped 1.6 cM distal to the SSR marker *Xcfd58.1* on chromosome arm 1AS (Fig. 2).

In an attempt to identify more markers linked to the *Snn4* locus, we developed PCR primers (Table 1) from ESTs mapped to the wheat 1AS3-0.86-1.00 deletion bin by the NSF-wheat EST project (http://wheat.pw.usda.gov/wEST/binmaps/). Primer sets were designed for 48 ESTs and eleven of these revealed polymorphisms between Arina and Forno, but only four pairs amplified fragments that mapped to chromosome 1A (Table 1; Fig. 2). The EST marker *XBE9590632* mapped 13.9 cM distal to the *Snn4* locus, and *XBG262267* and *XBG262975* co-segregated with each other at 0.9 cM distal to *Snn4*. Therefore, the *Snn4* locus is delineated to a 2.5 cM interval flanked by the EST markers *XBG262267/XBG262975* on the distal side and the SSR marker *Xcfd58.1* on the proximal side.

The role of a compatible *Snn4*–SnTox4 interaction in causing disease

Three replicates of the AF population along with the parents were inoculated with conidia of 1A7a and rated on a scale of 0–5 based on the severity of the disease (0 = highly resistant; 5 = highly susceptible) (Liu et al. 2004b). Average disease reaction types for Arina and Forno were 3.2 and 2.8, respectively (Table 2; Fig. 3). A Bartlett's χ^2 test for homogeneity of the three replicates indicated that they were homogeneous ($P = 0.134$), and therefore the combined means of the three replicates were used in all subsequent analysis. The average disease reaction types of the AF population followed a normal distribution and ranged from 1.0 to 4.0 with an overall mean of 2.6. The Pearson's product moment correlation between the two variables (average SNB disease and toxin reaction) was found to be 0.64 suggesting moderately high correlation between SNB disease and toxin sensitivity. RI lines sensitive to SnTox4 had a mean reaction type of 3.0 and ranged from 2.0 to 4.0, whereas the RI lines that were insensitive to SnTox4 had a mean reaction type of 2.2 and ranged from 1.0 to 3.5 (Table 2; Fig. 3).

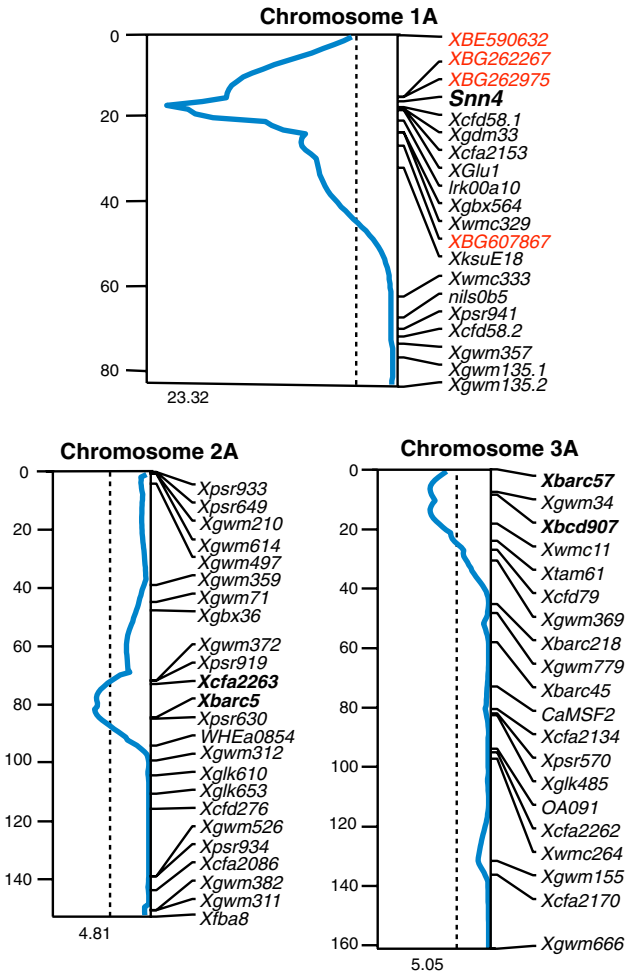

Fig. 2 Composite interval-regression maps of chromosomes 1A, 2A, and 3A generated in the Arina × Forno recombinant inbred population after inoculation of the population with conidia of the *Stagonospora nodorum* isolate 1A7a. A centiMorgan (*cM*) scale is shown to the *left* of the maps and the markers are shown to the *right*. A LOD scale is indicated along the *x*-axis, and the significant LOD threshold of 3.2 is indicated by the *dotted line*. Markers defining the QTL or QTL intervals are shown in *bold*. EST-derived markers developed in this research and mapping to chromosome 1A are shown in *red*

