University of Alberta

Characterization and silencing of differentially abundant proteins from *Pyrenophora tritici-repentis*

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Plant Science

Department of Agricultural, Food and Nutritional Science

Fall 2011
Edmonton, Alberta

Examining Committee

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Abstract

Tan spot, caused by *Pyrenophora tritici-repentis*, is an important foliar disease of wheat. Three genes (*stp*, *unp* and *glu*) encoding proteins previously found to be more abundant in pathogenic versus non-pathogenic fungal isolates were cloned, characterized, heterologously expressed, and silenced with the RNA silencing vector pSilent-1. The *unp* gene encoded a 16.9 kDa protein belonging to a superfamily of glycine-rich RNA-binding proteins, but growth and virulence of an *unp*-silenced strain of the fungus were not significantly different from the wild-type. The *stp* gene, which was not significantly silenced, encoded a 15.0 kDa protein homologous to a CipC-like antibiotic response protein. The *glu* gene encoded an exo-1,3-β-glucanase, 46.7 kDa in mass. Four *glu*-silenced strains were obtained. The strain in which silencing was strongest exhibited reduced growth, produced fewer appressoria, and caused less disease than the wild-type. The results suggest that exo-1,3-β-glucanase contributes to the development and virulence of *P. tritici-repentis.*

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Stephen Strelkov for the opportunity to study in his research group, the guidance and financial support throughout the program. I would also like to thank my advisory committee members, Dr. Nat Kav and Dr Michael Deyholos for their suggestions.

I gratefully acknowledge Dr. Tiesen Cao for technical help, assistance with the statistical analysis and for reviewing my thesis. I am also grateful to Dr. Reem Aboukhaddour and Mr. Yong Min Kim for technical help and valuable discussions. I wish to thank Xue Pan for assistance with the statistical analysis. I appreciated the assistance from all the members of Dr. Stephen Strelkov's laboratory and Dr. Nat Kav's laboratory. Help from Kelly Dunfield, Urmila Basu, Bruce Alexanda, Lynn Elmes and Laura Smith is also gratefully acknowledged.

Finally, I would like to thank my parents, my sisters and especially my wife Mihyun Kim for their understanding, encouragement and support during my studies.

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1.1 Tan spot of wheat

1.1.1 The problem and its significance

The fungus *Pyrenophora tritici-repentis* (Died.) Drechs. [syn. *P. trichostoma (Fr.),* anamorph: *Drechslera tritici-repentis* (Died.) Shoemaker; syn. *Helminthosporium tritici-repentis* (Died.)] is a homothallic ascomycete that causes tan spot (syn. yellow spot, yellow leaf blotch, eyespot) of wheat. As *P. tritici-repentis* is a stubble-borne pathogen, a shift in farming practices towards zero or minimum tillage for the purposes of soil conservation has resulted in an increase in the prevalence of tan spot in recent decades. The disease has been found in the major wheat-producing countries of the world ncluding Australia, Canada, Brazil, the United States and Argentina, as well as many other regions (Hosford Jr., 1982; Rees *et al*., 1988; Francl, 1997; Strelkov and Lamari, 2003; Lamari and Strelkov, 2010). While *P. tritici-repentis* has been mainly isolated from wheat, it also has been found on other cereals including barley, oats, rye, and various wild grasses (Krupinsky, 1992).

Symptoms of tan spot include the development of tan necrosis and/or extensive chlorosis on the leaves of wheat, with specific symptoms varying depending on the susceptibility/resistance of the host. The susceptible host response is characterized by the formation of either tan necrosis or chlorosis, with or without small dark brown to black spots on the leaf, whereas a

resistant host response is characterized by the appearance of small dark brown to black spots without, or with low levels, of tan necrosis or chlorosis (Lamari and Bernier, 1989a). Discoloration of the kernels, in the form of red smudge, black point, and/or dark smudge, represents the major symptoms observed on seeds infected with the tan spot pathogen (Fernández *et al*., 2001).

Tan spot can have a significant impact on wheat production. Depending on the level of disease severity, yield losses of 3 to 50% have been observed (Hosford Jr., 1982; Rees *et al*., 1982; Bhathal *et al*., 2003). In one case, yield losses of 75% were observed in a wheat field under extremely favorable conditions for the disease (Rees *et al*., 1982). In addition to yield losses, red smudge, black point and dark smudge caused by seed infection may reduce the seed quality (Fernández *et al*., 2001) and serve as a source of inoculum for further spread of tan spot. Long-distance transportation of infected seeds has been suggested to be responsible for the worldwide occurrence of tan spot (Schilder and Bergstrom, 1995). Recently, infection of the seed has been shown to be accompanied by the production of various mycotoxins including emodin and catenarin (Bouras and Strelkov 2008), which could pose a health concern.

1.1.2 Disease cycle

P. tritici-repentis is able to colonize stem tissue by saprophytic growth on the senescent leaves prior to harvest. At first the fungus is restricted to the

lesion spots, but after senescence of the leaves, it can extend its growth from the leaf sheath and then into the stem (Summerell and Burgess, 1988). After harvest, the sexual fruiting bodies (pseudothecia) start to develop on the infested wheat stubble and mature during fall and winter. Development of pseudothecia often requires light and a period of moist conditions. Pseudothecia overwinter on wheat or other host residues. Direct contact between the stubble and moist soil often facilities the development of pseudothecia, whereas buried stubble decomposes more rapidly and therefore supports the formation of less pseudothecia. Under favorable conditions in the spring, ascospores are released from the pseudothecia and serve as the primary inoculum. The optimal temperature for ascospore production in the pseudothecia is between 15-18^{o}C (Summerell and Burgess, 1988). These ascospores are airborne but do not travel long distances. After discharge from the pseudothecia, an ascospore lands on the leaf surface and begins to infect the young wheat seedling. Lesions develop on the infected wheat leaves, with the secondary inoculum, or conidia, developing on these lesions. Single conidia are borne on conidiophores. Sporulation is favored by high relative humidity and a period of light and darkness (Platt and Morrall, 1980a), with an optimal temperature of 21 ^{o}C (Francl, 1998).

