Genome characterization of *Pyrenophora tritici-repentis* isolates reveals high plasticity and independent chromosomal location of *ToxA* and *ToxB*

R. ABOUKHADDOUR¹, S. CLOUTIER², G. M. BALLANCE¹ AND L. LAMARI^{1,*}

¹Department of Plant Science, 66 Dafoe Rd., University of Manitoba, Winnipeg, MB, Canada, R3T 2N2

²Cereal Research Centre, Agriculture and Agri-Food Canada, 195 Dafoe Rd., Winnipeg, MB, Canada, R3T 2M9

SUMMARY

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker.], the causal agent of tan spot of wheat, is found in all major wheat-growing areas worldwide, where it infects wheat and numerous grass species (Krupinsky, 1982; Shoemaker, 1961). *Pyrenophora tritici-repentis* was first isolated from grasses in the 1850s and from wheat in the 1930s (Conners, 1937; Mitra, 1934). In North America, the fungal disease became a serious problem in wheat in the early 1970s, possibly as a consequence of changes in agronomic practices towards minimum and zero tillage (Bockus and Classen, 1992; Hosford, 1982; Rees and Platz, 1992; Sutton and Vyn, 1990). So far, eight races of this pathogen have been characterized, and each race has been shown to produce up to three host-selective toxins (Lamari *et al.*, 2003). These toxins, Ptr ToxA, Ptr ToxB and Ptr ToxC, are considered to be pathogenicity factors; Ptr ToxA induces necrosis, whereas Ptr ToxB and Ptr ToxC induce chlorosis, on susceptible hosts (Lamari *et al.*, 2003). On the host side, single dominant and independently inherited genes control the sensitivity to the toxins, one susceptibility gene for each toxin (Anderson *et al.*, 1999; Effertz *et al.*, 2002; Faris *et al.*, 1996; Friesen and Faris, 2004; Gamba *et al.*, 1998). The life cycle of the pathogen includes both sexual and asexual reproduction. Ascospores and conidia are disseminated by wind over limited distances (Schilder and Bergstrom, 1992). Strelkov and Lamari (2003) summarized the distribution of the different races as follows: Canada (races 1, 2, 3, 4), North Africa (races 1, 2, 5, 6), the Caucasus region (races 1, 2, 5, 7, 8) and the Fertile Crescent (races 1, 2, 3, 5, 7, 8). Recently, it has been proposed that the *ToxA* gene of *P. tritici-repentis* was acquired by horizontal transmission from *Stagonospora nodorum*, and it has been demonstrated that a region of 11 kb flanking the *Ptr ToxA* gene is essentially collinear with that in *St. nodorum* (Friesen *et al.*, 2006). In *P. tritici-repentis*, *ToxA* has been shown to be located on a 3-Mb chromosome in four isolates (Lichter *et al.*, 2002).

INTRODUCTION

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker.], the causal agent of tan spot of wheat, is found in all major wheat-growing areas worldwide, where it infects wheat and numerous grass species (Krupinsky, 1982; Shoemaker, 1961). *Pyrenophora tritici-repentis* was first isolated from grasses in the 1850s and from wheat in the 1930s (Conners, 1937; Mitra, 1934). In North America, the fungal disease became a serious problem in wheat in the early 1970s, possibly as a consequence of changes in agronomic practices towards minimum and zero tillage (Bockus and Classen, 1992; Hosford, 1982; Rees and Platz, 1992; Sutton and Vyn, 1990).

So far, eight races of this pathogen have been characterized, and each race has been shown to produce up to three host-selective toxins (Lamari *et al.*, 2003). These toxins, Ptr ToxA, Ptr ToxB and Ptr ToxC, are considered to be pathogenicity factors; Ptr ToxA induces necrosis, whereas Ptr ToxB and Ptr ToxC induce chlorosis, on susceptible hosts (Lamari *et al.*, 2003). On the host side, single dominant and independently inherited genes control the sensitivity to the toxins, one susceptibility gene for each toxin (Anderson *et al.*, 1999; Effertz *et al.*, 2002; Faris *et al.*, 1996; Friesen and Faris, 2004; Gamba *et al.*, 1998).

The life cycle of the pathogen includes both sexual and asexual reproduction. Ascospores and conidia are disseminated by wind over limited distances (Schilder and Bergstrom, 1992). Strelkov and Lamari (2003) summarized the distribution of the different races as follows: Canada (races 1, 2, 3, 4), North Africa (races 1, 2, 5, 6), the Caucasus region (races 1, 2, 5, 7, 8) and the Fertile Crescent (races 1, 2, 3, 5, 7, 8). Recently, it has been proposed that the *ToxA* gene of *P. tritici-repentis* was acquired by horizontal transmission from *Stagonospora nodorum*, and it has been demonstrated that a region of 11 kb flanking the *Ptr ToxA* gene is essentially collinear with that in *St. nodorum* (Friesen *et al.*, 2006). In *P. tritici-repentis*, *ToxA* has been shown to be located on a 3-Mb chromosome in four isolates (Lichter *et al.*, 2002).

*Correspondence: Tel.: 204-474-6071; Fax: +1 204 474 7528;
E-mail: L_Lamari@umanitoba.ca

The evolution of virulence involves the generation of new genetic variation, followed by selection. Genetic variation arises by mutation, chromosomal rearrangement, recombination, and inter- and intra-species hybridization (Burnett, 2003). Chromosome size and number variations have been reported for several fungal species (Coghlan *et al.*, 2005; Fierro and Martin, 1999; Kistler and Miao, 1992; Suga *et al.*, 2002; Zolan, 1995). In some pathogens, this variation is known to have played an important role in the evolution of virulence (Covert, 1998; Kistler *et al.*, 1996; Miao *et al.*, 1991a,b; VanEtten *et al.*, 1989). In *P. tritici-repentis*, chromosomal length polymorphism has been reported in a study comparing *Ptr ToxA* pathogenic and non-pathogenic isolates (Lichter *et al.*, 2002), but the extent of such polymorphism across and within all races is unknown.

The aims of this study were to characterize the structural organization of the *P. tritici-repentis* genome and the chromosomal location of the *ToxA* and *ToxB* pathogenicity genes in a large number of isolates representing all known races.

RESULTS

Karyotype patterns

Forty-seven isolates were included in this study (Table 1). In total, 14 different gel images were analysed. Each gel comprised the *Hansenula wingei* and *Schizosaccharomyces pombe* markers and a variable number of different *P. tritici-repentis* isolates. Independent chromosome preparations of the same isolate showed that isolates maintained an identical karyotype independent of the protoplast preparation or the electrophoresis run, indicating that the polymorphism observed among the different isolates was not an artefact of the experimental procedure and was reproducible (Fig. 1). Although gel-to-gel resolution varied slightly on the basis of the *H. wingei* and *S. pombe* marker standards on the different gels, the chromosome sizes were highly consistent.

A total of 15 distinct chromosomal bands was scored across all 47 isolates. The sizes of these bands were estimated to be 1.30, 2.00, 2.10, 2.20, 2.40, 2.70, 2.77, 2.80, 2.90, 3.00, 3.13, 3.50, 4.00, 4.20 and 5.70 Mb. In the already sequenced isolate Pt-1C-BFP (*Pyrenophora tritici-repentis* Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>), nine of the 11 chromosomes were found to range in size from 2.0 to 3.6 Mb, and thus our estimation of the chromosome sizes was consistent with these values. Twelve of the 15 observed bands occurred within the 1.3–3.5-Mb range. Karyotype analysis was based on these 12 bands because of the better chromosome size resolution in this size range. The results are summarized in Fig. 2 and Table 2. They revealed that the 47 isolates studied represented at least 29 different karyotypes.