The effects of the *Snn4*–SnTox4 interaction in disease caused by 1A7a were investigated by conducting QTL analysis. Simple linear regression and composite interval mapping (CIM) were used to identify molecular markers and genomic regions associated with SNB resistance. One major and two minor QTL were detected on three chromosomal regions. The major QTL designated *QSnb.fcu-1A* and a minor QTL designated *QSnb.fcu-3A* located on the chromosome arms 1AS and 3AS, respectively, were significantly associated with the SNB disease resistance contributed by Forno (Table 3; Fig. 2). A third QTL detected

Table 1 Expressed sequence tags (ESTs) mapped on chromosome arm 1AS in the Arina × Forno recombinant inbred population, the primers used to amplify them, and the annealing temperatures used

Marker	EST GenBank accession	PCR primers	Annealing temperature (°C)
XBE590632-1A	BE590632	AACGATGATCCATCCGTCTT	53
		TCCATCCTTCAACCACAACA	
XBG262267-1A	BG262267	CGTTACAACGATTGGTGCAT	53
		TCATCCAACCTCACCAACCA	
XBG262975-1A	BG262975	TCCAGTCAACAGCAACCATC	54
		CCAAACAGTGAAGCTGCAAA	
XBG607867-1A	BG607867	GTGGAAATGGAGGACGCTTA	54
		ATCTGTGACCGAGGCAGAAC	

Table 2 Average and range of disease reaction types of parents and recombinant inbred lines of the Arina × Forno population for the two allelic state combinations for *Snn4* after inoculation with conidia of *Stagonospora nodorum* isolate 1A7a

Genotype	Average disease reaction type	Reaction type range
Arina	3.2	2.5–3.5
Forno	2.8	2.0–3.5
Snn4/Snn4	3.0[a]	2.0–4.0
snn4/snn4	2.2[a]	1.0–3.5

[a] Average disease reaction type of *Snn4/Snn4* is significantly different from that of the *snn4/snn4* at the 0.05 level of probability

on the short arm of chromosome 2A, designated *QSnb.fcu-2A*, was significantly associated with resistance contributed by Arina. *QSnb.fcu-1A* peaked at the *Snn4* locus and explained 41% of the phenotypic variation. *QSnb.fcu-2A* peaked between the markers *Xcfa2263* and *Xbarc5*, and *QSnb.fcu-3A* peaked between the markers *Xbarc57* and *Xbcd907*. *QSnb.fcu-2A* and *QSnb.fcu-3A* explained 5.4 and 6.0% of the phenotypic variation, respectively. Together these three QTL explained 50% of the total phenotypic variation in the AF population. No significant QTL × marker interactions were observed at an α level of 0.0001. All the QTL associated with SNB disease resistance showed significant additive effects with *QSnb.fcu-1A* providing the largest effect (Table 3).

Partial characterization of SnTox4

Acetone precipitated active 1A7a culture filtrates were dialyzed and subjected to cation exchange chromatography. The eluted fractions were assayed on the differential line AF89. The toxin eluted between 140 and 180 mM NaCl with the center of the activity peak at 155 mM. The RI line AF89 was chosen as the differential line for SnTox4 because it appeared to harbor the sensitivity allele at the *Snn4* locus based on culture filtrate analysis and resistance alleles at the *QSnb.fcu-2A* and *QSnb.fcu-3A* QTLs based on spore inoculations. Infiltration of AF89 with the active

