RNA-mediated gene silencing of *ToxB* in *Pyrenophora tritici-repentis*

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SUMMARY

The fungus *Pyrenophora tritici-repentis* causes tan spot, a wheat leaf disease of worldwide importance. The pathogen produces three host-selective toxins, including Ptr ToxB, which causes chlorophyll degradation and foliar chlorosis on toxin-sensitive wheat genotypes. The *ToxB* gene, which codes for Ptr ToxB, was silenced in a wild-type race 5 isolate of the fungus thorough a sense- and antisense-mediated silencing mechanism. Toxin production by the silenced strains was evaluated in culture filtrates of the fungus via Western blotting analysis, and plant bioassays were conducted to test the virulence of the transformants *in planta*. The chlorosis-inducing ability of the silenced strains was correlated with the quantity of Ptr ToxB, and transformants in which toxin production was strongly decreased also caused very little disease on toxin-sensitive wheat genotypes. Cytological analysis of the infection process revealed that, in addition to a reduced capacity to induce chlorosis, the silenced strains with the greatest decrease in the levels of Ptr ToxB produced significantly fewer appressoria than the wild-type isolate, 12 and 24 h after inoculation onto wheat leaves. The results provide strong support for the suggestion that the amount of Ptr ToxB protein produced by fungal isolates plays a significant role in the quantitative variation in the virulence of *P. tritici-repentis*.

INTRODUCTION

The phytopathogenic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-repentis* (Died.) Shoem.] causes tan spot, an important leaf spot disease of wheat worldwide (Lamari and Strelkov, 2010). Cytological analysis of the infection of host tissues by this fungus has revealed that germinating conidia develop germ tubes, and that these tubes form appressoria and penetration pegs that penetrate through the epidermal cells and develop intracellular vesicles (Larez *et al.*, 1986). The growth of *P. tritici-repentis* continues intercellularly in the mesophyll layer, causing damage to the cells beyond the advancing hyphae, via the secretion of host-selective toxins (Larez *et al.*,

1986; Loughman and Deverall, 1986). The tan spot pathogen produces at least three different host-selective toxins, known as Ptr ToxA, Ptr ToxB and Ptr ToxC (Lamari and Strelkov, 2010), which interact in a highly specific manner with the host plant. The outcome of this interaction is the development of two distinct symptoms, tan necrosis and/or extensive chlorosis, which vary depending on the toxin(s) produced by the pathogen and the susceptibility gene(s) present in the infected host (Lamari *et al.*, 2003). Eight races of *P. tritici-repentis* have been described to date, which are defined by their ability to produce the various toxins (Lamari *et al.*, 2003). Ptr ToxA, the necrosis-inducing toxin, is a 13-kDa protein that is encoded by a single gene, *ToxA* (Balance *et al.*, 1996; Ciuffetti *et al.*, 1997). This gene is found only in races of *P. tritici-repentis* that possess Ptr ToxA activity. Ptr ToxB is a 6.6-kDa protein that induces chlorosis (Strelkov *et al.*, 1999) through the light-dependent degradation of chlorophyll, as a consequence of an inhibition of photosynthesis (Kim *et al.*, 2010; Strelkov *et al.*, 1998). Ptr ToxC also induces chlorosis, but on different wheat genotypes from Ptr ToxB. Unlike Ptr ToxA and Ptr ToxB, Ptr ToxC is not a protein, but instead appears to be a polar, low-molecular-mass molecule (Effertz *et al.*, 2002).

Although Ptr ToxA is encoded by a single gene in *P. tritici-repentis*, the gene coding for Ptr ToxB, *ToxB*, is found in multiple copies in many isolates of the fungus (Aboukhaddour *et al.*, 2009; Martinez *et al.*, 2004; Strelkov *et al.*, 2006). The *ToxB* loci characterized to date from isolates of *P. tritici-repentis* possessing strong Ptr ToxB activity have an identical 261-bp open reading frame (ORF) (Martinez *et al.*, 2004; Strelkov and Lamari, 2003), which codes for an 87-amino-acid protein with a 23-amino-acid signal peptide (Martinez *et al.*, 2001; Strelkov and Lamari, 2003). The signal peptide is cleaved to yield a mature protein of 64 amino acids in length. Homologues of *ToxB* have also been identified in isolates of races 3 and 4 that exhibit no Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov *et al.*, 2006; Strelkov and Lamari, 2003). The *ToxB* homologue found in race 3 differs from the wild-type *ToxB* in the first six nucleotides of the ORF, as well as in the upstream flanking sequence, which is unique to race 3 (Strelkov *et al.*, 2006), whereas the form of *ToxB* in race 4 (termed *toxb* by Martinez *et al.*, 2004) exhibits 86% homology with the wild-type over the length of the ORF (Martinez *et al.*, 2004; Strelkov and Lamari, 2003).