Table 1 Description of *Pyrenophora tritici-repentis* isolates including isolate name, race, host, origin and collection year.

Isolate name	Race	Host*	Origin†	Collection year
ASC1	1	BW	CAN	1986
SC14-2	1	DW	CAN	1999
SC24-2	1	DW	CAN	1999
SC25-2	1	DW	CAN	1999
SC7-2	1	DW	CAN	1999
SC14-1	1	DW	CAN	1999
SC30-2	1	DW	CAN	1999
SC25-3	1	DW	CAN	1999
I-31-3	1	DW	AZ	2001
I-31-4	1	DW	AZ	2001
I-35-28	1	DW	AZ	2001
I-35-35	1	DW	AZ	2001
I-34-10	1	DW	AZ	2001
I-33-4	1	BW	AZ	2001
86-124	2	BW	CAN	1986
SK102-1	2	DW	CAN	1999
SC19-2	2	DW	CAN	1999
SC27-3	2	DW	CAN	1999
SC5-1	2	DW	CAN	1999
SC29-1	3	DW	CAN	1999
90-2	4	BW	CAN	1990
I-17-1	5	DW	AZ	2001
I-17-2	5	DW	AZ	2001
I-17-8	5	DW	AZ	2001
I-36-1	5	DW	AZ	2001
I-36-2	5	DW	AZ	2001
I-73-4	5	DW	SYR	2001
ALG3-24	5	DW	ALG	1993
ALG3-X1	5	DW	ALG	1993
ALG4-X1	5	DW	ALG	1993
ALGH2	6	DW	ALG	1997
I-17-10	7	DW	AZ	2001
I-17-5	7	DW	AZ	2001
I-36-4	7	DW	AZ	2001
I-35-5	7	DW	AZ	2001
I-35-24	7	DW	AZ	2001
I-34-1	8	DW	AZ	2001
I-34-2	8	DW	AZ	2001
I-73-1	8	DW	SYR	2001
I-31-1	8	DW	AZ	2001
I-17-11	8	DW	AZ	2001
I-35-16	8	DW	AZ	2001
I-35-13	8	DW	AZ	2001
I-35-14	8	DW	AZ	2001
I-35-17	8	DW	AZ	2001
I-35-18	8	DW	AZ	2001
I-35-19	8	DW	AZ	2001

*BW, bread wheat/*Triticum aestivum*; DW, durum wheat/*Triticum durum*.

†ALG, Algeria; AZ, Azerbaijan; CAN, Canada; SYR, Syria.

Fig. 1 Ten independent chromosomal preparations of the same isolates, using two different single-spore-derived cultures and separated on different gels, are displayed side by side, demonstrating the consistency of the karyotyping methods. (A) I-17-2; (B) 17-10; (C) I-17-11; (D) I-35-5; (E) I-35-14; (F) I-35-35; (G) I-73-1; (H) ALG4-X1; (I) ALGH2; (J) ASC1. Black arrows on the side of each panel point to the 5.7-Mb chromosome.

Fig. 2 Diagram representing the 29 karyotypes of the 47 *Pyrenophora tritici-repentis* isolates analysed. Karyotypes 1–29 are indicated on top, races 1–8 are marked below. Molecular sizes (Mb) are on the left. Co-migrating chromosomes are drawn as thicker bands. Chromosomal bands hybridizing to *ToxA* and *ToxB* are denoted by A and B, respectively, above the chromosomal band. *K8 karyotype occurs for race 1 and 2 isolates, but is only shown among race 1. ***ToxA* and *ToxB* appear to locate on the same band in K27.

Table 2 Distribution of *Pyrenophora tritici-repentis* isolates among the different karyotypes in relation to race, haploid number and genome size.

Karyotype	Race	Isolate	Excluding chromosomes > 5.7		Including chromosomes > 5.7	
			Chromosome number	Genome size	Chromosome number	Genome size*
K1	1	ASC1	9	32.8	9	32.8
K1	1	SC24-2	9	32.8	9	32.8
K1	1	SC25-2	9	32.8	9	32.8
K2	1	SC7-2	9	30.5	9	30.5
K2	1	SC14-1	9	30.5	9	30.5
K2	1	SC30-2	9	30.5	9	30.5
K3	1	SC25-3	9	29.3	9	29.3
K4	1	SC14-2	9	29.3	9	29.3
K5	1	I-31-3	9	31.6	10	37.3+
K5	1	I-31-4	9	31.6	10	37.3+
K6	1	I-35-35	9	33.7	10	39.4+
K7	1	I-35-28	8	33.7	9	39.4+
K8	1	I-33-4	8	28.5	10	39.9+
K8	2	86-124	8	28.5	10	39.9+
K9	1	I-34-10	11	39.1	11	39.1
K10	2	SK102-1	8	26.7	8	26.7
K11	2	SC19-2	8	25.5	8	25.5
K12	2	SC27-3	8	25.5	8	25.5
K13	2	SC5-1	10	35.1	10	35.1
K14	3	SC29-1	11	41.9	11	41.9
K15	4	90-2	9	26.0	9	26.0
K16	5	I-17-1	9	30.9	10	36.6+
K16	5	I-17-2	9	30.9	10	36.6+
K16	5	I-17-8	9	30.9	10	36.6+
K16	5	I-36-1	9	30.9	10	36.6+
K16	5	I-36-2	9	30.9	10	36.6+
K17	5	I-73-4	10	34.9	10	34.9
K18	5	ALG3-24	9	29.2	10	34.9
K19	5	ALG3-X1	11	39.5	11	39.5
K20	5	ALG4-X1	11	37.5	11	37.5
K21	6	ALGH2	9	28.3	9	28.3
K22	7	I-17-10	9	29.3	9	29.3
K23	7	I-17-5	8	26.5	9	26.5
K24	7	I-36-4	9	28.9	9	28.9
K25	7	I-35-5	10	32.1	10	32.1
K26	7	I-35-24	9	29.4	9	29.4
K27	8	I-34-1	9	36.6	11	48.0+
K27	8	I-34-2	9	36.6	11	48.0+
K27	8	I-73-1	9	36.6	9	36.6
K28	8	I-31-1	9	29.7	9	29.7
K28	8	I-17-11	9	29.7	9	29.7
K29	8	I-35-13	9	29.1	9	29.1
K29	8	I-35-14	9	29.1	9	29.1
K29	8	I-35-16	9	29.1	9	29.1
K29	8	I-35-17	9	29.1	10	34.8+
K29	8	I-35-18	9	29.1	9	29.1
K29	8	I-35-19	9	29.1	9	29.1

*Genome size followed by '+' means that the genome size is larger than that value.