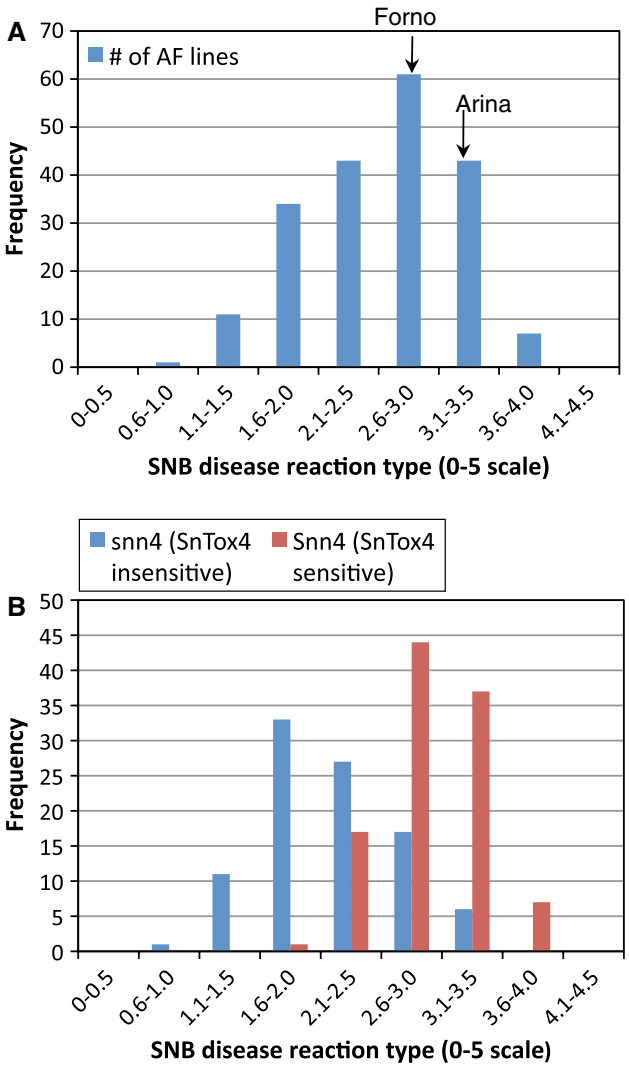

Fig. 3 Histograms demonstrating the average SNB disease reaction type (obtained from the three replicates of 1A7a conidial inoculations) versus frequency. **a** Average disease reaction types of the AF population. **b** Average disease reaction types of the SnTox4 sensitive and insensitive AF lines

SnTox4 sample treated with water alone resulted in the development of necrosis within 72 h, whereas no reaction occurred after infiltrating AF89 with a pronase-treated

Table 3 QTLs for seedling resistance to Stagonospora nodorum blotch caused by the isolate 1A7a in the Arina × Forno recombinant inbred population detected by composite interval mapping using the combined means of three replicates

Chromosome arm	QTL designation	Marker or marker interval	Chromosome peak position (cM)	Source of resistance	R^2	LOD	Additive effect
1AS	*QSnb.fcu-1A*	*Snn4*	17.0	Forno	0.41	23.32	0.39
2AS	*QSnb.fcu-2A*	*Xcfa2263-Xbarc5*	78.0	Arina	0.05	4.81	0.17
3AS	*QSnb.fcu-3A*	*Xbarc57-Xbcd907*	6.0	Forno	0.06	5.05	0.17

The chromosomal locations, associated markers, peak positions, R^2, LOD, and additive values are given

SnTox4-containing sample (Fig. 1). AF89 plants infiltrated with pronase alone showed no visible reaction.

To further confirm this result, we infiltrated pronase-treated and untreated acetone precipitated samples on a subset of 80 AF RI lines, which included 40 SnTox4 sensitive lines and 40 SnTox4 insensitive lines. No reaction was observed on any of the 80 lines when infiltrated with the pronase-treated sample. The 40 SnTox4 sensitive lines showed necrosis when infiltrated with the sample lacking pronase and the 40 insensitive lines remained insensitive. QTL analysis demonstrated that the effects on the AF lines infiltrated with untreated SnTox4-containing sample were due to the *Snn4* locus, whereas the AF lines infiltrated with the pronase-treated sample showed no effect of *Snn4* (Fig. 4). These results indicate that SnTox4 is most likely a protein.

To determine the approximate size of SnTox4, the active culture filtrates were subjected to ultrafiltration using 30 and 10 kDa Amicon molecular weight cutoff filters. Both the flow-through and the concentrates were infiltrated on AF89. Using active culture filtrates, both the 30 kDa flow-through and the concentrate caused necrosis (Fig. 1). However, only the concentrate and not the flow-through of the 10 kDa filter caused necrosis (Fig. 1). This indicates that the size range of SnTox4 is between 10 and 30 kDa.