The *ToxB* homologues in races 3 and 4 of *P. tritici-repentis* are transcribed in conidia (Amaike *et al.*, 2008; Strelkov *et al.*, 2006) and, at least in race 4, in mycelia (Amaike *et al.*, 2008) of the

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fungus, but at much lower levels than in wild-type isolates. The number of *ToxB* loci varies among isolates, even within the same race (Strelkov *et al.*, 2006). For instance, although 8–10 copies of *ToxB* were estimated to be present in a highly pathogenic race 5 isolate of the fungus (Alg3-24), only two copies were detected in a weakly pathogenic isolate of race 5 (92-171R5) from western Canada (Strelkov *et al.*, 2006). A single copy of the *ToxB* homologue is found in isolates of races 3 and 4 that lack Ptr ToxB activity (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). A higher *ToxB* copy number is positively correlated with an increased quantity of *ToxB* transcript and greater chlorosis symptom development on toxin-sensitive host plants (Amaike *et al.*, 2008). The amount of *ToxB* transcript present is also positively correlated with the number of appressoria produced by isolates of *P. tritici-repentis*. Therefore, it has been suggested that Ptr ToxB may have additional role(s) in the basic pathogenic or parasitic ability of the fungus, beyond its capacity to induce chlorosis on toxin-sensitive wheat leaves (Amaike *et al.*, 2008). This suggestion appears to be supported by the identification of *ToxB*-like sequences in sister species of *P. tritici-repentis* and other fungi, including *Magnaporthe grisea*, which are not pathogens of wheat (Andrie *et al.*, 2008). To further investigate the role of Ptr ToxB in parasitic fitness and the quantitative variation in the virulence of *P. tritici-repentis*, *ToxB*-silenced transformants expressing variable amounts of the toxin were generated through sense- and antisense-mediated gene silencing. In the context of this article, the terminology of Shaner *et al.* (1992) is employed, wherein pathogenicity is a comprehensive term referring to the ability of a microorganism to cause disease, and results from the virulence (degree of pathogenicity on specific host genotypes) and general parasitic ability of the microorganism. The impact of a decrease in Ptr ToxB production on the growth of *P. tritici-repentis* and on fungal pathogenesis was examined *in planta* and via cytological analysis.

RESULTS

Selection and screening of *ToxB*-silenced transformants

The silencing of *ToxB* was achieved by the transformation of the sense and antisense constructs together with the pSilent-1 vector encoding the hygromycin B resistance gene. The hyphal tips of the colonies that emerged on the hygromycin-amended medium were excised and transferred onto V8-potato dextrose agar (V8-PDA) plates containing 150 µg/mL hygromycin B. A total of five different transformants grew on this medium and were denoted as tf1, tf2, tf4, tf5 and tf6.

Morphology and growth rate of transformants

All transformants exhibited colony morphologies similar to that of the wild-type isolate Alg3-24 (Fig. 1A). Likewise, the growth rates

of the transformants tf1, tf2, tf4 and tf6 were not significantly different from the wild-type over a time course of 6 days (Fig. 1B). The growth rate of tf5, however, was significantly lower. The average colony diameter for cultures of tf5 was 33% less than that for the wild-type after 6 days of growth (Fig. 1B).

Evaluation of Ptr ToxB production

The amount of Ptr ToxB produced by Alg3-24 and the silenced strains of *P. tritici-repentis* was evaluated by Western blotting analysis using polyclonal antibodies raised against histidine (His)-tagged Ptr ToxB (Cao *et al.*, 2009). These antibodies reacted with a protein with a mass of approximately 6 kDa, corresponding to the expected size of Ptr ToxB, in culture filtrates (21 days old) of the five transformants and the wild-type isolate (Fig. 2). The amount of Ptr ToxB, however, was considerably decreased in all of the transformants relative to the wild-type. The greatest decrease in Ptr ToxB production, as assessed by the intensity of the toxin band, was observed in tf1, which produced a band with an intensity of only 15% of that in Alg3-24. The next greatest decrease in the quantity of Ptr ToxB was observed in the transformant tf4, with a toxin band intensity 23% of the wild-type, followed by tf5, with an intensity of 64%, and tf2 and tf6, with intensities of 76% and 81%, respectively, relative to the wild-type isolate (Fig. 2).

Infection process

Conidia of all silenced strains and the wild-type isolate germinated within 3 h of inoculation onto the susceptible host genotype '6B662', with germination rates of 90%–100% observed by this time. By 12 h, the germination rates for all strains ranged from 96% to 100% (data not shown). Similarly, the average number of germ tubes formed per conidium was not significantly ($P < 0.001$) different between strains at any time point after inoculation (data not shown). At 3 and 6 h, the number of appressoria that had developed per conidium in the wild-type and the silenced strains was also not significantly ($P < 0.05$) different (Table 1). At 12 h after inoculation, however, the silenced strain tf1 had produced significantly less appressoria than the wild-type. At 24 h, both tf1 and tf4 had formed significantly fewer numbers of appressoria than Alg3-24 and the other silenced strains (Table 1).