Dispersal of conidia can be divided into three successive phases: liberation, dissemination and deposition. Populations of mature conidia were found to be highest in July and August. Conidia of *P. tritici-repentis* are

generally liberated in the afternoon under dry conditions after a night period with free moisture from rain and dew (Platt and Morrall, 1980b; Francl, 1995). A wind flow of 3.3 m/s is sufficient to result in 100% conidial liberation. Moreover, variable rather than constant humidity tends to favor conidial liberation (Platt and Morrall, 1980b). After landing on wheat leaves, the conidia germinate and form appressoria, and then penetrate the plant epidermal cells and leaf mesophyll, causing the typical tan spot symptoms (Loughman and Deverall, 1986; Dushnicky *et al*., 1998a). The optimal temperature for disease development is 18-28℃, depending on the specific wheat cultivar (da Luz and Bergstrom, 1986).

The disease cycle can also start with an infected seed, on which *P. tritici-repentis* can survive as dormant mycelium. Under favorable conditions, the dormant mycelium resumes growth from the infected pericarp and begins to colonize the coleoptile. The pathogen then invades the first and second leaves of the seedlings (Schilder and Bergstrom, 1995).

1.1.3 Management of tan spot

In the 1970s, as a consequence of high oil prices and concerns about soil erosion, farmers in North America began to adopt conservation tillage systems, which replaced stubble-burning or conventional tillage as routine agronomic practices (Bockus and Shroyer, 1998). Although conservation tillage reduces soil erosion, conserves energy (since the field does not have to be worked as

many times), and increases soil moisture and crop yields, it also retains crop stubble on the soil surface, creating favorable environments for the survival of stubble-borne pathogens such as *P. tritici-repentis*. Thus, the incidence of tan spot began to increase due to these changes in farming practices. Tan spot of wheat is often associated with other leaf spot diseases of wheat, such as Stagonospora blotch, Septoria blotch, and spot blotch, which makes disease management more complicated (Bockus and Shroyer, 1998; De Wolf *et al*., 1998). Cultural control, chemical control, and biological control measures, along with the use of genetically resistant wheat cultivars, are the major strategies used for the management of tan spot.

1.1.3.1 Cultural management methods

Tillage can help destroy stubble on the soil surface and is effective against tan spot disease (Bockus, 1998). Buried residues tend to be degraded more quickly than residues on the soil surface because the speed of residue decomposition is decreased with reduced moisture and low temperatures. Buried residues also are subject to microbial degradation in addition to high moisture (Bailey and Duczek, 1996). Summerell *et al*. (1988) found that the chances of recovering *P. tritici-repentis* from crop stubble were very low after the stubble had been buried in the soil for 26 weeks. In contrast, the chances of recovering *P. tritici-repentis* from stubble was only reduced by 50% if the stubble remained exposed to the air on the soil surface for 104 weeks

(Summerell and Burgess, 1988; Summerell and Burgess, 1989).

Moreover, buried residues tend to reduce the survival and formation of pseudothecia because of the antagonistic activity of other soil-borne microflora under moist conditions (Pfender *et al*., 1991; Zhang and Pfender, 1993; Bockus and Shroyer, 1998). In general, decreased tillage is associated with an increase in leaf spot diseases (Summerell and Burgess, 1989). However, Sutton and Vyn (1990) reported that a decrease in tillage may not necessarily mean an increase in disease severity. Disease severity can be affected by many other factors. The amount of residues left in one season may not be a good indication of disease severity in the next season. Factors such as the age of the residue, herbicide treatment, and environmental conditions can also influence the importance of plant residues as an inoculum source. The application of herbicides appeared to reduce the production of pseudothecia, while more pseudothecia were produced on two-year old versus one-year old plant residues. Disease severity could be low in a growing season with high temperatures and low humidity (Sharma *et al*., 1989; Bailey and Duczek, 1996).

Crop rotation with non-hosts of *P .triciti-repentis* can help reduce the amount of wheat residue on the soil surface and thus reduce the severity of tan spot. Bockus and Claassen (1992) found that rotation to sorghum from wheat resulted in reduced tan spot disease under conventional tillage conditions. Bhathal and Loughman (2001) also reported that a crop rotation as short as

one year could reduce the severity of both tan spot and *Septoria nodorum* blotch by greatly reducing early infection from the local carry-over. Sutton and Vyn (1990) studied the foliar disease severity under three different crop rotation regimes, soybean-wheat-wheat, corn-barley-wheat, and alfalfa-alfalfa-wheat under conventional, minimum, and zero tillage conditions, and found that the crop order in the rotation as well as tillage may affect individual wheat diseases, but did not affect the total severity of the foliar diseases. Krupinsky and Tanaka (2001) reported that adequate nitrogen fertilization could effectively decrease tan spot. Cultivation of mixed cultivars with different resistance genes has been reported to have potential for reducing disease severity caused by multiple pathogens, by increasing the durability of the resistance genes (Cox *et al*., 2004).

1.1.3.2 Chemical management methods

The use of fungicides is an effective way to control tan spot. Colson *et al*. (2003) studied the effectiveness of 12 fungicides against tan spot in Australia, and found that application of flusilazol, prochloraz, propiconazole, or tebuconzaole could significantly increase yields under high disease pressure. These authors suggested that the optimal timing of fungicide applications is prior to or immediately after a rain or irrigation event, since these are conditions that may promote infection of the flag leaf (Colson *et al*., 2003). A single application of fungicide resulted, on average, in a 36% reduction of tan

spot under high disease pressure. However, the cost of fungicide application may be too high relative to the expected yield gains if the disease severity is less than 38%. A single application of fungicide is most effective between the bolting and fully headed developmental stages. Even the application of fungicides during the milky ripening stage proves to be more effective than during flag leaf emergence (Bockus, 1998).

The infection of wheat seeds by *P. tritici-repentis* is very rare in Alberta (Turkington *et al*., 2002), but seed transmission of the pathogen is nonetheless plausible (Da Luz *et al*., 1998). Treatment of the seeds with triadimenol, a systemic fungicide, has been reported to be effective against early tan spot infection. Under greenhouse conditions, treatment with triadimenol protected the seedlings from infection by *P. tritici-repentis* for up to 30 days. Under field conditions, fungicide-treatment of seeds increased yields by 20% compared to non-treated seeds infected by *P. tritici-repentis* (Da Luz and Bergstrom, 1986).