Twenty isolates had unique karyotypes, six isolates shared the same karyotype (K29) and the remaining 21 isolates were represented by eight other karyotypes. Karyotype variations were as pronounced within as across races. The 14 race 1 isolates alone clustered into nine different karyotypes. However, with the exception of karyotype K8, which included isolates from both races 1 and 2, all of the other karyotypes represented isolates from a single race. The smallest chromosomal band of 1.3 Mb was present only in ALG4-X1 (race 5, K20) (Fig. 2). Isolates collected from the same field displayed as many as five different karyotypes (isolates from K6, K7, K25, K26 and K29). Overall, the geographical origins of isolates were unrelated to chromosomal polymorphism. The 15 North American isolates were grouped into 11 different karyotypes (isolate/karyotype = 1.36), whereas the 32 isolates from the Old World (Azerbaijan, Syria and Algeria) were grouped into 19 different karyotypes (isolate/karyotype = 1.68).

Chromosome number

The chromosome number was estimated to vary among isolates in the range 8–11 (Table 2). This number was slightly underestimated when chromosomes larger than 5.7 Mb were not included (Table 2). However, these estimates did take into consideration the co-migration of chromosomes of similar size. Indeed, several bands displayed a higher intensity than their neighbouring bands, indicative of two or more co-migrating bands. Densitometric scans were used to estimate the number of co-migrating chromosomes. Validation of the densitometric measurement was performed by resolution of co-migrating chromosomes using modified electrophoresis conditions with isolates SC29-1 and I-73-1. For example, the densitometric scan for the 3.5–5.7-Mb chromosomal region of isolate I-73-1 estimated the presence of at least two and four co-migrating chromosomes in two densiton peaks (Fig. 3A,B). The modified electrophoresis conditions improved the resolution of this size range and revealed the presence of six distinct bands (Fig. 3C), in agreement with the densitometric measurement.

Genome size

An estimate of the genome size was obtained by adding the sizes of all individual chromosomal bands in each isolate. A total of 16 isolates had one or two chromosomes with a size over 5.7 Mb (Table 2). However, because no commercial marker with a chromosome larger than 5.7 Mb was available, the estimation of the size of genomes with these larger chromosomes was less accurate. A value of 5.7+ was given to each chromosome larger than 5.7 Mb and, in these cases, the genome size was probably underestimated (Table 2). The estimated genome size of the isolates ranged from 25.5 to 48.0 Mb. The genome size of the sequenced isolate Pt-1C-BFP from race 1, which has 11 chromosomes, is approximately 38 Mb (*Pyrenophora tritici-repentis*

Fig. 3 Densitometric measurements of isolate I-73-1 chromosomal bands separated by pulsed field gel electrophoresis (PFGE) using standard separation conditions as well as conditions to preferentially resolve larger chromosomes. (A) Separation under standard conditions and densitometric analysis of the separated chromosomes show higher density bands in the 3.5–5.7-Mb range, indicative of unresolved co-migrating chromosomes. The numbers beside the peaks indicate the relative intensity of each band. The numbers beside the bands represent the estimated number of co-migrating chromosomes based on the densitometric values. (B) Enhanced view of isolate I-73-1 resolved side-by-side with the molecular standard *Schizosaccharomyces pombe*. The separation conditions were the same as in (A). (C) Improved resolution of isolate I-73-1 large chromosomes using modified electrophoresis conditions (2 V; 1200–1800 s; 72 h) clearly shows the presence of multiple chromosomes in the 3.5–5.7-Mb range. Resolved bands are indicated by arrows, and brackets indicate the corresponding 3.5–5.7-Mb range. Corresponding fragments between (B) and (C) are marked by joined lines.

Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>). The genome size was found to be correlated with the chromosome number ($r^2 = +0.8$).

ToxA and ToxB chromosomal location

Southern blot analysis was carried out to determine the chromosomal location of *ToxA* and *ToxB* genes among the different races of *P. tritici-repentis*. No hybridization signal was observed with *ToxA* for isolates from races 3, 4, 5 and 6 which do not possess *ToxA*, and a single hybridization signal was observed with blots of isolates from races 1, 2, 7 and 8. An illustration of the results with isolates from races 7 and 8 is provided in Fig. 4, with the overall summary for all isolates in Fig. 2.

The *ToxA*-carrying chromosome varied in size as much across all Ptr *ToxA*-producing isolates as among isolates within a given race (Fig. 2). The *ToxA* probe hybridized to chromosomal bands of five different molecular sizes (2.8, 2.9, 3, 3.5 and 5.7 Mb), of which four size polymorphisms were found among race 1 isolates alone (Fig. 2). In total, 36 isolates carried the *ToxA* gene, and 21 isolates had this gene on a 2.9-Mb chromosomal band.

The size of chromosomes on which *ToxB* occurred was equally polymorphic. The *ToxB* probe hybridized to chromosomal bands with molecular sizes of 2.2, 2.4, 2.7, 2.8, 3.5 and 5.7 Mb in isolates from races 3, 5, 6, 7 and 8 (Fig. 2). In each case, a single

chromosome band signal was observed, except for the race 5 isolate, ALG4-X1, represented by karyotype K20, in which signals were observed on two separate bands. No *ToxB* hybridization signal was observed with isolates of races 1 and 2. The chromosomal location of *ToxB* in isolate 90-2 from race 4 was not determined because of the low intensity of the chromosomal bands in this isolate. The presence of a *ToxB* homologous sequence has been detected previously in isolate 90-2 using restricted genomic DNA of this isolate (Strelkov *et al.*, 2002).

In isolates of races 7 and 8, in which *ToxA* and *ToxB* genes co-exist, probes for the respective genes hybridized to different chromosomal bands for all the isolates. In three isolates (I-34-1, I-34-2 and I-73-1) from race 8, *ToxA* and *ToxB* genes appeared to co-locate on a large 5.7-Mb chromosomal band. However, further analysis of the isolate I-73-1 under different separation conditions, to improve the resolution of the larger chromosomes, demonstrated the independent chromosomal location of the two genes. *ToxA* was located on a 5.7-Mb chromosome, whereas *ToxB* was located on a 4.6-Mb chromosome (Fig. 5). Where multiple isolates shared a common karyotype, the specific band location of *ToxA* or *ToxB* was consistent for all isolates with that karyotype. In several cases, the *ToxA* or *ToxB* probe hybridized to a chromosomal band that appeared to be composed of co-migrating chromosomes (Fig. 2) and, in these cases, the specific band location of a gene between co-migrating chromosomes could not be made.

Fig. 4 Electrophoretic karyotype of seven isolates of *Pyrenophora tritici-repentis* and identification of the chromosomes that carry *ToxA* and *ToxB*. (A) Pulsed field gel electrophoresis (PFGE) was performed using the conditions described previously (Zhong *et al.*, 2002). The isolates are labelled on top of the lanes and the karyotypes are in parentheses. The molecular standards *Hansenula wingei* and *Schizosaccharomyces pombe* are shown in the first and last lane, respectively. Molecular sizes (Mb) are indicated on the left. Hybridization with *ToxA* (B) and *ToxB* (C) shows that the two genes are located on different chromosomes.

Fig. 5 Electrophoretic karyotype and Southern blot analysis of *Pyrenophora tritici-repentis* isolate I-73-1 under different separation conditions. (A) Contour-clamped homogeneous electric field (CHEF) gel (left) and Southern blot with *ToxA* (middle) and *ToxB* (right) of isolate I-73-1 (lane 1) and *Schizosaccharomyces pombe* (lane 2). Separation conditions: 2 V, 1200–960 s, 24 h; followed by 2.5 V, 960–480 s, 96 h. (B) CHEF gel (left) and Southern blot with *ToxA* (middle) and *ToxB* (right) of isolate I-73-1 (lane 1) and *S. pombe* (lane 2). Separation conditions: 2 V, 1200–1800 s, 72 h.