In general, the infection process was similar for all silenced strains and the wild-type isolate. At 3 h after inoculation, the majority of conidia had germinated and appressoria were visible on the leaf surface (Fig. 3A,B). Although either a single appressorium or multiple appressoria could form from a single germ tube, the development of multiple appressoria was more common in the wild-type isolate Alg3-24 and the silenced strains tf2, tf5 and tf6 (Fig. 3C). In the case of tf1 and tf4, in which silencing of Ptr ToxB

Fig. 1 Characterization of *ToxB*-silenced transformants of *Pyrenophora tritici-repentis*. (A) Morphology of colonies of the wild-type isolate Alg3-24 and the hygromycin-resistant transformants tf1, tf2, tf4, tf5 and tf6 on V8-potato dextrose agar medium after 6 days of growth. (B) Growth rate of all tested strains expressed in colony diameter in millimetres per day. Data points represent the mean of four replications with bars indicating the standard deviation of the mean.

was strongest, no multiple appressoria were observed on a single germ tube even at 24 h after inoculation, and germ tubes were often observed to grow over the leaf surface unable to form any appressoria at all (Fig. 3D). Appressoria developed mainly on the epidermal cell junctures, but were occasionally also observed on the stomatal complex (Fig. 3E). Following appressorium formation, penetration of the host epidermal cells could be seen as early as 6 h after inoculation with the wild-type isolate and the silenced strains. Penetration resulted in the formation of intracellular vesicles by the fungus in the host epidermal cells (Fig. 3F). The formation of vesicles was followed by the development of intracellular hyphae in the epidermal cells (Fig. 3G). At 6 h, the fungus also initiated intercellular growth in the leaf mesophyll layer (Fig. 3G) and, by 12 h, there was significant ingress of the hyphae into the mesophyll (Fig. 3H). At 24 h, damaged mesophyll cells stained deeply with aniline blue, indicating the occurrence of major physiological changes (Fig. 3I).

Symptom development

The wild-type race 5 isolate Alg3-24 and all silenced transformants induced typical chlorosis symptoms on the toxin-sensitive wheat line 6B662, but none caused chlorosis on the toxin-insensitive wheat cv. Salamouni (Fig. 4). All silenced strains, however, caused significantly less symptoms than the wild-type on 6B662. The tf1 and tf4 strains, in which *Ptr ToxB* production was lowest, caused minimal chlorosis on 6B662, which was limited to the development of very small chlorotic lesions around the penetration sites (Fig. 4A). Evaluation with image analysis software (Lamari, 2008) revealed that inoculation with Alg3-24 caused the most severe chlorosis, with an average ($\pm$ standard deviation) of $57\% \pm 3.9\%$ of the leaf surface turning chlorotic 7 days after inoculation with this isolate (Fig. 4B). Inoculation with tf2 and tf6 resulted in $43\% \pm 4.8\%$ and $46\% \pm 3.5\%$ of leaf chlorosis, respectively, whereas inoculation with tf5 resulted in chlorosis on $38\% \pm 3.1\%$ of the

Fig. 2 Western blot analysis of protein from *ToxB*-silenced transformants and the wild-type isolate Alg3-24 of *Pyrenophora tritici-repentis* with polyclonal antibodies specific to Ptr ToxB. Lane 1, kaleidoscope prestained standards (Bio-Rad, Mississauga, ON, Canada; cat. no. 161-0324); lane 2, transformant tf1; lane 3, tf2; lane 4, tf4; lane 5, tf5; lane 6, tf6; lane 7, wild-type isolate Alg3-24. Samples consisted of 5 µg of total protein from concentrated 21-day-old culture filtrates of each fungal strain, which were resolved via sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) prior to transfer to a polyvinylidene fluoride membrane. The number under each band represents its intensity relative to the wild-type isolate, as measured using the densitometry function of Alpha Imager software (Alpha Innotech, San Leandro, CA, USA).

Table 1 Mean number of appressoria per fungal conidium after inoculation of wheat line 6B662 with the *ToxB*-silenced transformants tf1, tf2, tf4, tf5 and tf6, and the wild-type isolate Alg3-24, of *Pyrenophora tritici-repentis*.