Aside from fungicides, the foliar application of xanthan gum together with a suspension of heat-inactivated conidia to wheat leaves was shown to induce local and systemic protection against tan spot disease. After application with this mixture, the treated plants expressed a defense response and accumulated pathogenesis-related (PR) proteins, with a concurrent decrease in the number of foliar lesions (Bach *et al*., 2003).

1.1.3.3 Biological control

Under greenhouse conditions, *Aspergillus niger*, *Penicillium lilacinum*, *Rhodotorula rubra*, *Bacillus* species, and *Chaetomium globosum* can inhibit tan spot disease development as biological control agents. These antagonistic microorganisms may compete with *P. tritici-repentis* for nutrients or space by direct exclusion of the pathogen, or by producing antibiotic-like substances or other metabolites that inhibit growth of the pathogen (Perello *et al*., 2001; Perello *et al*., 2002). In another study, the biological control agents *Trichoderma aureoviride* and *T. koningii* inhibited mycelial growth of *P. tritici-repentis* by 50% and 74%, respectively, on potato dextrose agar, and induced plasmolysis of the conidia and hyphae of the pathogen (Perello *et al*., 2003). Under greenhouse conditions, the application of *T. aureoviride* and *T. koningii* significantly reduced tan spot disease severity. While *Trichoderma* species are thought to inhibit *P. tritici-repentis* by competing with this fungus for nutrients, they have also been suggested to secret lytic enzymes to attack *P. tritici-repentis* (Perello *et al*., 2003). By using alginate/milled bran based inoculum containing *Limonomyces roseipellis*, Pfender *et al*., (1993) obtained significant reductions in the formation of *P. tritici-repentis* pseudothecia in 3 years of a 4 year experiment. Biological control agents are also effective when applied to infested seeds, because they can reduce the growth of the pathogen or may even eradicate it from the seeds, and thus increase seedling emergence and yield (Levy *et al*., 1992). Although many biological control agents are

effective *in vitro* or under greenhouse conditions, their effectiveness in the field is still unknown, given the fact that additional factors such as temperature, moisture, and wind could affect control efficiency (Perello *et al*., 2003). Therefore, while biological control of tan spot may be possible in the future, it is still not a practical or commonly used approach in commercial wheat production.

1.1.3.4 Genetic resistance

Relative to other control methods, the use of resistant cultivars is the most economic and environmentally friendly way to manage tan spot. Generally, resistant cultivars should meet three criteria: 1) presence of resistance gene(s) in the cultivar; 2) high levels of resistance in the cultivar; and 3) durable resistance in the cultivar (Bockus, 1998). Although the deployment of genetically resistant cultivars has many advantages, only a few cultivars have high levels of resistance to *P. tritici-repentis* (De Wolf *et al*., 1998). The tan spot resistance genes Tsr1, Tsr2, Tsr3, Tsr4, Tsr5, and Tsr6 have been reported to be responsible for resistance to different races of *P. tritici-repentis*. A number of quantitative trait loci (QTL) on different chromosomes have also been discovered for resistance to tan spot (Singh *et al*., 2010). Recently, Singh *et al*., (2006) evaluated 126 durum and spring wheat cultivars and lines, and found that only ten genotypes showed high levels of resistance to the multiple races of *P. tritici-repentis* and its toxins. Ali *et al*., (2008) screened 164 wheat

genotypes and found three wheat genotypes with resistance to both tan spot and *Stagonospora nodorum* blotch, while 13 genotypes were resistant to tan spot and *Septoria tritici* blotch, and four wheat genotypes were highly resistant to tan spot, *Stagonospora nodorum* blotch and *Septoria tritici* blotch of wheat (Ali *et al*., 2008). While tan spot resistant wheat cultivars may not be widely available at the present, the identification of resistance genes and QTLs will benefit resistance breeding efforts in the future.

1.2. The infection process

1.2.1 Pre-penetration events

The landing of *P. tritici-repentis* conidia on the wheat leaf surface is followed by conidial germination, germ tube elongation and the development of appressoria (Larez *et al*., 1986). Germ tubes are formed from basal and intercalary cells of the conidia (Dushnicky *et al*., 1998b). Previous observations indicated that 65% of the conidia had germinated within 3 hr after inoculation, and more than 95% of the conidia had germinated after 6 hr (Larez *et al*., 1986; Amaike *et al*., 2008). Germ tube number per conidium varies from 1-6, with most of the conidia producing 2-4 germ tubes (Dushnicky *et al*., 1996). Larez *et al*., (1986) reported an average of 2.5 germ tubes per conidium between 6 and 72 hr after inoculation. According to Larez and coworkers (1986), the percentage of germinated conidia and the number of germ tubes per conidium were not related to host resistance. Appressoria

often formed at the end of germ tubes prior to penetration. The time it took for appressorium formation appeared to depend on the virulence of specific isolates of *P. triciti-repentis*, with a more virulent isolate requiring less time than weakly virulent or avirulent isolates (Amaike *et al*., 2008). The extent of appressorium formation was not related to the resistance of the wheat genotype (Larez *et al*., 1986; Amaike *et al*., 2008). Conidial germination, the number of germ tubes per conidium, length of the germ tube, and appressorium production also appeared to increase with increasing temperature and wet period (Hosford *et al*., 1987). Amaike *et al*., (2008) reported that appressorium formation was positively correlated with the expression levels of *ToxB*, a gene encoding for a host-specific toxin (see below), suggesting that Ptr ToxB may play some role in pre-penetration processes (Amaike *et al*., 2008). The appressoria of *P. tritici-repentis* are usually round or club-shaped, and form on epidermal cell junctures, the anticlinal or periclinal walls of epidermal cells, or on the stomatal complex (Dushnicky *et al*., 1996).