Occurrence of *ToxA*-carrying chromosome among Ptr *ToxA*-producing and *ToxA*-non-producing isolates of *P. tritici-repentis*

To obtain a better characterization of the chromosome on which *ToxA* resides, Southern blot analysis with six different chromosome-specific single-copy probes (P1, P2, P3, P4, P5 and P6) was carried out with selected Ptr *ToxA*-producing (ASC1, I-35-35,

I-35-28, I-35-5 and I-73-1) and *ToxA*-non-producing (SC29-1 and ALG4-X1) isolates. The probes were selected to be unique and to be distributed along the chromosome (Fig. 6) known to carry *ToxA* in the sequenced isolate (*Pyrenophora tritici-repentis* Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>). In the selected Ptr *ToxA*-producing isolates, *ToxA* was located on chromosomal bands of different sizes from 2.9 to 5.7 Mb (Fig. 2), whereas isolates SC29-1 and ALG4-X1 lacked the *ToxA* gene (no signal).

Hybridization with the P1 chromosome-specific probe showed a single chromosome signal with each tested isolate (Fig. 7). The remaining chromosome-specific probes showed the same pattern, and all six probes co-located on the chromosomal band that carried the *ToxA* gene with two exceptions. First, in isolate I-73-1, in which *ToxA* was localized on a 5.7-Mb chromosome, these probes were all located to a 3.5-Mb chromosome. Therefore, in this isolate, *ToxA* appeared to have been translocated from the 3.5- to the 5.7-Mb chromosome (Fig. 7G). Second, in isolates SC29-1 and ALG4-X1, Ptr *ToxA*-non-producing isolates, these probes were located on chromosomes of 3.5 and 2.9 Mb, respectively (Fig. 7E,F).

DISCUSSION

Chromosomal polymorphism

To investigate the chromosomal variability in the tan spot fungus, the karyotypes of 47 *P. tritici-repentis* isolates were analysed. These isolates represented all eight races and came from a range

Fig. 6 Diagram representing the location of *ToxA* and the chromosome-specific single-copy probes (P1–P6), and their physical distance on chromosome 6 of isolate Pt-1C-BFP (*Pyrenophora tritici-repentis* Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>). The numbers above the arrows indicate the distance from *ToxA* in base pairs.

Fig. 7 Occurrence of the chromosome-specific probe (P1) in *Pyrenophora tritici-repentis* *ToxA*-producing and *ToxA*-non-producing isolates. For each pair of images from A to F, the left lane represents the contour-clamped homogeneous electric field (CHEF) gel and the right lane represents the Southern blot with probe P1 for ASC1, I-35-28, I-35-35, I-35-5, ALG4-X1 and SC29-1, respectively. (G) CHEF gel (left) and Southern blot with P1 (middle) and with *ToxA* probe (right) for isolate I-73-1. Standard separation conditions (2 V, 1200–960 s, 24 h; followed by 2.5 V, 960–480 s, 96 h) were used in all the above isolates, except for SC29-1 (2 V, 1200–1800 s, 27 h).

of geographical origins. The collection of isolates enabled the observation of extensive chromosomal polymorphism within and between races and among isolates from different parts of the world and from the same field.

Previous work on the karyotypes of *P. tritici-repentis* has shown major karyotype polymorphism between pathogenic and non-pathogenic isolates and, to a lesser extent, within each of the two groups (Lichter *et al.*, 2002). This observation led the authors to conclude that greater genomic rearrangements have occurred between, rather than within, the pathogenic or non-pathogenic isolates. However, the results of Lichter *et al.* (2002) were based on a limited number of isolates and a smaller representation of races. Therefore, their results may have superimposed the karyotype variability on pathogenic ability. In this paper, there was no relationship between karyotype and pathogenicity or geographical origin. A similar conclusion, i.e. the

absence of a relationship between karyotype and pathotype, has been reported for other pathogenic fungi, namely *Septoria nodorum* (Cooley and Caten, 1991), *Magnaporthe grisea* (Talbot *et al.*, 1993) and *Alternaria alternata* (Zolan, 1995). Our results with *P. tritici-repentis* corroborate these earlier findings. The genetic structure of a large collection of *P. tritici-repentis* isolates analysed using amplified fragment length polymorphism, coupled with sequence data from the internal transcribed spacer region, has revealed no genetic grouping according to race or geographical origin (Friesen *et al.*, 2005). The fact that *P. tritici-repentis* karyotypes could not be grouped on the basis of their pathogenicity was anticipated, because the ability of this pathogen to establish a compatible interaction with its host is controlled by a small number of genes, namely the toxin genes *ToxA*, *ToxB* and putative *ToxC*. These small and discrete genetic elements are functional in nature and are likely to maintain their

functions even when large-scale structural changes occur, such as translocations or other chromosomal rearrangements. Indeed, in isolate I-73-1, a translocation of *ToxA* to another chromosome is strongly suggested without affecting *ToxA*-related pathogenicity.

The karyotypes of *P. tritici-repentis* displayed two types of polymorphism, namely chromosome length and haploid chromosome number. Both types of polymorphism have been found among and within the different races. This fungal genome appears to have an extreme plasticity and can tolerate large-scale structural changes. Furthermore, considerable variation in karyotype was displayed among isolates collected from the same field, further indicative of the great genetic diversity of this organism. Chromosomal polymorphism has been reported in a number of fungal plant pathogenic species. Karyotypes of the rice blast fungus *M. grisea* were as variable within as between pathotype groups, although this variability was described mainly for chromosomal length (Talbot *et al.*, 1993). Fifteen spores of *Leptosphaeria maculans* collected from a single field revealed the presence of 10 different karyotypes (Plummer and Howlett, 1993). Similarly, substantial chromosome length polymorphism existed in a population of *Septoria tritici* collected from a 40 × 40-m section of a single wheat field (McDonald and Martinez, 1991). Structural changes that do not affect the expression of the essential fungal genes are likely to be tolerated (Zolan, 1995).

The haploid number of the *P. tritici-repentis* isolates studied varied from eight to 11 chromosomes, irrespective of the race to which they belonged or their geographical origin (Table 1). The *P. tritici-repentis* genome size ranged from 25.5 to 48.0 Mb, with an average of 33.5 Mb. This range is consistent with the recently sequenced isolate Pt-1C-BFP from race 1, which has 11 chromosomes and is approximately 38 Mb in size (*Pyrenophora tritici-repentis* Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>).

Polyploidization, aneuploidy and repetitive DNA can all contribute to genome size increases. In *P. tritici-repentis*, the directly proportional relationship ($r^2 = 0.8$) between chromosome number and genome size suggests aneuploidy as one driving force. Isolates I-34-1 and I-34-2, for example, have 11 chromosomes and are 48.0 Mb in size. This is almost twice the genome size of isolates SC19-2 and SC27-3, which have eight chromosomes and are 25.5 Mb in size (Table 2). Another potential source of variation in genome size is the proliferation of repetitive DNA. However, it is unlikely that such a large difference in genome size could be solely attributed to this mechanism, as fungal genomes have been reported to contain 3%–20% repetitive DNA (Daboussi and Capy, 2003). Aneuploidy has been described in a number of plant pathogenic fungi, including *M. grisea* (Talbot *et al.*, 1993; Valent and Chumley, 1991), *Ustilago maydis* (Kinscherf and Sally, 1988) and *Colletotrichum gleosporioides* (Masel *et al.*, 1990). Limited

evidence of aneuploidy in fungal species could simply reflect its difficult detection (Brasier, 1987).