Fungal strain	Time after inoculation (h)			
	3	6	12	24
tf1	1.33*a	1.47a	1.63a	2.06a
tf2	1.27a	2.53a	2.97b	3.30b
tf4	1.13a	1.53a	2.07ab	2.73a
tf5	1.60a	1.83a	1.87ab	3.57b
tf6	1.23a	1.93a	2.50ab	3.27b
Alg3-24	1.20a	1.20a	2.83b	4.03b

*The mean number of appressoria was assessed for a total of 30 randomly selected conidia at different times after inoculation of line 6B662 with the *ToxB*-silenced transformants or the wild-type isolate. Means followed by a common letter in the same column are not significantly different ($P < 0.05$) as determined by Tukey's honestly significant difference (HSD) test.

leaf surface 7 days after inoculation. The silenced strains tf1 and tf4 caused the lowest levels of chlorosis, at $5.0\% \pm 2.0\%$ and $17\% \pm 2.8\%$, respectively (Fig. 4B). Decreases in chlorosis symptom development were well correlated with reductions in Ptr ToxB production in the silenced strains.

DISCUSSION

The *ToxB* gene, encoding the host-selective toxin Ptr ToxB, occurs in multiple copies in wild-type isolates of *P. tritici-repentis* (Martinez *et al.*, 2004; Strelkov *et al.*, 2006). Copies of *ToxB* have been detected in chromosomes varying in size from 2.2 to 5.7 Mb and, in some cases, on two different chromosomes (Aboukhaddour *et al.*, 2009; Martinez *et al.*, 2001). It is unknown whether the physical arrangement of the multiple copies of *ToxB* affects transcription levels. Nonetheless, the cloning and sequencing of three

of the 8–10 copies of *ToxB* in the wild-type race 5 isolate Alg3-24 from Algeria revealed that they possessed an identical ORF (Strelkov *et al.*, 2006), which was also identical to the ORF in six copies of *ToxB* sequenced from another race 5 isolate from North Dakota (Martinez *et al.*, 2004). The identical ORF found in the multiple copies of *ToxB* suggests that each copy encodes for the active toxin. Therefore, gene manipulation to knock out all the individual copies of *ToxB* would represent an inherently more difficult approach to study gene function than the silencing strategy utilized in the current investigation. Indeed, the identical nature of the ORF found in the multiple copies of *ToxB* in Alg3-24 enabled the knockdown of all copies of the gene, thereby facilitating the analysis. As suppression of gene expression by RNA silencing may be partial (Nakayashiki *et al.*, 2005; Shafran *et al.*, 2008), a number of silenced strains were generated, each producing varying levels of Ptr ToxB (Fig. 2). This spectrum of Ptr ToxB-producing phenotypes is similar to the situation observed in naturally occurring isolates of *P. tritici-repentis* (Amaike *et al.*, 2008; Strelkov *et al.*, 2002).

All transformants showed a decrease in the amount of Ptr ToxB produced relative to that of the wild-type isolate Alg3-24. Despite this decline in levels of the toxin protein, all silenced strains exhibited colony morphologies similar to that of the wild-type and, with the exception of tf5, also exhibited growth rates very similar to that of the wild-type (Fig. 1). It is interesting to note that tf5 produced more Ptr ToxB than did tf1 and tf4 (Fig. 2), which grew 'normally', suggesting that the slower growth of tf5 was not related to a decreased level of this protein. Conidial germination rates were also not significantly different between the silenced strains and the wild-type isolate, nor were the numbers of germ tubes produced per conidium. In contrast, the extent of the decrease in the amount of Ptr ToxB produced by each silenced strain was well correlated with the ability of these strains to induce chlorosis on a Ptr ToxB-sensitive host genotype (Fig. 4). Moreover, the two strains (tf1 and tf4) producing the least Ptr ToxB were also the strains in which appressorium formation was lowest (Table 1). These results suggest that, although a decrease in the amount of Ptr ToxB had a significant impact on the pathogenicity of the silenced strains, it had little, if any, effect on other biological parameters, such as the germination rates and growth on axenic medium.

The observation that strains deficient in Ptr ToxB production generally grew normally is not surprising, as isolates from races 1, 2 and 3 of *P. tritici-repentis*, in which the *ToxB* locus is completely absent, also grow normally. Indeed, Strelkov and Lamari (2003) stated that the Ptr toxins do not seem to have any critical biological function apart from their role in pathogenesis, a suggestion that is supported by the current study. In contrast, the Ptr ToxB deficiency in the silenced strains resulted in significantly lower levels of chlorosis on the toxin-sensitive wheat genotype 6B662. This finding provides strong support for the suggestion that the