1.2.2 Invasion and growth

Penetration of the epidermal cell walls by *P. tritici-repentis* appears to have two mechanisms: enzymatic hydrolysis and mechanical puncture (Dushnicky *et al*., 1996). Initial penetration involves enzymatic hydrolysis followed by mechanical puncture. A penetration peg is formed below the

appressorium, which allows entry into a host epidermal cell. Alternatively, but less commonly, the penetration pegs can also enter guard cells or subsidiary cells, and hyphae can subsequently grow into the stomatal chamber. Extracellular sheaths were observed to spread out from the appressoria onto the leaf surface (Larez *et al*., 1986). Penetration usually takes place 3 hr after the spores land on the leaf surface. If the first attempt at penetration fails, then another infection peg or appressorium will be formed from an extended germ tube (Dushnicky *et al*., 1996).

After penetration, the fungus usually produces one or two intracellular vesicles from the infection peg, from which one or more secondary hyphae will be derived (Larez *et al*., 1986; Loughman and Deverall, 1986; Dushnicky *et al*., 1996; Dushnicky *et al*., 1998a). Under some circumstances, no vesicles develop after penetration, but hyphae may still be able to grow in two or more directions inside the epidermal cell (Larez *et al*., 1986). Penetration or attempted penetration may sometimes result in papilla formation by the host, which represents a defense mechanism. The number of papillae is dependent on the specific host, but a high percentage of papillae is observed beneath those appressoria with a low percentage of colonization (Larez *et al*., 1986). Secondary hyphae may be able to extend to the neighboring epidermal cells or penetrate the lower epidermal cell wall and extend into the intercellular spaces of the mesophyll within 24 hours after inoculation. Penetration of the mesophyll cells has never been observed (Dushnicky *et al*., 1996; Dushnicky

et al., 1998a; Dushnicky *et al*., 1998b). In a resistant host, growth of the intercellular hyphae in the mesophyll tissue is restricted and less widespread than in the susceptible host, thus fewer lesions are observed in the resistant host (Loughman and Deverall, 1986). Ingress of the fungus into resistant hosts is usually only stopped after the invasion of the mesophyll tissue is initialized (Lamari and Bernier, 1989b; Dushnicky *et al*., 1996; Dushnicky *et al*., 1998a).

Lateral growth of the fungus in the mesophyll tissue continues until the hyphae reach the large vascular mid-veins of the host (Larez *et al*., 1986; Dushnicky *et al*., 1998b). The xylem and phloem are not invaded by the fungus, although the bundle sheath cells surrounding the large and small mid-veins are often disrupted (Dushnicky *et al*., 1998b). Colonization of host cells by the fungus is often associated with a collapse of the cell membranes and sometimes a disintegration of the chloroplast (Larez *et al*., 1986).

1.3 Virulence in *Pyrenophora tritici-repentis*

1.3.1 Race structure of the pathogen

Initially, isolates of *P. tritici-repentis* were classified into four pathotypes based on their ability to induce necrosis and/or chlorosis on a wheat differential set: pathotype 1 (nec+chl+), pathotype 2 (nec+chl-), pathotype 3 (nec-chl+), and pathotype 4 (nec-chl-) (Lamari and Bernier, 1989a, 1989b). In this wheat differential set, the cv. Glenlea developed only necrosis, line 6B365 only chlorosis, and cv. Salamouni developed neither symptom. This

symptom-based classification system allowed for a maximum of four pathotypes, and could not accommodate isolates that produced the same symptoms but on different wheat hosts (Strelkov and Lamari, 2003). This short-coming was illustrated when Lamari *et al*. (1995) reported a new isolate from Algeria that could induce chlorosis on wheat line 6B662, but was not able to induce chlorosis on wheat line 6B365. Thus, while this isolate was technically pathotype 3 (nec-chl+), it possessed a very distinct virulence pattern from other isolates classified as pathotype 3.

The finding of this new isolate led to a new, race classification system, which was based on the virulence patterns of pathogen isolates on a new wheat differential set. Thus, line 6B662 was added to the differentials (Lamari *et al*., 1995), which allowed the new race classification system to accommodate more races (Lamari *et al*., 2003). The original pathotypes 1, 2, 3, and 4 were replaced by races 1, 2, 3, and 4, respectively, while race 5 included the isolate from Algeria that can caused chlorosis on 6B662 (Lamari *et al*., 1995). Later, race 5 isolates were also reported from the United States (Ali *et al*., 1999), Canada (Strelkov *et al*., 2002), Syria and Azerbaijan (Lamari *et al*., 2005). Isolates representing a new race (race 6) were also subsequently identified from North African collections of *P. tritici-repentis*. Race 6 isolates combined the virulence patterns of races 3 and 5 (Strelkov *et al*., 2002). Another two new races of the pathogen were discovered in the Fertile Crescent and the Caucasus region of central Asia. Race 7 combines the virulence of races 2 and 5,

whereas race 8 combines the virulence patterns of races 2, 3, and 5 (Lamari *et al*., 2003).

To date, only races 1, 2, 3, 4, and 5 have been found in North America, with races 1 and 2 being the most prevalent (Lamari and Strelkov, 2010). Race 1 is predominant in the United States (Ali and Francl, 2001; Ali and Francl, 2003). In western Canada between 1984 and 1987, race 1 was the dominant race and race 2 was rare (Lamari and Bernier, 1989b). However, in leaf samples collected from 1991-1994, race 2 comprised 38% to 50% of the total population (Lamari *et al*., 1998). Samples collected from 2000 to 2002 suggested that 62% to 84% of the isolates collected were race 2 (Singh *et al*., 2007). Singh *et al*., (2007) suggested that race 2 had a selective advantage over race 1 in western Canada, but this may require further study. In addition to these eight races, additional races have been suggested by different research groups. Combining phenotypic and genotypic characterization (Andrie *et al*., 2007), Lepoint *et al*. (2010) found 9 isolates that might belong to new races. One isolate (MUCL 42316) produced small, dark-brown to black spots sometimes surrounded by tan necrotic and chlorotic zones on ‘Glenlea’ and 6B365, but 6B365 was used as a necrosis-insensitive wheat line in the wheat differential set (Lepoint *et al*., 2010). Using the same method, Ali *et al*. (2010) also found some isolates that might represent novel races. The identification of possible new races suggests that the addition of new wheat lines to the differential set should be considered, so as to help identify additional races

(Singh *et al*., 2010). Race classification based on molecular assays such as rapid amplified polymorphisms (RAPD) and amplified fragment length polymorphisms (AFLP) was not successful, as all isolates had unique banding patterns regardless of their race or geographic origin (Friesen *et al*., 2005; Singh and Hughes, 2006; Leisova *et al.*, 2008).