Concentrates and filtrates of both the 10 and 30 kDa subjected active culture filtrates were used to screen the same subset of 80 AF lines to verify the presence of SnTox4. QTL analysis indicated the presence of SnTox4 in both the concentrate and the filtrate of the 30 kDa filter and only in the concentrate of the 10 kDa filter (data not shown). This result provides further evidence that SnTox4 is in the range of 10–30 kDa.

Light has been shown to be essential for compatibility of other wheat–*S. nodorum* toxin interactions. Therefore, to determine if a compatible *Snn4*–SnTox4 interaction is also light dependent, we infiltrated AF89 with partially purified SnTox4 under dark conditions. As a control, AF89 plants were infiltrated under normal lighting conditions using the same partially purified SnTox4 sample. Infiltrated AF89 plants kept in the dark developed no symptoms, whereas the plants subjected to normal lighting showed necrosis after 48 h (Fig. 1M, N). This result indicates that a compatible *Snn4*–SnTox4 interaction is dependent on light.

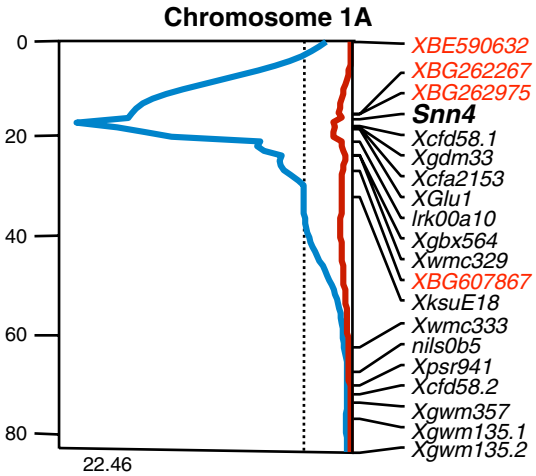

Fig. 4 Interval-regression analysis of chromosome 1A of the Arina × Forno population after infiltration of 80 recombinant inbred lines with partially purified SnTox4 cultures treated with pronase (*red line*) and without pronase (*blue line*). Markers are shown to the *right* of the map and a centiMorgan (*cM*) scale is indicated to the *left*. The critical LOD threshold is indicated by the *dotted line*

Discussion

The host–toxin interactions in the wheat–*S. nodorum* pathosystem can be described as the inverse of Flor's (1956) classic gene-for-gene model. In the wheat–*S. nodorum* system, the direct or indirect interaction between the effector proteins (HSTs) produced by the pathogen and the products of the host genes results in disease susceptibility. If either the toxin or the corresponding host sensitivity gene is not present, an incompatible interaction occurs resulting in a resistant response. In a classical gene-for-gene system, the interaction between an effector (Avr gene product) and a corresponding host resistance (R) gene leads to localized host cell death, which is characterized by a hypersensitive response that occurs via programmed cell death. If either the Avr gene or the corresponding host R gene is not present, a compatible reaction occurs, which results in a susceptible reaction. Therefore, the wheat–*S. nodorum* system follows an inverse gene-for-gene scenario

at the host–toxin interface. However, host–toxin interactions usually show strong additive effects when multiple compatible interactions operate in the same background (Friesen et al. 2007, 2008b, 2009). This leads to disease resistance being inherited in a quantitative manner and no resemblance of a gene-for-gene system.

Previous studies on host–toxin interactions in the wheat–*S. nodorum* pathosystem, namely *Snn1*–SnTox1 (Liu et al. 2004b), *Tsn1*–SnToxA (Friesen et al. 2006; Liu et al. 2006), *Snn2*–SnTox2 (Friesen et al. 2007) and *Snn3*–SnTox3 (Friesen et al. 2008b), have demonstrated that compatible host–toxin interactions play key roles in the development of SNB. The current work describes the identification and characterization of a fifth HST produced by *S. nodorum* and demonstrates that a compatible *Snn4*–SnTox4 interaction is a significant factor in the development of disease. This work further broadens the understanding and characterization of the wheat–*S. nodorum* pathosystem. Like the previous four *S. nodorum* toxins that were partially characterized, SnTox4 is proteinaceous in nature. The estimated size of SnTox4 is between 10 and 30 kDa, which is similar to SnTox1 (Liu et al. 2004a) and SnTox3 (Friesen et al. 2008b). Our results indicate that a compatible *Snn4*–SnTox4 interaction is light dependent as are compatible *Tsn1*–ToxA, *Snn1*–SnTox1, and *Snn2*–SnTox2 interactions (Manning and Ciuffetti 2005; Friesen et al. 2007; T. L. Friesen et al., unpublished data).