Fig. 3 Fluorescence micrographs of infection of leaves of the Ptr ToxB-sensitive/race 5-susceptible wheat line 6B662 by ToxB-silenced transformants and the wild-type isolate Alg3-24 of *Pyrenophora tritici-repentis*. Samples were stained with aniline blue. (A) Germinated conidium (c) of silenced strain tf5 on the leaf surface, 3 h after inoculation. Note the presence of appressoria (a) at the end of the germ tube (g). (B) Multiple germ tubes with appressoria developing from a single conidium of silenced strain tf2, 3 h after inoculation. (C) Germinated conidium of Alg3-24, 6 h after inoculation. Note the formation of multiple appressoria from a single germ tube; v, vesicle. (D) Germinated conidium of silenced strain tf4, 24 h after inoculation. Note the germ tube growing over the leaf surface without the formation of any appressoria. (E) Germinated conidium of silenced strain tf1 on the leaf surface, 6 h after inoculation. Note the presence of an appressorium on a stomatal complex (s). (F) Formation of an intracellular vesicle (v) in a host epidermal cell, 6 h after inoculation with tf2; intracellular hyphae (ih) are also visible within the epidermal cell. (G) Intercellular hyphae (ih/me) of silenced strain tf6 in the host mesophyll layer, 6 h after inoculation. (H) Further ingress of hyphae of tf6 in the host mesophyll, 12 h after inoculation. (I) Damaged mesophyll cells (dme), 24 h after inoculation with the wild-type isolate Alg3-24. Note the deep staining of the cells with aniline blue, which is indicative of cell damage.

amount of toxin protein produced by fungal isolates plays a significant role in the quantitative variation in the virulence of *P. tritici-repentis*. This suggestion was made previously in a comparison of pathogenic (race 5) and nonpathogenic (race 4) isolates of the fungus (Amaike *et al.*, 2008), but the pivotal role of Ptr ToxB

could not be demonstrated conclusively in that study as isolates of races 4 and 5 exhibit numerous other major differences at the proteome level (Cao *et al.*, 2009). The results of the present study, in which all silenced strains were derived from the same pathogenic isolate, clearly indicate that, in addition to serving as a

Fig. 4 Reactions of wheat line 6B662 and cv. Salamouni to inoculation with *ToxB*-silenced transformants and the wild-type race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis*. (A) Appearance of the Ptr *ToxB*-sensitive/race 5-susceptible line 6B662 and the Ptr *ToxB*-insensitive/race 5-resistant cv. Salamouni 7 days after inoculation with the silenced strains tf1, tf2, tf4, tf5 and tf6, and the wild-type isolate Alg3-24, of the fungus. Note the varying levels of chlorosis on line 6B662; the necrotic flecking on 'Salamouni' is a typical resistance reaction. (B) Percentage of total leaf area turning chlorotic in line 6B662 in response to infection by the wild-type isolate and the silenced transformants. Leaves were harvested 7 days after inoculation, and total leaf and total lesion areas were measured using Assess 2.0 Image Analysis Software (Lamari, 2008). Error bars indicate the standard deviation from five repetitions of one run of the experiment.

pathogenicity factor (*sensu* Yoder, 1980) for *P. tritici-repentis*, Ptr *ToxB* may also be regarded as a virulence factor for the fungus. These findings are consistent with an earlier report by Friesen and Faris (2004), who found that a toxin-insensitivity gene, *tsc2*, is sufficient for susceptibility to race 5 of *P. tritici-repentis*, but that additional minor factors affect resistance or susceptibility. Collectively, it would appear that, although Ptr *ToxB* may be critical for the development of tan spot on toxin-sensitive wheat, the amount of Ptr *ToxB* produced is also important for the severity of the disease.

Another component of pathogenicity, in addition to virulence on specific host genotypes, pertains to the parasitic ability of a particular microorganism (Shaner *et al.*, 1992). As the abundance of *ToxB* transcript in naturally occurring pathogenic, weakly pathogenic and nonpathogenic isolates of *P. tritici-repentis* was

found to be positively correlated with the development of appresoria by these isolates, Amaike *et al.* (2008) suggested that Ptr *ToxB* could have a role(s) in basic pathogenic/parasitic fitness and/or prepenetration processes. As in the case of chlorosis development, however, this earlier analysis was confounded by the fact that, in addition to differences in *ToxB* expression, 133 other differentially abundant proteins have been identified in the proteome of pathogenic and nonpathogenic isolates of the fungus. A number of these differentially abundant proteins have also been implicated in microbial virulence in other pathosystems (Cao *et al.*, 2009). In the current study, all silenced strains were derived from the same pathogenic isolate, Alg3-24, facilitating direct comparisons between strains producing different amounts of toxin. It is therefore interesting that, in the two strains, tf1 and tf4, in which toxin production was most strongly silenced, the formation of

appressoria also declined significantly (Table 1). Although the molecular mechanisms involved in the formation of these structures by *P. tritici-repentis* are unknown, the relationship between the amounts of Ptr ToxB produced and appressorium formation is likely to be indirect and noncausative, as isolates producing Ptr ToxA, but lacking *ToxB*, are fully pathogenic. Nonetheless, the current findings support some indirect function for Ptr ToxB in the basic parasitic ability of *P. tritici-repentis*. Indeed, the recent identification of *ToxB*-like sequences in *Pyrenophora bromi* and other ascomycetes suggests that Ptr ToxB or Ptr ToxB-like proteins could have other functions, which are yet to be defined (Andrie *et al.*, 2008).