1.3.2 Toxin involvement in disease development

Host-specific toxins (HSTs) are pathogen effectors that induce toxicity and promote disease only in the specific host species and in genotypes of that host expressing a specific and often dominant susceptibility gene. In non-host plants, they are not toxic (Friesen *et al*., 2008). HSTs are generally structurally complex and chemically diverse low molecular mass secondary metabolites or proteins. These HSTs are often essential determinants of pathogenicity or virulence, and in most cases disease does not occur without the HSTs (Wolpert *et al*., 2002). Currently, HSTs have been found in more than 20 pathogens and most of these pathogens are necrotrophic or facultative saprophytic fungi (Markham and Hille, 2001). The best known HST-producing species are *Cochliobolus*, *Alternaria, Pyrenophora*, and *Stagonospora.* Most of these species belong to the order Pleosporales (Friesen *et al*., 2008). *Cochliobolus victoriae* is the causal agent of Victoria blight of oats and it produces victorin (Meehan and Murphy, 1946). *Cochliobolus heterostrophus* race t is the causal agent of southern corn leaf blight and it produces T-toxin (Tatum, 1971).

Cochliobolus carbonum causes northern leaf spot and ear rot of maize and produces HC-toxin (Walton *et al*., 1982). *Alternaria alternata* can cause various diseases on pear, strawberry, apple and tomato, and produces AK-toxin, AF-toxin, AM-toxin, and AAL-toxin, respectively, on these hosts (Wolpert *et al*., 2002). *S. nodorum* is known to produce at least five HSTs, some of which are proteinaceous in nature (Abeysekara *et al*., 2009).

In the case of *P. tritici-repentis*, three HSTs have been reported and more HSTs have been suggested (Lamari and Strelkov, 2010). These three toxins are termed Ptr ToxA, Ptr ToxB, and Ptr ToxC. These toxins are responsible for the necrosis and/or chlorosis symptoms that develop on susceptible wheat lines after infection by toxin-producing isolates of *P. tritici-repentis* (Strelkov and Lamari, 2003). The sensitivity of susceptible wheat genotypes to different races results from a sensitivity to one or more of these toxins (Ciuffetti *et al*., 2010). These toxins act as pathogenicity (Strelkov and Lamari, 2003) or virulence factors (Friesen *et al*., 2003). The Ptr toxins are sufficient to cause disease symptoms on sensitive wheat lines when applied by themselves, and expression of a toxin gene in a non-pathogenic isolate makes it pathogenic (Ciuffetti *et al*., 1997). Among the eight races of *P. tritici-repentis*, races 2, 3, and 5 are regarded as basic races as they produce only one toxin each (Ptr ToxA, Ptr ToxB, and Ptr ToxC, respectively), whereas races 1, 6, 7, and 8 each can produce combinations of two or three toxins and thus are considered as composite races (Strelkov and Lamari, 2003; Lamari and Strelkov, 2010).

For each HST, there is a corresponding sensitivity gene in the host. The recognition of these corresponding genes between the host and pathogen leads to a compatible (susceptible) response, resulting in a mirror image of the classical gene-for-gene system (Strelkov and Lamari, 2003).

1.3.2.1 Ptr ToxA

Ptr ToxA was the first purified and well-characterized HST from *P. tritici-repentis*, and is able to induce necrosis on sensitive wheat genotypes (Ballance *et al*., 1989; Tomás *et al*., 1990; Tuori *et al*., 1995; Zhang *et al*., 1997). The gene encoding for Ptr ToxA (termed *ToxA*) was later cloned (Ballance *et al*., 1996; Ciuffetti *et al*., 1997). Ptr ToxA is a 13.2 kDa secreted protein derived from a preproprotein, which has an additional 23 amino acid signal peptide and a 4.3 kDa pro-domain for proper folding (Tuori *et al*., 2000). The Ptr ToxA protein consists mainly of β-sheets with little α-helix (Zhang *et al*., 1997). *ToxA* is a single copy gene present in all Ptr ToxA-producing races of *P. tritici-repentis* (Ciuffetti *et al*., 1997; Lamari *et al*., 2003). Ptr ToxA is regarded as a pathogenicity factor given the fact that transformation of a non-Ptr ToxA producing isolate with the *ToxA* gene made the isolate pathogenic (i.e., able to cause necrosis on a sensitive wheat genotype) (Ciuffetti *et al*., 1997; Ciuffetti *et al*., 2010).

Wheat sensitivity to Ptr ToxA is located on the 5BL chromosome and is conditioned by a recessive gene *Tsn1* (Lamari *et al*., 1991; Stock *et al*., 1996;

Gamba *et al*., 1998; Anderson *et al*., 1999). Analysis of the mature Ptr ToxA sequence revealed that it contains an arginyl-glycyl-aspartic (RGD) motif at positions 140-142 of the amino acid chain, in a solvent-exposed loop region of the protein (Sarma *et al*., 2005). The RGD motif is believed to bind to an integrin-like plasma membrane receptor protein. Mutations of RGD to RAD or RGE led to a loss of the toxin activity (Meinhardt *et al*., 2002). The binding of the RGD motif to a putative receptor on the wheat mesophyll cell membrane of a Ptr ToxA-sensitive but not of a Ptr ToxA-insensitive host has been reported (Mannig and Ciuffetti, 2005). Interactions between the toxin and the receptor were hypothesized to lead to the internalization of Ptr ToxA (Manning and Ciuffetti, 2005; Manning *et al*., 2007; Manning *et al*., 2008). Co-treatment of Ptr ToxA with another RGD containing peptide may inhibit the interaction of the RGD motif and receptor, thus resulting in a decrease in necrosis symptoms and less internalized Ptr ToxA (Manning *et al*., 2008). Pandelova *et al*. (2009) also reported the up-regulation of a possible RGD motif binding receptor in Ptr ToxA infiltrated toxin-sensitive wheat leaf tissue. Once internalized, Ptr ToxA is localized to chloroplasts and interacts with a chloroplast protein termed ToxA binding protein 1 (ToxABP1). A vitronectin-like loop of Ptr ToxA (137-146) is responsible for the interaction. Mutation from T to A at base 137 resulted in a failure of the interaction (Manning *et al*., 2007). Silencing of *ToxABP1* could reduce the necrosis symptoms (Manning *et al*., 2010). Interaction of Ptr ToxA with ToxABP1 was