Occurrence of *ToxA*-carrying chromosome among *Ptr ToxA*-producing and *ToxA*-non-producing isolates of *P. tritici-repentis*

To date, *ToxA* has been detected as a single-copy gene present only in isolates that produce *Ptr ToxA* (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). The sequenced genome of *P. tritici-repentis* isolate Pt-1C-BFP (race 1) confirmed the presence of a single *ToxA* copy on a 2.8-Mb chromosome (*Pyrenophora tritici-repentis* Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>). Transformation of a non-pathogenic isolate with the cloned *ToxA* gene has demonstrated that *ToxA* is a pathogenicity factor (Ciuffetti *et al.*, 1997). This single-copy pathogenicity factor, whilst providing an advantage to the fungus with regard to its pathogenicity, remains a non-essential gene.

The co-occurrence of *ToxA* and the single-copy probes P1, P2, P3, P4, P5 and P6 on the same chromosome in a number of *Ptr ToxA*-producing isolates (ASC1, I-35-28, I-35-35, I-35-5) means that the *ToxA*-encoding chromosomes in these isolates and, more generally, in most isolates are homologous. This conclusion is based on the high frequency of *ToxA* on a 2.9-Mb chromosome (33 of 47 isolates), and that isolates with *ToxA* on other sized chromosomes were sampled and the *ToxA*-carrying chromosomes were shown to be homologous in all except one case. In the above isolates, *ToxA*-carrying chromosomes varied in size from 2.9 to 3.5 Mb, revealing that chromosomal length differences of as much as 20% (up to 600 kb) occurred for these homologous chromosomes in *P. tritici-repentis*. Size differences in homologous chromosomes have been reported in other fungal species, such as *Septoria tritici* (McDonald and Martinez, 1991). Moreover, the hybridization of P1, P2, P3, P4, P5 and P6 to chromosomes of 3.5 and 2.9 Mb in SC29-1 and ALG4-X1, respectively, both *Ptr ToxA*-non-producing isolates, strongly suggests that the chromosome carrying *ToxA* is of an essential nature in the *P. tritici-repentis* genome. This conclusion is consistent with the prediction of Lichter *et al.* (2002) that the differences between the pathogenic and non-pathogenic isolates can be explained by the pathogenic capacity of the isolate and the toxin gene, rather than reflecting a simple molecular event (such as the involvement of a supernumerary chromosome).

Interestingly, in isolate I-73-1, in which *ToxA* was on a 5.7-Mb chromosome, P1, P2, P3, P4, P5 and P6 were located on a 3.5-Mb chromosome. These probes were designed to span the central 1.2 Mb of the 2.8-Mb chromosome in Pt-1C-BFP (Fig. 6), and their co-occurrence represents a significant portion of this specific chromosome. This indicates a translocation of the *ToxA* gene to the 5.7-Mb chromosome from the central portion of a

chromosome now sized at 3.5 Mb. The mechanism of this translocation remains unknown, but may involve ectopic recombination between repeated sequences on other chromosomes (Zolan, 1995). Alternatively, a transposon might be involved in the *ToxA* translocation. An 11-kb region flanking *ToxA* was found to harbour a transposase sequence of the hAT family (Friesen *et al.*, 2006). Transposons have been suggested to provide substrates for chromosomal rearrangements in fungi (Fierro and Martin, 1999).

Chromosomal location of *ToxB*

Hybridization of a *ToxB* probe to chromosomes of *P. tritici-repentis* revealed the location of this gene on chromosomes of 2.2–5.7 Mb in the different isolates. *ToxB* has been shown to be present in multiple copies in races 5, 6, 7 and 8 (Martinez *et al.*, 2004; Strelkov *et al.*, 2003). The chromosomal location of *ToxB* in one isolate has been investigated previously (Martinez *et al.*, 2004); the authors found that *ToxB* copies were located on two chromosomes, of 2.7 and 3.5 Mb, with the majority of copies located on the 2.7-Mb chromosome. In the current study, the *ToxB* probe hybridized to 27 different isolates. In 10 of these isolates, a single hybridization signal was detected on a chromosomal band estimated to be composed of one chromosome. In 16 of these isolates, the *ToxB* probe hybridized to a chromosomal band estimated to be composed of co-migrating chromosomes. In ALG4-X1, a race 5 isolate, *ToxB* showed two clear, albeit of different intensity, hybridization signals on 2.8- and 3.5-Mb chromosomal bands. The intensity of the hybridization signals suggested that a larger number of copies were located on the 2.8-Mb chromosomal band, as observed previously (Martinez *et al.*, 2004). A clustered or dispersed organization of the *ToxB* copies on these chromosomes was not revealed in this study, because hybridizations were carried out on intact chromosomes.

A recent study has revealed the presence of the *ToxB* sequence and its homologous sequences in *P. bromi*, the closest relative to *P. tritici-repentis*, and in a broad range of plant pathogenic ascomycetes, suggesting that *ToxB* was transmitted vertically through an early ascomycete ancestor (Andrie *et al.*, 2007). In *P. bromi*, the peptide sequence of Pb ToxB is 88 amino acids in length, compared with 87 for Ptr ToxB, corresponding to the insertion of one codon. The vertical transmission of this pathogenicity factor is consistent with the hypothesis of Strelkov and Lamari (2003) that *P. tritici-repentis*, with its various toxins, evolved on grass species and was present for millions of years on all continents before moving to its wheat host.

This is the first large-scale karyotype study of *P. tritici-repentis* including isolates from all eight standard races and from diverse geographical origins. The *P. tritici-repentis* genome shows extensive chromosomal polymorphism indicative of its plasticity. Karyotypes are as diverse within as across races. Chromosomal polymorphism of toxin-carrying chromosomes has been observed

for both chromosome size and number (one or two). *ToxA* and *ToxB* have been shown to be carried on different chromosomes. Moreover, the chromosome on which *ToxA* normally localizes is homologous among isolates and appears to be of an essential nature. Finally, a translocation of *ToxA* to another chromosome has been observed in one isolate.

EXPERIMENTAL PROCEDURES

Terminology

In this report, the concept of virulence is adapted from Green (1975) and is meant to describe the condition by which a race of a pathogen is able to establish a compatible interaction with a specific host, and implies race/cultivar specificity.

Fungal isolates

Forty-seven isolates of *P. tritici-repentis* were included in this study. These were selected from globally distributed pathogen populations representing North America, the Fertile Crescent, the Caucasus region and North Africa (Lamari *et al.*, 2005). The collection year, host origin, geographical origin and race for each isolate are listed in Table 1.

Inoculum preparation and inoculation

Inoculum was produced from monoconidial isolates of *P. tritici-repentis* on V8-PDA, as described previously (Lamari and Bernier, 1989). Inoculation of the differential set was carried out to confirm the race identity of the isolates, as described previously (Lamari and Bernier, 1989). The three wheat accessions of the differential set each contain a single gene conferring sensitivity to a single Ptr toxin: 'Glenlea' (Ptr ToxA), 6B662 (Ptr ToxB) and 6B365 (Ptr ToxC) (Lamari *et al.*, 2003).