Once the strongly and weakly silenced strains of *P. tritici-repentis* had penetrated the host tissues, the infection process itself did not seem to be qualitatively different from that observed in the wild-type isolate (Fig. 4). Rather, differences in fungal development and the effects on the host appeared to be of a quantitative nature, with those strains producing the most Ptr ToxB being the most virulent. This observation is, again, likely to be a consequence of the amount of Ptr ToxB released by the invading hyphae in the host tissue, with more toxin yielding more severe symptoms of disease.

There exists one other report in which RNA silencing was used to disrupt the production of a host-selective toxin in *Alternaria alternata* (Miyamoto *et al.*, 2008). To our knowledge, however, this is the first study in which a host-selective toxin was silenced in *P. tritici-repentis*. The results indicate that the use of vectors that generate a hairpin RNA of the target gene is a useful approach to study the role of host-selective toxins in this and probably other fungal pathogens. The observation that Ptr ToxB may serve not only as a pathogenicity factor essential for the successful infection of toxin-sensitive wheat, but also as a virulence factor contributing quantitatively to symptom severity, suggests that the role of this toxin is more complex than originally thought.

EXPERIMENTAL PROCEDURES

Fungal isolate and growth conditions

All experimental procedures and silencing studies were conducted using a highly pathogenic race 5 isolate of *P. tritici-repentis*, Alg3-24, originally collected from eastern Algeria (Lamari *et al.*, 1995). This isolate produces Ptr ToxB and is estimated to possess 8–10 copies of the wild-type *ToxB* gene (Strelkov *et al.*, 1999, 2006). Conidia of Alg3-24 were stored as 25% glycerol stock at -80°C . Small plugs, 0.5 cm in diameter, from a 7-day-old culture of Alg3-24 were transferred singly to 9-cm-diameter Petri dishes containing 30 mL of V8-PDA medium [V8-juice (150 mL), Difco PDA (10 g), CaCO_3 (3 g), Bacto agar (10 g) and distilled water (850 mL)] (Lamari and Bernier, 1989). The cultures were incubated at 20°C in the dark until they reached 4 cm in diameter. To induce sporulation, the cultures were flooded with sterile distilled water and the mycelium was flattened with the bottom of a flamed test tube. After the water had been decanted, the

cultures were incubated at room temperature for 18 h under light, followed by an incubation of 24 h in the dark at 15°C (Lamari and Bernier, 1989). The conidia were harvested by flooding the cultures with sterile distilled H_2O , and gently dislodging the spores with a sterilized wire loop. The collected conidia were used either to prepare protoplasts or to inoculate the plants as described below.

Vector construction

The plasmid pSilent1 (Nakayashiki *et al.*, 2005) was obtained from the Fungal Genetics Stock Center (Kansas City, MO, USA; McCluskey, 2003). A 432-bp fragment from the *ToxB* gene, including the entire 261-bp ORF, was cloned into pSilent1 in sense and reverse/complementary orientations on both sides (*XhoI/HindIII* sites and *StuI/ApaI* sites) of the 147-bp intron 2 of the cutinase gene from *Microdochium oryzae*, driven by the PtrpC promoter. The coding region, in the sense orientation, was amplified by polymerase chain reaction (PCR) with the primers *ToxBXhoI* (5'-CCACT CGAGTACAGTAATCTCTTCTACGCT-3') and *ToxBHindIII* (5'-CGAAAGCTT CCCTATACCTAATGTAGGG-3'). The coding region, in the antisense orientation, was amplified with the primers *ToxBApaI* (5'-AAAGGGCCCTAC AGTAATCTCTTCTACGCT-3') and *ToxBStuI* (5'-ACCAGGCCTCCCTATACCT AATGTAGGG-3'). Integration of both the sense and antisense strands of *ToxB* was confirmed by sequencing the purified plasmid (Macrogen, Rockville, MD, USA) using Big Dye Terminator cycling conditions on a 3730XL DNA Analyser (Applied Biosystems, Foster City, CA, USA). A total of 3 μg of plasmid was incubated with protoplasts of Alg3-24 for fungal transformation as described below.