postulated to be involved in photosystem (PS) II biogenesis/degradation and/or thylakoid formation and the interaction finally lead to a light-dependent accumulation of reactive oxygen species (ROS) and cell death (Manning *et al*., 2009).

A *ToxA* homolog has also been identified in *S. nodorum,* causal agent of *Stagonospora nodorum* blotch of wheat (Friesen *et al*., 2006). Friesen *et al*. (2006) compared 95 *S. nodorum SnToxA* and 54 *P. tritici-repentis ToxA* amplicons from different regions, and found only one haplotype in *P. tritici-repentis* but 11 *S. nodorum* haplotypes. Therefore, they suggested that the *ToxA* gene from *P. tritici-repentis* originated from *S. nodorum* and was obtained through interspecific gene transfer. Since the necrosis symptoms of tan spot were not widely reported until the 1940s, they suggested that gene transfer could have happened at that time (Friesen *et al*., 2006).

1.3.2.2 Ptr ToxB

In addition to Ptr ToxA, *P. tritici-repentis* also produces another proteinaceous HST named Ptr ToxB. Ptr ToxB is a 6.6 kDa protein that can cause chlorosis on susceptible wheat genotypes (Strelkov *et al*., 1999). This HST is encoded by the *ToxB* gene, which encodes for a 87 amino acid preprotein that contains a 23 amino acid signal peptide (Martinez *et al*., 2001; Strelkov and Lamari, 2003; Martinez *et al*., 2004). Ptr ToxB has been identified from races 5, 6, 7, and 8 of *P. tritici-repentis* (Strelkov *et al*., 2002;

Lamari *et al*., 2003; Strelkov *et al*., 2006). Although races 3 and 4 do not produce active Ptr ToxB, they also possess *ToxB* gene homologs (Strelkov and Lamari, 2003; Martinez *et al*., 2004; Strelkov *et al*., 2006). The homolog of *ToxB* in race 4 (termed *toxb* by Martinez *et al*., 2004) shares 86% similarity with the *ToxB* gene in race 5 (Strelkov and Lamari, 2003; Martinez *et al*., 2004) and is transcribed at a very low level (Amaike *et al*., 2008). Unlike the protein encoded by the 'wild-type' *ToxB* gene from race 5, heterologously expressed Ptr ToxB produced from the *ToxB* homolog of a race 4 isolate had only trace activity (Kim and Strelkov, 2007), suggesting that the differences in the DNA sequence of this gene were responsible for the loss of activity. The flanking up-stream sequence of the *ToxB* homolog in race 3 is different from *ToxB* in race 5, which results in differences in the putative signal peptide but in an identical mature protein (Strelkov *et al*., 2006).

Races 3 and 4 possess only one copy of *ToxB*, while 2 to 10 copies of the *ToxB* gene are present in races of *P. tritici-repentis* possessing Ptr ToxB activity. This suggests a correlation between virulence and gene copy number, with isolates with more copy numbers usually being more virulent (Lamari *et al*., 2003; Martinez *et al*., 2004; Strelkov *et al*., 2006). Amaike *et al*. (2008) compared two race 5 isolates with different virulence levels and found that the level of *ToxB* transcript was correlated with the extent of chlorosis induced on a toxin-sensitive host as well as with greater and more rapid development of appressoria, providing evidence that *ToxB* plays an important role during the

infection process.

Compared with Ptr ToxA, the mechanism for Ptr ToxB action in sensitive wheat genotypes is largely unknown. Recently, Kim *et al*. (2010) examined the effect of Ptr ToxB on susceptible wheat leaves by two-dimensional gel electrophoresis (2DE) and tandem mass spectrometry, and found 66 differentially expressed proteins of the toxin treatment, nearly all of which matched chloroplast-localized proteins. The toxin was also found to inhibit photosynthesis, but only in the Ptr ToxB-sensitive wheat genotype. As such, Kim *et al*., (2010) suggested that Ptr ToxB causes a disruption of the photosynthetic machinery, leading to the generation of ROS and oxidative stress, which finally leads to plant cell death. Indeed, in an earlier study, Strelkov *et al*. (1998) found that the addition of ROS scavengers to Ptr ToxB-treated leaves prevented the development of chlorosis. A Ptr ToxB-insensitivity gene (Tsc2) is located on chromosome arm 2BS and accounts for 69% of phenotypic variation in resistance to race 5 (Friesen and Faris, 2004).

As is the case with *ToxA*, *ToxB* gene homologs have also been found in other ascomycetes including *P. bromi*, *P. teres*, and *P. graminea*, *Cochliobolus* species, *Alternaria* species, and *Magnaporthe grisea*, suggesting that they may share the same ancestor within the ascomycota (Andrie *et al*., 2008). The role of these homologs in these other species, or even in races of *P. tritici-repentis* that lack Ptr ToxB activity, remains to be fully understood.

1.3.2.3 Ptr ToxC

The third HST produced by *P. tritici-repentis* has been termed Ptr ToxC. It is reported to be a non-ionic, polar, low molecular-mass molecule (Effertz *et al*., 2002). It has been partially purified by gel filtration, ion exchange and subsequently reversed-phase chromatography but is not fully characterized. It also causes chlorosis symptoms on wheat, but on different host genotypes than Ptr ToxB. Sensitivity to Ptr ToxC is controlled by a gene located on chromosome arm 1AS, which accounts for 26 to 64% of the variation in host resistance (Effertz *et al*., 2001).