Protoplast production and whole chromosome preparation

Protoplasts of *P. tritici-repentis* were prepared using a protocol adapted and modified from Ciuffetti *et al.* (1997). Conidia were harvested from 7-day-old cultures and inoculated into 1-L glass flasks containing 500 mL of 0.25 × Difco potato dextrose broth to a final concentration of 5×10^5 conidia per millilitre. The flasks were placed on a rotary shaker (100 r.p.m.) and incubated for 18 h at room temperature (20–22 °C). The germinated conidia were filtered onto one layer of miracloth tissue and washed with 500 mL of sterile distilled water. The germinated spores (3 g fresh weight) were spread gently in 5-cm plastic Petri plates, and 8 mL of filter-sterilized osmotic magnesium (OM) buffer was added. The OM buffer (1.2 M MgSO₄, 10 mM potassium phosphate at

pH 5.8) contained 140 mg of lysing enzyme L1412 (Sigma, St. Louis, MO, USA), 40 mg of driselase D9515 (Sigma), 1500 U of β -glucuronidase type H1-G0751 (Sigma) and 50 mg of yatalase (Takara Mirus Bio, Madison, WI, USA). The germinated spores were then incubated at 33 °C for 3 h with gentle agitation (50 r.p.m.) in the dark to release the protoplasts. The suspension was filtered through double Nitex filters of 80 and 30 μ m, washed twice with approximately 35 mL of 1 M sorbitol (Sigma) and centrifuged at approximately 1900 *g* for 20 min at 8 °C in a CS-6R centrifuge (Beckman, Fullerton, CA, USA). The pellet was collected and resuspended in 2 mL of 1 M sorbitol, and centrifuged in a microfuge for 10 min at 3000 *g* and 8 °C. The final pellet was resuspended in 40 μ L of 1 M sorbitol. The protoplast concentration was evaluated using a haemocytometer. The volume was adjusted to obtain a concentration of 1.5×10^8 – 2×10^8 protoplasts/mL. An equal volume of 1.2% low-melting-point agarose (Bio-Rad, Hercules, CA, USA) was added to a final concentration of 0.75×10^8 – 1×10^8 protoplasts/mL. In general, one or two 75–80- μ L plugs were obtained from each protoplast preparation.

Solidified plugs were incubated in washing buffer [0.5 M ethylenediaminetetraacetic acid (EDTA), pH 9, 1% sarkosyl, 1 mg/mL proteinase K; Invitrogen, Burlington, ON, Canada] at 50 °C for 48 h with two buffer changes. Plugs were further washed with 0.5 M EDTA, pH 9 for 3 h at 50 °C and stored in 0.05 M EDTA, pH 9 at 4 °C until needed. Prior to loading, the plugs were equilibrated in 1 $\times$ TAE buffer (40 mM Tris-acetate, 1 mM EDTA) for 1 h on ice. Whole plugs were loaded on to the electrophoresis gel to ensure sufficient DNA for visualization and Southern transfer.

Pulsed field gel electrophoresis (PFGE)

Chromosome separation was performed by PFGE on a contour-clamped homogeneous electric field (CHEF) DR II system (Bio-Rad) using 0.8% agarose MP gel (Roche Diagnostics, Laval, QC, Canada) in 1 $\times$ TAE buffer cooled to 14 °C. Standard electrophoresis conditions involved a 1200–960 s switch time for 24 h at 2 V/cm, followed by 960–480 s for 96 h at 2.5 V/cm at a constant 14 °C (Zhong *et al.*, 2002). These conditions had been optimized to provide good resolution in the 2–3.5-Mb size range (Zhong *et al.*, 2002). The gels were stained with SYBR® Gold (Invitrogen) for 30 min and photographed under UV light (365 nm). Conditions that would preferentially resolve the larger chromosomes (2 V/cm; 1200–1800 s; 72 h at 14 °C) were also used to test the validity of the densitometric estimation of the number of co-migrating larger chromosomes when separated under standard conditions.

Chromosome size estimation

In this context, a chromosome or chromosomal band refers to a band visualized on the CHEF gel. In some cases, the band was composed of co-migrating chromosomes.

The chromosome sizes of *P. tritici-repentis* were estimated on the basis of their migration relative to the chromosomal DNA of commercially prepared chromosomes of *S. pombe* and *H. wingei* (Bio-Rad), using the molecular weight point-to-point function of an Alpha Imager (Alpha Innotech, San Leandro, CA, USA).

Two independent chromosomal preparations were made for 10 different isolates, using two different single-spore-derived cultures of each isolate. The two independent chromosomal preparations for each isolate were resolved on different gels. The band sizes of the separated chromosomes of these isolates were determined independently for each gel and compared across gels. This allowed us to estimate that chromosomes smaller than 3.5 Mb could be sized with a precision of ± 50 kb. Therefore, chromosomes in this size range that differed by less than 50 kb were classified as being of the same size. The 50-kb error size did not apply in two instances: (i) when two chromosomes from a single isolate were clearly resolved as two bands; and (ii) when chromosomes from different isolates with size differences of less than 50 kb were separated side by side on the same gel and could be visually identified as being of different size. Using the same comparison principle as described above, the resolution of bands larger than 3.5 Mb was estimated to be less accurate under the PFGE conditions used in this study. Therefore, chromosomes in the 3.5–5.7-Mb range with size estimates that differed by less than 125 kb were considered to be of the same size. Any chromosomes larger than 5.7 Mb were assigned a size of 5.7+ Mb, and the estimation of the chromosome number and genome size was carried out with and without the chromosomes of 5.7+ Mb.

Karyotype patterns were defined by comparing the 12 chromosomal bands and size polymorphisms recorded in the 1.3–3.5-Mb range. This range, although conservative, was selected because it provided the most accurate resolution.

Chromosome number estimation

Visual observation of the PFGE karyotype clearly showed that some bands were more intensely stained than others, indicating that they probably corresponded to multiple co-migrating chromosomes. To estimate the chromosome number of *P. tritici-repentis*, the densitometric function of the Alpha Imager was used for all distinct fragments of each isolate (Alpha Innotech). The intensity of the stain in each distinguishable chromosomal band was revealed as a peak, and the integrated area under each peak represented the band intensity. The relative intensity represented the contribution of each peak to the total intensity. Therefore, peaks with high relative intensity values implied the co-migration of more than one chromosome. In order to validate this estimate, different electrophoresis conditions (2 V; 1200–1800 s; 72 h) were used to better resolve the 3.5–5.7-Mb size range and to separate the co-migrating chromosomes of isolates I-73-1 and SC29-1.