In contrast to the other *S. nodorum* toxins, SnTox4 is unique in that it causes a mottled necrotic reaction as compared to the severe and extensive necrosis caused by the other four toxins. It is possible that the different toxins produced by *S. nodorum* may have different levels of affinity for their host receptors or recognition factors. In this case, it might be possible that toxins with high affinity for host recognition could result in a relatively severe reaction, i.e. severe necrosis, whereas toxins with moderate or low affinity for host recognition may lead to less severe symptoms such as light or mottled necrosis. The isolation of the *Snn4* gene and the gene encoding SnTox4 will allow studies to characterize the interaction and associated pathways at the molecular level, which would provide knowledge regarding the fundamental basis of different host–toxin interactions.

The AF population was evaluated for reaction to Stagonospora glume blotch under natural infestation in the field (Schnurbusch et al. 2003), but it has not previously been evaluated for reaction to Stagonospora nodorum leaf blotch in either the field or the greenhouse. In related research, Friesen et al. (2009) showed that the HSTs SnToxA and SnTox2 were important factors in conferring leaf blotch on adult plants under field conditions. Here, we chose to evaluate the AF population for reaction of seedlings to leaf blotch caused by a Swiss isolate to determine if host–toxin interactions were associated with leaf blotch susceptibility in the population and if the QTLs for glume blotch resistance reported by Schnurbusch et al. (2003) might coincide with toxin sensitivity loci or resistance QTLs associated with leaf blotch in seedlings. Our results demonstrate that host–toxin interactions are in fact associated with susceptibility to leaf blotch, but neither the *Snn4* locus nor the minor QTLs on chromosomes 2A and 3A coincided with the major QTLs for glume blotch on chromosome arms 3BS, 4BL, and 5BL. This would suggest that different loci and possibly different mechanisms are associated with leaf and glume blotch. However, our research was conducted using a single isolate, whereas the glume blotch QTLs were identified under natural infestation. It is possible that other isolates produce different toxins that could be associated with glume blotch susceptibility.

Arina was susceptible to SNB caused by isolate 1A7a with an average disease reaction type of 3.2 and Forno was moderately resistant with an average reaction type of 2.8. Although the difference in parental reaction types was only 0.4, extreme transgressive segregation was observed in the population, which had reaction types ranging from 1.0 to 4.0 indicating that different resistance/susceptibility genes were contributed by both parents. A significant portion of the population was more resistant to 1A7a than was Forno, which indicates that Arina contributed a significant degree of resistance (or Forno contributed susceptibility). However, only one resistance QTL (*QSnb.fcu-2A*) contributed by Arina was detected, and it explained only 5.4% of the phenotypic variation. It is possible that additional resistance QTLs contributed by Arina went undetected either due to their effects being too minor to detect, or the possibility that they lie within genomic regions not adequately covered by markers in this population. On the contrary, the resistance effects contributed by Forno are significantly accounted for by *QSnb.fcu-1A* and *QSnb.fcu-3A*, which explain 41.0 and 6.0% of the variation, respectively.

The *Snn4*–SnTox4 interaction, which was responsible for the effects of the QTL *QSnb.fcu-1A*, explained most of the variation between Arina and Forno. Because Arina is sensitive to SnTox4 and carries the dominant *Snn4* allele for sensitivity, we consider *QSnb.fcu-1A* to be a "susceptibility" QTL contributed by Arina. It is possible that the effects of the QTLs on chromosome arms 2AS and 3AS could also be the result of host–toxin interactions not yet identified. It is also possible that HSTs are not the only disease determinants in the *S. nodorum* system, and the minor resistance QTLs could be the result of other non-HST associated resistance mechanisms. Further work examining fractions of 1A7a culture filtrates that lack SnTox4 is needed to determine if additional host–toxin

interactions are involved in conferring disease in this population.

Preliminary studies on this population with other toxin producing *S. nodorum* isolates have indicated that this population also segregates for *Tsn1*, the gene that confers sensitivity to SnToxA, and that Forno harbors the dominant *Tsn1* allele for SnToxA sensitivity (unpublished data). Because the Swiss isolate 1A7a does not possess the *SnToxA* gene (data not shown), the *Tsn1*–SnToxA interaction is not relevant in conferring susceptibility to this isolate. However, it is possible that Arina and/or Forno contain other HST sensitivity genes besides *Snn4* and *Tsn1*, and they may be revealed when the population is screened for reaction to culture filtrates of other isolates that produce additional toxins.