1.3.3 Other factors contributing to the virulence of *P. tritici-repentis*

In addition to Ptr ToxA, Ptr ToxB, Ptr ToxC, other HSTs may also contribute to the virulence of *P. tritici-repentis* (Lamari and Strelkov, 2010). Friesen *et al*. (2002) derived three mutant wheat lines harboring insensitivity to Ptr ToxA from the hard red spring wheat cv. Kulm (which contains a single dominant gene for Ptr ToxA sensitivity) and found that while one of these lines was insensitive to Ptr ToxA, it was only partially resistant to the Ptr ToxA/Ptr ToxC producing race 1. Thus, they concluded that necrosis factors other than Ptr ToxA may also contribute to necrosis symptom development (Friesen *et al*., 2002). Lepoint *et al*. (2010) identified four isolates of *P. tritici-repentis* that could induce necrosis symptoms on sensitive wheat

genotypes, but did not appear to harbor the *ToxA* gene (based on PCR analysis). Ali *et al*., (2010) also found 13 *ToxA* gene-deficient isolates that caused cause necrosis on 'Glenlea' and 6B365 (Ali *et al*., 2010). These studies all suggest the presence of other factors that could contribute to necrosis symptoms.

Besides the HSTs, *P. tritici-repentis* is also known to produce a number of host non-specific toxins such as triticone A, triticone B (Sugawara *et al*., 1988), catenarin, emodin, and islandicin (Bouras and Strelkov, 2008). Catenarin accumulation in wheat kernels can lead to the red discoloration associated with smudge, but this compound appears to have antifungal properties as well (Wakuliński *et al*., 2003; Bouras and Strelkov, 2008).

Since the discovery of the first HST in *P. tritici-repentis* (Ballance *et al*., 1989), most research on tan spot pathogenesis and fungal virulence seems to have been focused on the discovery and understanding of these HSTs. Less attention has been paid to factors other than the toxins which may also contribute to the virulence of *P. tritici-repentis*. Recently, Cao *et al*., (2009) compared the proteome of a pathogenic race 5 isolate (Alg3-24, which possesses wild-type *ToxB* and Ptr ToxB activity) and a non-pathogenic race 4 isolate (90-2, which possesses a *ToxB* homolog but no Ptr ToxB activity), and found 133 differentially abundant proteins. Of those proteins up-regulated in the pathogenic isolate, some had been previously reported to have roles in the pathogenicity of other fungi. These proteins included several secreted proteins

such as α-mannosidase, exo-1,3-β-glucanase, heat-shock proteins, BiP proteins, and a number of other metabolic enzymes. Cao *et al*., (2009) also found that in addition to Ptr ToxB, two other protein spots were unique to the pathogenic isolate. These findings suggest the involvement of other factors that may contribute to virulence or pathogenicity in *P. tritici-repentis*.

1.4 Research objectives

The toxins of *P. tritici-repentis* are responsible for the necrosis and chlorosis symptoms that develop on toxin-sensitive wheat leaves. The sensitivity to these toxins and the susceptibility to toxin-producing isolates of *P. tritici-repentis* are under the control of the same, dominant genes in the host (Larmari and Bernier, 1989b; Larmari *et al.*, 1991). Therefore, the Ptr toxins serve as pathogenicity factors for the tan spot pathogen. However, information on other factors that could contribute to the virulence and/or basic pathogenic ability of isolates of *P. tritici-repentis* is still lacking (Lamari and Strelkov, 2010). A proteome-level comparison of a pathogenic race 5 isolate (Alg3-24, Ptr ToxB-producing) with a non-pathogenic race 4 isolate (90-2, toxin non-producing) revealed significant differences in the quantities of many proteins. Of particular interest were two proteins identified only from the pathogenic isolate, as well as proteins with putative functions in fungal pathogenesis that were more abundant in the pathogenic isolate (Cao *et al*., 2009). The identification of these proteome-level differences between the

isolates raises the question of whether any of these differentially abundant proteins contribute to their differential virulence on wheat.

Thus, the objectives of the present study were: (1) to characterize two proteins identified only in the proteome of the pathogenic race 5 isolate, (2) to characterize an exo-1,3-β-glucanase secreted by both the pathogenic and non-pathogenic isolates, but more abundant in the former, and (3) to examine the effect of silencing of the genes coding these proteins on the virulence of the race 5 isolate on wheat. We hypothesize that while the HSTs may be the most important determinants of virulence in *P. tritici-repentis*, other factors may contribute to the general pathogenic ability of isolates, and silencing of these proteins may result in reduced disease severity. Information obtained in the current study will expand our knowledge of the wheat - *P. tritici-repentis* pathosystem, and may in the long run contribute to sustainable management of tan spot.

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Chapter 2. Characterization and Silencing of Differentially Abundant Proteins from *Pyrenophora tritici-repentis*

2.1 Introduction

Tan spot, caused by the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph: *Drechslera tritici-repentis* (Died.) Shoem), is an important foliar disease of wheat worldwide. Yield losses due to tan spot can be as high as 50% when infection is severe (Hosford, 1982). Tan spot is associated with the appearance of two distinct symptoms on the leaves of susceptible wheat genotypes: tan necrosis and/or extensive chlorosis (Lamari and Bernier, 1989). The development of these symptoms results from the activity of host-specific toxins (HSTs), which are differentially produced by isolates of *P. tritici-repentis* (Strelkov and Lamari, 2003). Three HSTs have been characterized to date: Ptr ToxA, Ptr ToxB, and Ptr ToxC (Lamari and Strelkov, 2010). The Ptr toxins serve as pathogenicity factors for the fungus, and when applied to sensitive leaves, the toxins can reproduce the symptoms associated with fungal infection (Strelkov and Lamari, 2003).