Southern hybridization

The following probes were used in hybridization experiments: *ToxA*, *ToxB* and six *ToxA* chromosome-specific single-copy probes (P1–P6) amplified from the ASC1 (race 1) isolate. The *ToxA* probe was a 901-bp cDNA of Ptr *ToxA* from isolate 86-124 (race 2), and the *ToxB* probe was a 200-bp cDNA of Ptr *ToxB* from isolate ALG3-24 (race 5). Primers for P1, P2, P3, P4, P5 and P6 were selected from chromosome 6 of the *P. tritici-repentis* isolate Pt-1C-BFP (*Pyrenophora tritici-repentis* Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA, USA; <http://www.broad.mit.edu>). P1, P2 and P3 are 772 749, 724 146 and 430 185 bp, respectively, upstream of the *ToxA* gene, and P4, P5 and P6 are 219 805, 521 224 and 928 487 bp, respectively, downstream of the *ToxA* gene (Fig. 6). The sequences of the chromosome-specific probes (P1, P2, P3, P4, P5 and P6) used in this study are provided as Supporting Information.

Chromosomal DNA separated by PFGE was depurinated in 0.25 M HCl for 30 min, denatured in 0.5 M NaOH, 1.5 M NaCl for 30 min and neutralized in 1 M Tris-Cl (pH 7.5), 1 M NaCl for 25 min. Gels were vacuum-blotted onto Hybond N⁺ membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) with 20 × SSPE (1 × SSPE: 0.15 M NaCl, 10 mM NaH₂PO₄ and 1 mM EDTA, pH 7.7) using a VacuGene™XL instrument (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for 90 min at 5 kPa. The DNA was cross-linked by UV irradiation (245 nm).

Prehybridization was performed for 3 h in a solution containing 20% formamide, 6 × SSPE, 5 × Denhardt's, 0.5% sodium dodecylsulphate (SDS) and 20 µg/mL denatured salmon sperm DNA. Probes were ³²P-radiolabelled by random primer labelling. Hybridization was carried out overnight with 25 ng of ³²P-labelled probe at 50 °C. The membranes were washed at 65 °C for 10 min in 5 × standard saline citrate (SSC), 0.1% SDS, twice for 10 min in 2.5 × SSC, 0.1% SDS, and once for 10 min in 1 × SSC, 0.1% SDS. They were then exposed to X-ray film (Kodak, Rochester, NY, USA) with intensifying screens for 1 week at –70 °C. After autoradiography, the membranes were stripped of labelled probe by washing in 95 °C water containing 0.05% SDS, and then re-probed as needed. Routine molecular manipulations, not otherwise mentioned in this report, followed Sambrook *et al.* (1989).

ACKNOWLEDGEMENTS

The authors thank Mr Andrzej Walichnowski for a review of the manuscript. Grateful appreciation is extended to Richard Smith, Ralph Kowatsch, Andrzej Walichnowski and Elsa Reimer for technical help, to Bert Luit and Mike Shillinglaw for their support with the graphics and to Joanne Schiavoni for assistance with manuscript preparation. Financial assistance from the University of Manitoba Graduate Fellowship to Reem Aboukhaddour and

research support from the Natural Sciences and Engineering Research Council of Canada (NSERC) to L. Lamari are also acknowledged.

REFERENCES

- Anderson, J.A., Effertz, R.J., Faris, J.D., Franc, L.J., Meinhardt, S.W. and Gill, B.S. (1999) Genetic analysis of sensitivity to *Pyrenophora tritici-repentis* necrosis-inducing toxin in durum and common wheat. *Phytopathology*, **89**, 293–297.
- Andrie, R.M., Schoch, C.L., Hedges, R., Spatafora, J.W. and Ciuffetti, L.M. (2007) Homologs of *ToxB*, a host-selective toxin gene from *Pyrenophora tritici-repentis*, are present in the genome of sister-species *Pyrenophora bromi* and other members of the Ascomycota. *Fungal Genet. Biol.* **45**, 363–377.
- Ballance, G.M., Lamari, L., Kowatsch, R. and Bernier, C.C. (1996) Cloning expression and occurrence of the gene encoding the Ptr necrosis toxin from *Pyrenophora tritici-repentis*. *Mol. Plant Pathol.* On-line www.bspp.org.uk/mppol/1996/1209ballance.
- Bockus, W.W. and Classen, M.M. (1992) Effects of crop rotation and residue management practices on severity of tan spot of winter wheat. *Plant Dis.* **76**, 633–636.
- Brasier, C.M. (1987) The dynamics of fungi. In: *Evolutionary Biology of Fungi* (Rayner, A.D.M., Brasier, C.M. and Moore, D., eds), pp. 231–260. Cambridge: Cambridge University Press.
- Burnett, J. (2003) *Fungal Populations Species*. New York: Oxford University Press.
- Ciuffetti, L.M., Tuori, R.P. and Gaventa, J.M. (1997) A single gene encodes a selective toxin causal to the development of tan spot of wheat. *Plant Cell*, **9**, 135–144.
- Coghlan, A., Eichler, E.E., Oliver, S.G., Paterson, A.H. and Stein, L. (2005) Chromosome evolution in eukaryotes: a multi-kingdom perspective. *Trends Genet.* **21**, 673–682.
- Connors, L.L. (1937) *17th Annual Report. Canadian Plant Diseases Survey*, p. 5. Guelph, ON: Canadian Department of Agriculture, Division of Botany and Plant Pathology.
- Cooley, R.N. and Caten, C.E. (1991) Variation in electrophoretic karyotype between strains of *Septoria nodorum*. *Mol. Gen. Genet.* **228**, 17–23.
- Covert, S.F. (1998) Supernumerary chromosomes in filamentous fungi. *Curr. Genet.* **33**, 311–319.
- Daboussi, M. and Capy, P. (2003) Transposable elements in filamentous fungi. *Annu. Rev. Microbiol.* **57**, 275–299.
- Effertz, R.J., Meinhardt, S.W., Anderson, J.A., Jordahl, J.G. and Franc, L.J. (2002) Identification of a chlorosis-inducing toxin from *Pyrenophora tritici-repentis* and the chromosomal location of an insensitivity locus in wheat. *Phytopathology*, **92**, 527–533.
- Faris, J.D., Anderson, J.A., Franc, L.J. and Jordahl, J.G. (1996) Chromosomal location of a gene conditioning insensitivity in wheat to a necrosis-inducing culture filtrate from *Pyrenophora tritici-repentis*. *Phytopathology*, **86**, 459–463.
- Fierro, F. and Martin, J.F. (1999) Molecular mechanisms of chromosomal rearrangement in fungi. *Crit. Rev. Microbiol.* **25**, 1–17.
- Friesen, T.L. and Faris, J.D. (2004) Molecular mapping of resistance to *Pyrenophora tritici-repentis* race 5 and sensitivity to Ptr ToxB in wheat. *Theor. Appl. Genet.* **109**, 464–471.
- Friesen, T.L., Ali, S., Klein, K.K. and Rasmussen, J.B. (2005) Population genetic analysis of a global collection of *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat. *Phytopathology*, **95**, 1144–1150.
- Friesen, T.L., Stukenbrock, E.H., Liu, Z., Meinhardt, S., Ling, H., Faris, J.D., Rasmussen, J.B., Solomon, P.S., McDonald, B.A. and Oliver, R.P.