The *Snn4* locus is located near the distal end of the short arm of wheat chromosome 1A. The placement of four EST-based markers onto the AF chromosome 1A linkage map demonstrates that *Snn4* is located within the wheat 1AS3-0.86-1.00 deletion bin. This bin is known to be one of the most gene-rich regions of the wheat genome (Sandhu et al. 2001; Peng et al. 2004; Qi et al. 2004) and it contains many important disease resistance genes (see Erayman et al. 2004 for review) including the tan spot chlorosis resistance QTL *QTsc.ndsu-1A* (Faris et al. 1997, 1999) and the *Tsc1* gene, which confers sensitivity to the tan spot chlorosis-inducing toxin Ptr ToxC (Effertz et al. 2002). Although, *Tsc1* and *Snn4* both confer sensitivity to toxins, they are not likely to be the same gene. We tested the wheat cultivar Opata 85, which carries the *Tsc1* gene and is sensitive to Ptr ToxC, with partially purified SnTox4 cultures and found it to be insensitive. Also, SnTox4 is likely a protein whereas Ptr ToxC is not. Therefore, the two toxins likely have different targets and disease is induced by separate mechanisms.

Previously identified toxin sensitivity genes *Tsn1*, *Snn1*, *Snn2* and *Snn3* have been mapped to wheat chromosomes 5BL, 1BS, 2DS and 5BS, respectively. *Snn1*, which confers sensitivity to SnTox1, is located near the distal end of the short arm of chromosome 1B (Liu et al. 2004a; Reddy et al. 2008). It is possible that *Snn1* and *Snn4* are homoeoallelic. However, there are no common markers between the maps generated by Liu et al. (2004a) or Reddy et al. (2008) and the map of chromosome 1A developed in the AF population. The addition of more EST-based markers to the AF map will allow for better comparisons to be made between the AF chromosome 1A map and the 1B map developed by Reddy et al. (2008), which will help determine if *Snn1* and *Snn4* are homoeoalleles and possibly derived from a common origin.

The wheat–*S. nodorum* pathosystem now consists of five genetically well characterized host–toxin interactions. All five interactions are similar in that single dominant genes in the host confer sensitivity to the toxins, and all five play prominent roles in disease development. However, differences among the interactions exist regarding light dependence, toxin size, and now with the characterization of the *Snn4*–SnTox4 interaction, the type of symptoms that develop. Therefore, there is likely diversity in the mechanisms exploited by *S. nodorum* to cause disease. It is not yet known how many different host–toxin interactions may be involved in the wheat–*S. nodorum* pathosystem, but it is the system with the most HST–host gene interactions characterized to date and may serve well as a model for other pathosystems involving necrotrophic fungal pathogens.

Acknowledgments This research was supported by USDA-ARS CRIS projects 5442-21000-033 and 5442-22000-037, and Swiss SNF grant 105620.

References

Ballance GM, Lamari L, Bernier CC (1989) Purification of a host-selective necrosis toxin from *Pyrenophora tritici-repentis*. Physiol Mol Plant Pathol 35:203–213

Barthe P, Pujade-Renaud V, Breton F, Gargani D, Thai R, Roumestand C, de Lamotte F (2007) Structural analysis of cassiicolin, a host-selective protein toxin from *Corynespora cassiicola*. J Mol Biol 367:89–101

Bostwick DE, Ohm HW, Shaner G (1993) Inheritance of Septoria nodorum blotch resistance in wheat. Crop Sci 33:439–443

Du CG, Nelson LR, McDaniel ME (1999) Diallel analysis of gene effects conditioning resistance to *Stagonospora nodorum* (Berk) in wheat. Crop Sci 39:686–690

Effertz RJ, Meinhardt SW, Anderson JA, Jordahl JG, Francl LJ (2002) Identification of chlorosis-inducing toxin from *Pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat. Phytopathology 92:527–533

Erayman M, Sandhu D, Sidhu D, Dilbirligi M, Baenziger PS, Gill KS (2004) Demarcating the gene-rich regions of the wheat genome. Nucleic Acids Res 32:3546–3565

Faris JD, Friesen TL (2009) Reevaluation of a tetraploid wheat population indicates that the *Tsn1*-ToxA interaction is the only factor governing Stagonospora nodorum blotch susceptibility. Phytopathology 99:906–912