Fungal transformation

Conidia from a sporulating culture of *P. tritici-repentis* isolate Alg3-24 were collected and inoculated into 1-L Erlenmeyer flasks containing 500 mL of $0.25 \times$ Difco potato dextrose broth (PDB) to a final concentration of 5×10^5 conidia/mL. The liquid cultures were incubated overnight in a rotary shaker at 100 rpm at room temperature. Germinated conidia were filtered through one layer of miracloth and washed with sterile distilled H_2O . Protoplasts were generated by the addition of 8 mL of a filter-sterilized solution containing 140 mg of Lysing enzyme, 40 mg of Driselase and 50 mg of Yatalase in a solution of 1.2 M MgSO_4 , 10 mM potassium phosphate, pH 5.8 (Aboukhaddour *et al.*, 2009). The protoplasts were washed once in STC (1.2 M sorbitol in 10 mM Tris-HCl, pH 7.5, and 50 mM CaCl_2), quantified on a haemocytometer, and diluted to a final concentration of 1×10^7 protoplasts/mL in STC, polyethylene glycol 4000 and dimethylsulphoxide (DMSO) (80 : 20 : 1, v/v/v), and kept on ice or stored at -20°C for up to 4 months (Ciuffetti *et al.*, 1997). The fungal transformation protocol was adapted from Ciuffetti *et al.* (1997). Briefly, 100 μL of a 1×10^7 protoplasts/mL suspension in STC buffer was mixed with 3 μg of plasmid DNA and 1 μL of 0.1 M spermidine, and incubated on ice for 1 h. One millilitre of polyethylene glycol 4000 (50 mM Tris, pH 8.0, and 50 mM CaCl_2) was added to the above mix, gently mixed and incubated for 20 min at room temperature. Four millilitres of regeneration medium (RM) (1.2 M sorbitol, 0.1% yeast extract, 0.1% casein hydrolysate and 0.8% agar) were added to the mixture. The mixture containing the protoplasts was then spread evenly on the top of 9-cm-diameter Petri dishes containing 20 mL of solidified RM (1.2 M sorbitol, 0.1% yeast extract, 0.1% casein hydrolysate and 1.5% agar). The Petri dishes were incubated overnight in the

dark at room temperature. The protoplasts were then overlaid with 5 mL of RM-top agar (0.8% agar) containing 4.5 g of hygromycin B (Invitrogen, Burlington, ON, Canada) to yield a final concentration of 150 µg hygromycin/mL. After 5–8 days of incubation in the dark at room temperature, hygromycin B-resistant colonies of *P. tritici-repentis* were individually transferred to 9-cm-diameter Petri dishes filled with V8-PDA medium amended with 150 µg/mL hygromycin B. The growth of selected transformants was monitored daily by measuring the colony diameter in two directions at right angles to each other. The treatments were replicated four times and an average diameter was calculated for each treatment.

Confirmation of construct integration into transformants

PCR analysis was performed on selected transformants to evaluate whether the silencing construct had been integrated into the genome of *P. tritici-repentis*. The forward primer PF1 (5'-ACGACCGGTCATACCTTCT-3') and reverse primer PR1 (5'-ATGGCCAACAAATCTCCAGT-3') amplified a fragment of the plasmid construct containing the *ToxB* gene ligated in the sense direction. The primers PF2 (5'-AAAACACACAGCCAGGGAAC-3') and PR2 (5'-CTGACATCGACACCAACGAT-3') amplified a fragment of the plasmid construct containing the *ToxB* gene integrated in the antisense direction. PCR conditions consisted of 95 °C for 2 min; 30 cycles of 95 °C for 50 s, 55 °C for 50 s and 72 °C for 50 s; and 72 °C for 7 min. The amplicons were sequenced as described above.

Pathogenicity assays

Two hexaploid wheat genotypes, line 6B662 and cv. Salamouni, were inoculated with the wild-type isolate Alg3-24 of *P. tritici-repentis* or the silenced strains. Line 6B662 is susceptible to race 5 of the fungus and sensitive to Ptr ToxB, whereas cv. Salamouni is resistant to all races and insensitive to all known toxins produced by the fungus. Seeds were sown in 10-cm-diameter plastic pots filled with Sunshine potting mix (W.R. Grace and Co., Fogelsville, PA, USA) at a rate of six seeds per pot. The seedlings were maintained in a glasshouse at 20 °C/18 °C (day/night) with a 16-h photoperiod at 250 µmol/m²/s (natural light supplemented with artificial lighting) until inoculation at the two- to three-leaf stage. The seedlings were inoculated with a suspension of 3000 conidia/mL [to which 10 drops of Tween-20 (polyoxyethylene sorbitan monolaurate) per litre were added] using a sprayer connected to an air line. Leaves were sprayed until run-off. Immediately following inoculation, the plants were placed in the dark in a misting chamber (relative humidity, ≥95%) for a 24-h period, with continuous wetness provided by an ultrasonic humidifier. After incubation under high humidity, the plants were transferred to a growth chamber and kept at 20 °C/18 °C (day/night) with a 16-h photoperiod (180 µmol/m²/s) and 60% relative humidity. Experiments were independently repeated twice with three replicates (pots) per treatment at each time point. The second leaves were collected 7 days after inoculation and five leaves were chosen randomly from each treatment and scanned on a flatbed scanner (Epson Perfection 2480 Photo, Epson Canada Ltd., Markham, ON, Canada). Total leaf and lesion areas were measured using Assess 2.0 Image Analysis Software (Lamari, 2008).