- (2006) Emergence of a new disease as a result of interspecific virulence gene transfer. *Nature*, **38**, 953–956.
- Gamba, F.M., Lamari, L. and Brûlé-Babel, A.L. (1998) Inheritance of race specific necrotic and chlorotic reactions induced by *Pyrenophora tritici-repentis* in hexaploid wheats. *Can. J. Plant Pathol.* **20**, 401–407.
- Green, G.J. (1975) Virulence changes in *Puccinia graminis* f. sp. *tritici* in Canada. *Can. J. Bot.* **53**, 1377–1386.
- Hosford, R.M. Jr. (1982) Tan spot developing knowledge 1902–1981, virulent races and wheat differentials, methodology, rating systems, other leaf diseases, literature. In: *Tan Spot of Wheat and Related Diseases Workshop* (Hosford, R.M. Jr, ed.), pp. 1–24. Fargo, ND: North Dakota Agricultural Experiment Station.
- Kinscherf, T.G. and Sally, A.L. (1988) Molecular analysis of the karyotype of *Ustilago maydis*. *Chromosoma*, **96**, 427–433.
- Kistler, H.C. and Miao, V.P.W. (1992) New modes of genetic change in filamentous fungi. *Annu. Rev. Phytopathol.* **30**, 131–152.
- Kistler, H.C., Meinhardt, L.W. and Benny, U. (1996) Mutants of *Nectria haematococca* created by a site-directed chromosome breakage are greatly reduced in virulence toward pea. *Mol. Plant–Microbe Interact.* **9**, 804–809.
- Krupinsky, J.M. (1982) Observation of the host range of isolates of *Pyrenophora trichostoma*. *Can. J. Plant Pathol.* **4**, 42–46.
- Lamari, L. and Bernier, C.C. (1989) Evaluation of wheat lines and cultivars to tan spot *Pyrenophora tritici-repentis* based on lesion type. *Can. J. Plant Pathol.* **11**, 49–56.
- Lamari, L., Strelkov, S.E., Yahyaoui, A., Orabi, J. and Smith, R.B. (2003) The identification of two new races of *Pyrenophora tritici-repentis* from the host center of diversity confirms a one-to-one relationship in tan spot of wheat. *Phytopathology*, **93**, 391–396.
- Lamari, L., Strelkov, S.E., Yahyaoui, A., Amedov, M., Saidov, M., Djunusova, M. and Koichibayev, M. (2005) Virulence of *Pyrenophora tritici-repentis* in the countries of the Silk Road. *Can. J. Plant Pathol.* **27**, 383–388.
- Lichter, A., Gaventa, J.M. and Ciuffetti, L.M. (2002) Chromosome-based molecular characterization of pathogenic and non-pathogenic wheat isolates of *Pyrenophora tritici-repentis*. *Fungal Genet. Biol.* **37**, 180–189.
- Martinez, J.P., Oesch, N.W. and Ciuffetti, L.M. (2004) Characterization of the multiple copy host selective toxin gene, *ToxB*, in pathogenic and nonpathogenic isolates of *Pyrenophora tritici-repentis*. *Mol. Plant–Microbe Interact.* **17**, 467–474.
- Masel, A., Braithwaite, K., Irwin, J. and Manners, J. (1990) Highly variable molecular karyotypes in the plant pathogen *Colletotrichum gleosporioides*. *Curr. Genet.* **18**, 81–86.
- McDonald, B.A. and Martinez, J.P. (1991) Chromosomal length polymorphism in a *Septoria tritici* population. *Curr. Genet.* **19**, 265–275.
- Miao, V.P., Covert, S.F. and Van Etten, H.D. (1991a) A fungal gene for antibiotic resistance on a dispensable ('B') chromosome. *Science*, **254**, 1773–1776.
- Miao, V.P., Matthews, D.E. and Van Etten, H.D. (1991b) Identification and chromosomal locations of a family of cytochrome P-450 genes for pisatin detoxification in the fungus *Nectria haematococca*. *Mol. Gen. Genet.* **226**, 214–223.
- Mitra, M. (1934) A leaf spot disease of wheat caused by *Helminthosporium thosporium tritici-repentis* Died. *Indian J. Agric. Sci.* **4**, 692–700.
- Plummer, K.M. and Howlett, B.J. (1993) Major chromosomal length polymorphisms are evident after meiosis in the phytopathogenic fungus *Leptosphaeria maculans*. *Curr. Genet.* **24**, 107–113.
- Rees, R.G. and Platz, G.J. (1992) Tan spot and its control: some Australian experience. In: *Advances in Tan Spot Research* (Francel, L.J., Krupinsky, J.M. and McMullen, M.P., eds), pp. 1–9. Fargo, ND: North Dakota Agricultural Experiment Station.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Schilder, A.M.C. and Bergstrom, G.C. (1992) The dispersal of conidia and ascospores of *Pyrenophora tritici-repentis*. In: *Proceedings from the Second International Tan Spot Workshop* (Francel, L.J., Krupinsky, J.M. and McMullen, M.P., eds), pp. 96–99. Fargo, ND: North Dakota Agric. Exp. Station, Fargo.
- Shoemaker, R.A. (1961) *Drechslera* Ito. *Can. J. Bot.* **40**, 809–836.
- Strelkov, S.E. and Lamari, L. (2003) Host–parasite interactions in tan spot *Pyrenophora tritici-repentis* of wheat. *Can. J. Plant Pathol.* **25**, 339–349.
- Strelkov, S.E., Lamari, L., Sayoud, R. and Smith, R.B. (2002) Comparative virulence of chlorosis-inducing races of *Pyrenophora tritici-repentis*. *Can. J. Plant Pathol.* **24**, 29–35.
- Strelkov, S.E., Kowatsch, R.F., Ballance, G.M. and Lamari, L. (2003) Occurrence and expression of *ToxB* in races of *Pyrenophora tritici-repentis*. In: *Proceedings from the Fourth International Wheat Tan Spot and Spot Blotch Workshop* (Rasmussen, J.B., Friesen, T.L. and Ali, S., eds), pp. 15–18. Fargo, ND: North Dakota Extension Services.
- Suga, H., Ikeda, S., Taga, M., Kageyama, K. and Hyakumachi, M. (2002) Electrophoretic karyotyping and gene mapping of seven formae speciales in *Fusarium solani*. *Curr. Genet.* **41**, 254–260.
- Sutton, J.C. and Vyn, T.J. (1990) Crop sequences and tillage practices in relation to diseases of winter wheat in Ontario. *Can. J. Plant Pathol.* **12**, 358–368.
- Talbot, N.J., Salch, Y.P., Ma, M. and Hamer, J.E. (1993) Karyotypic variation within clonal lineages of the rice blast fungus, *Magnaporthe grisea*. *Appl. Environ. Microbiol.* **59**, 585–593.
- Valent, B. and Chumley, F.G. (1991) Molecular genetic analysis of the rice blast fungus, *Magnaporthe grisea*. *Annu. Rev. Phytopathol.* **29**, 443–467.
- VanEtten, H.D., Matthews, D.E. and Matthews, P.S. (1989) Phytoalexin detoxification: importance for pathogenicity and practical implications. *Annu. Rev. Phytopathol.* **27**, 143–164.
- Zhong, S., Steffenson, B.J., Martinez, J.P. and Ciuffetti, L.M. (2002) A molecular genetic map and electrophoretic karyotype of the plant pathogenic fungus *Cochliobolus sativus*. *Mol. Plant–Microbe Interact.* **15**, 481–492.
- Zolan, M.E. (1995) Chromosome-length polymorphism in fungi. *Microbiol. Rev.* **59**, 686–698.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Sequences of the chromosome-specific probes (P1, P2, P3, P4, P5 and P6) used in this study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.