Faris JD, Anderson JA, Francl LJ, Jordahl JG (1997) RFLP mapping of resistance to chlorosis induction by *Pyrenophora tritici-repentis* in wheat. Theor Appl Genet 94:98–103

Faris JD, Li WL, Liu DJ, Chen PD, Gill BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. Theor Appl Genet 98:219–225

Faris JD, Haen KM, Gill BS (2000) Saturation mapping of a gene-rich recombination hotspot region in wheat. Genetics 154:823–835

Flor HH (1956) The complementary genetic systems in flax and flax rust. Adv Genet 8:29–54

Fried PM, Meister E (1987) Inheritances of leaf and head resistance of winter wheat to *Septoria nodorum* in a diallel cross. Phytopathology 86:459–463

Friesen TL, Stukenbrock EH, Liu Z, Meinhardt S, Ling H, Faris JD, Rasmussen JB, Solomon PS, McDonald BA, Oliver RP (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. Nat Genet 38:935–956

Friesen TL, Meinhardt SW, Faris JD (2007) The *Stagonospora nodorum*-wheat pathosystem involves multiple proteinaceous

host-selective toxins and corresponding host sensitivity genes that interact in an inverse gene-for-gene manner. Plant J 51:681–692

Friesen TL, Faris JD, Solomon PS, Oliver RP (2008a) Host-specific toxins: effectors of necrotrophic pathogenicity. Cell Microbiol 10:1421–1428

Friesen TL, Zhang Z, Solomon PS, Oliver RP, Faris JD (2008b) Characterization of the interaction of a novel *Stagonospora nodorum* host-selective toxin with a wheat susceptibility gene. Plant Physiol 146:682–693

Friesen TL, Chu CG, Liu ZH, Xu SS, Halley S, Faris JD (2009) Host-selective toxins produced by *Stagonospora nodorum* confer disease susceptibility in adult plants under field conditions. Theor Appl Genet 118:1489–1497

Haen KM, Lu HJ, Friesen TL, Faris JD (2004) Genomic targeting and high-resolution mapping of the *Tsn1* gene in wheat. Crop Sci 44:951–962

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Liu ZH, Faris JD, Meinhardt SW, Ali S, Rasmussen JB, Friesen TL (2004a) Genetic and physical mapping of a gene conditioning sensitivity in wheat to a partially purified host-selective toxin produced by *Stagonospora nodorum*. Phytopathology 94:1056–1060

Liu ZH, Friesen TL, Rasmussen JB, Ali S, Meinhardt SW, Faris JD (2004b) Quantitative trait loci analysis and mapping of seedling resistance to Stagonospora nodorum leaf blotch in wheat. Phytopathology 94:1061–1067

Liu Z, Friesen TL, Ling H, Meinhardt SW, Oliver RP, Rasmussen JB, Faris JD (2006) The *Tsn1*-ToxA interaction in the wheat-*Stagonospora nodorum* pathosystem parallels that of the wheat-tan spot system. Genome 49:1265–1273

Lu HJ, Faris JD (2006) Macro- and microcolinearity between the genomic region of wheat chromosome 5B containing the *Tsn1* gene and the rice genome. Funct Integr Genomics 6:90–103

Lu HJ, Fellers JP, Friesen TL, Meinhardt SW, Faris JD (2006) Genomic analysis and marker development for the *Tsn1* locus in wheat using bin-mapped ESTs and flanking BAC contigs. Theor Appl Genet 112:1132–1142

Manly KK Jr, Cudmore HH, Meer JM (2001) MAP MANAGER QTX, cross platform software for genetic mapping. Mamm Genome 12:930–932

Manning VA, Ciuffetti LM (2005) Localization of Ptr ToxA produced by *Pyrenophora tritici-repentis* reveals protein import into wheat mesophyll cells. Plant Cell 17:3203–3212

McDonald BA, Linde C (2002) The population genetics of plant pathogens and breeding strategies for durable resistance. Euphytica 124:163–180

Paillard S, Schnurbusch T, Winzeler M, Messmer M, Sourdille P, Abderhalden O, Keller B, Schachermayr G (2003) An integrative genetic linkage map of winter wheat (*Triticum aestivum* L). Theor Appl Genet 107:1235–1242

Panaccione DG, Scott-Craig JS, Pocard JA, Walton JD (1992) A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. Proc Natl Acad Sci USA 89:6590–6594