Microscopy

Fungal penetration of epidermal cell walls, vesicle formation, colonization of the epidermal cells and colonization of the mesophyll layer were observed microscopically on inoculated leaves. Tissue was collected from the middle of the second leaf of seedlings of line 6B662 at 3, 6, 12 and 24 h after inoculation. The leaf tissue was cut into pieces of 1 cm in length, immersed in 1 M KOH for 24 h at room temperature and then autoclaved at 120 °C for 15 min. The autoclaved leaf segments were stored at 4 °C until analysis, and samples were placed at room temperature for at least 30 min prior to microscopic observation. Each specimen was rinsed three times in double-distilled H₂O and mounted in a staining solution consisting of 0.05% (w/v) aniline blue in 0.067 M K₂HPO₄, pH 9.0, as described by Hood and Shew (1996). This method resulted in a high degree of contrast between the host tissue and the fungal structures. A total of 30 randomly selected conidia per sample were examined for percentage germination, number of germ tubes per conidium and number of appressoria per conidium. All samples were examined under high magnification with a Zeiss Axio Vision AX10 fluorescence microscope (Carl Zeiss Imaging Solutions GmbH, Gottingen, Germany) fitted with an ultraviolet excitation filter (BP 546/12). Images were captured with a Zeiss Axio Cam HRm camera (Carl Zeiss Imaging Solutions GmbH) using the microscope operating and image analysis software, Axio Vision 4.6. Analysis of variance for multiple comparisons [Tukey's honestly significant difference (HSD) test] was conducted using R Software (R Development Core Team, 2008).

Production of culture filtrates

To test for the production of Ptr ToxB by the transformants and the wild-type isolate Alg3-24, cultures of each isolate were grown on V8-PDA (Lamari and Bernier, 1989) until they were 4–5 cm in diameter. Five plugs, 1 cm in diameter, were excised from each colony and transferred to 300-mL Erlenmeyer flasks filled with 100 mL of modified Fries medium (Dhingra and Sinclair, 1986) amended with 0.1% yeast extract and containing only 0.955 mM KH₂PO₄ and 1.49 mM K₂HPO₄. The cultures were incubated in the dark without agitation at 20 °C for 21 days. Culture filtrates were collected by vacuum filtration through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, UK) and then passed through 0.45-µm cellulose nitrate filters. The culture filtrates were lyophilized in a freeze-drier and stored at –20 °C until needed. The lyophilized culture filtrates were redissolved in 20 mM sodium acetate buffer (pH 4.6) and centrifuged at 17 400 *g* for 10 min. The supernatant was collected and dialysed overnight against water, concentrated again by freeze-drying, redissolved in 100 µL of sterile distilled H₂O, and then tested for toxin production via Western blotting analysis as described below.

Protein estimation and electrophoresis

Five micrograms of total soluble protein from concentrated filtrates of 21-day-old cultures of the wild-type isolate Alg3-24 and the transformants were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was estimated by the method of Bradford (1976) using a Bio-Rad protein assay kit according to the manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada). PAGE was run under denaturing conditions with SDS in a Mini-Protein II

electrophoresis cell (Bio-Rad) using a Tris–tricine buffer system (Schägger and von Jagow, 1987).

Western blotting analysis

The polyacrylamide gels and polyvinylidene fluoride (PVDF) membranes (Bio-Rad) were equilibrated for 15 min in transfer buffer [48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20% (v/v) methanol]. The proteins were then transferred from the gel to the PVDF membrane at 15 V for 30 min in a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). After transfer, the blots were incubated overnight at 4 °C with agitation in Tris-buffered saline (TBS) (150 mM NaCl and 50 mM Tris, pH 7.5) containing 5% (w/v) nonfat dry milk. They were then rinsed three times in TTBS [0.05% (v/v) Tween-20 in TBS] and incubated for 1 h with rabbit polyclonal antibodies raised against heterologously expressed His-tagged Ptr ToxB (Cao *et al.*, 2009), diluted 1 : 3000 in antibody buffer [1% (w/v) nonfat dry milk in TTBS]. Blots were washed three times at 5 min per wash in TTBS, and then incubated with goat antirabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Bio-Rad), diluted 1 : 3000 in antibody buffer. Blots were washed again, and the membranes were developed with a tetramethyl benzidine substrate kit for peroxidase (Vector Laboratories, Burlingame, CA, USA), according to the manufacturer's instructions. Blot images were recorded with a GS-800 Calibrated Densitometer (Bio-Rad).

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