Parada RY, Sakuno E, Mori N, Oka K, Egusa M, Kodama M, Otani H (2008) *Alternaria bassicae* produces a host-specific protein toxin from germinating spores on host leaves. Phytopathology 98:458–463

Peng JH, Zadeh H, Lazo GR, Gustafson JP, Chao S, Anderson OD, Qi LL, Echalier B, Gill BS, Dilbirligi M, Sandhu D, Gill KS, Greene RA, Sorrells ME, Akhunov ED, Dvorak J, Linkiewicz AM, Dubcovsky J, Hossain KG, Kalayacharla V, Kianian SF, Mohmoud AA, Miftahudin, Conley EJ, Anderson JA, Pathan MS, Nguyen HT, McGuire PE, Qualset CO, Lapitan NLV (2004) Chromosome bin map of expressed sequence tags in homoeologous group 1 of hexaploid wheat and homoeology with rice and *Arabidopsis*. Genetics 168:609–623

Qi LL, Echalier B, Chao S, Lazo GR, Anderson OD, Akhunov ED, Dvorak J, Linkiewicz AM, Ratnasiri A, Dubcovsky J, Bermudez-Kandianis CE, Greene RA, Kantety R, La Rota CM, Munkvold JD, Sorrells SF, Sorrells ME, Dilbirligi M, Sidhu D, Eryman M, Randhawa HS, Sandhu D, Bondareva SN, Gill KS, Mahmoud AA, Ma X-F, Miftahudin, Gustafson JP, Conley EJ, Nduati V, Gonzalez-Hernandez JL, Anderson JA, Peng JH, Lapitan NLV, Hossain KG, Kalavacharl V, Kianian SF, Pathan MS, Zhang DS, Nguyen HT, Choi D-W, Close TJ, McGuire PE, Qualset CO, Gill BS (2004) A chromosome bin map of 16, 000 EST loci and distribution of genes among the three genomes of polyploid wheat. Genetics 168:701–712

Reddy L, Friesen TL, Meinhardt SW, Chao S, Faris JD (2008) Genomic analysis of the *Snn1* locus on wheat chromosome arm 1BS and the identification of candidate genes. Plant Genome 1:55–66

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers Methods. Mol Biol 132:365–386

Sandhu D, Champoux JA, Bondareva SN, Gill KS (2001) Identification and physical localization of useful genes and markers to major gene-rich regions on wheat group 1S chromosomes. Genetics 157:1735–1747

Sarpeleh A, Wallwork H, Catcheside DEA, Tate ME, Able AJ (2007) Proteinaceous metabolites from *Pyrenophora teres* contribute to symptom development of barley net blotch. Phytopathology 97:907–915

Schnurbusch T, Paillard S, Fossati D, Messmer M, Schachermayr G, Winzeler M, Keller B (2003) Detection of QTLs for Stagonospora glume blotch resistance in Swiss winter wheat. Theor Appl Genet 107:1226–1234

Schnurbusch T, Bossolini E, Messmer M, Keller B (2004) Tagging and validation of a major quantitative trait locus for leaf rust resistance and leaf tip necrosis in winter wheat cultivar Forno. Phytopathology 94:1036–1041

Strelkov SE, Lamari L, Balance GM (1998) Induced chlorophyll degradation by a chlorosis toxin from *Pyrenophora tritici-repentis*. Can J Plant Pathol 20:428–435

Tomas A, Feng GH, Reeck GR, Bockus WW, Leach JE (1990) Purification of a cultivar-specific toxin from *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. Mol Plant Microbe Interact 3:221–224

Tommasini L, Schnurbusch T, Fossati D, Mascher F, Keller B (2007) Association mapping of Stagonospora nodorum blotch resistance in modern European winter wheat varieties. Theor Appl Genet 115:697–708

Tuori RP, Wolpert TJ, Ciuffetti LM (1995) Purification and immunological characterization of toxic components from cultures of *Pyrenophora tritici-repentis*. Mol Plant Microbe Interact 8:41–48

Wolpert TJ, Dunkle LD, Ciuffetti LM (2002) Host-selective toxins and avirulence determinants: what's in a name? Annu Rev Phytopathol 40:251–285

Xu SS, Friesen TL, Cai X (2004) Sources and genetic control of resistance to Stagonospora nodorum blotch in wheat. Recent Res Dev Genet Breed 1:449–469