Ptr ToxA is a 13.2 kDa protein encoded by a gene termed *ToxA*, and causes the development of necrosis on sensitive wheat genotypes (Ballance *et al.*, 1996; Ciuffetti *et al.*, 1997). Ptr ToxB is a 6.61 kDa protein encoded by the *ToxB* gene, and causes chlorosis on sensitive wheat genotypes (Strelkov *et al.*, 1999; Lamari *et al.*, 2003; Strelkov *et al*. 2006). Ptr ToxC is also able to

induce chlorosis, but on different host genotypes than Ptr ToxB (Strelkov and Lamari, 2003). Unlike the other Ptr toxins, Ptr ToxC appears to be a low molecular mass, polar, non-ionic compound (Effertz et al., 2002), but has yet to be fully characterized. Isolates of *P. tritici-repentis* are classified into races based on the reaction of a set of wheat differential hosts, which reflects the ability of the isolates to produce the different HSTs, alone or in combination (Strelkov and Lamari, 2003). Eight races of the fungus have been identified, corresponding to the expected number of virulence patterns expected from three HSTs matching three susceptibility genes in the host (Lamari *et al*., 2003; Strelkov and Lamari, 2003).

While the *ToxA* gene occurs as a single copy in races 1, 2, 7 and 8 of *P. tritici-repentis*, *ToxB* occurs as a multi-copy gene in isolates representing races 5, 6, 7 and 8 of the fungus, with 2 to 10 copies of *ToxB* per genome (Lamari *et al*., 2003; Strelkov *et al*., 2006). The virulence of Ptr ToxB-producing isolates has been found to be positively correlated with *ToxB* gene copy number, with those isolates possessing more copies causing greater levels of disease (Lamari *et al*., 2003; Strelkov *et al*., 2006; Amaike *et al.*, 2008). In addition to the wild-type *ToxB* found in Ptr ToxB-producing isolates of *P. tritici-repentis*, *ToxB* homologs have also been found in isolates of races 3 and 4 that do not posses Ptr ToxB activity (Strelkov and Lamari, 2003; Strelkov *et al*., 2006). In non-pathogenic race 4 isolates of *P. tritici-repentis*, the *ToxB* homolog (termed *toxb* by Martinez *et al.*, 2004) shares 86% similarity with the wild-type *ToxB*

(Strelkov and Lamari, 2003; Martinez *et al.*, 2004; Strelkov *et al*. 2006). However, the quantity of *toxb* transcript in race 4 was found to be much lower than the quantity of *ToxB* transcript in a race 5 isolate of the fungus, which possesses strong chlorosis-inducing activity (Amaike *et al.*, 2008), and heterologous expression of the *toxb* gene in *Escherichia coli* yielded a protein with only a trace level of Ptr ToxB activity (Kim and Strelkov, 2007). These investigations indicate the importance of the *ToxB* gene and its copy number in the ability of *P. tritici-repentis* to cause disease. However, in addition to the production of Ptr ToxB, a recent proteome-level comparison revealed that 133 other proteins were differentially abundant in a pathogenic race 5 isolate and a non-pathogenic race 4 isolate of *P. tritici-repentis* (Cao *et al.*, 2009).

A number of the proteins found to be differentially abundant between the pathogenic and non-pathogenic isolates have been implicated in microbial virulence in other pathosystems, while the role of others in pathogenicity, if any, is not clear or unknown (Cao *et al.*, 2009). Two proteins, matching the proteins BC1G_05503 and BC1G_05706 from *Botryotinia fuckeliana*, were identified only in the mycelial proteome of the pathogenic race 5 isolate. The biological functions of these two proteins in *P. tritici-repentis*, however, are not known. Another protein, the enzyme exo-1,3-β-glucanase, was found to be present in the secretomes of races 4 and 5, but its abundance was more than six-fold greater in the pathogenic isolate (Cao *et al.*, 2009). Exo-1,3-β-glucanases cleave glucose residues from the non-reducing terminus of a

β-glucan chain (Martin *et al.*, 2007), a glucose polymer that is widely present in plants as a cell wall component (Stone and Clarke, 1992).

Fungal β-glucanases are thought to play important roles in morphogenesis, mobilization of β-glucans in response to carbon and energy metabolism, cell wall growth and extension, autolysis, and starvation (Martin *et al.*, 2007). Reuveni and coworkers (2007) revealed that the virulence of *Alternaria alternata* isolates causing moldy-core disease of apples is related to their capacity to produce glucanase. Similarly, Huser and coworkers (2009) found that exo-1,3-β-glucanase mutants of *Colletotrichum higginsianum* were less virulent than the wild-type fungus in causing anthracnose disease on *Arabidopsis* seedlings. However, whether or not exo-1,3-β-glucanase has a role in the virulence or pathogenicity of *P. tritici-repentis* has not been evaluated.

Gene knock-out, a strategy to disrupt a target gene with a homologous gene, has been frequently employed in gene function studies. However, Moriwaki and coworkers (2007) have pointed out that gene knock-out via homologous recombination has a low efficiency and is also time-consuming. RNA-mediated gene silencing (RNA silencing) has been shown to be a powerful tool for gene targeting in fungi (Nakayashiki *et al.*, 2005). Double-stranded RNA (dsRNA) is introduced into the organism of interest, where it interferes with the target mRNA either by triggering its degradation or by inhibiting its transcription or translation (Nakayashiki *et al.*, 2005;

Moriwaki *et al*., 2007). In the current study, the RNA silencing vector pSilent-1 (Nakayashiki *et al*., 2005) was used to construct silencing vectors for the genes encoding the hypothetical proteins BC1G_05503, BC1G_05706 and exo-1,3-β-glucanase, in order to explore their functions in *P. tritici-repentis,* particularly in relation to virulence and pathogenicity. It is hypothesized that other proteins, in addition to the Ptr toxins, may contribute to basic parasitic ability in isolates of *P. tritici-repentis*.

2.2 Materials and methods

2.2.1 Fungal material

Three isolates of *P. tritici-repentis*, representing races 4 and 5 of the fungus, were used in the current study. Race 4 isolate 90-2, which was originally obtained from the Canadian prairies, is a non-pathogenic isolate (Lamari *et al*., 1989b) that contains a single copy of the *ToxB* homolog *toxb* (Strelkov and Lamari, 2003). Race 5 isolate Alg3-24 was collected from Algeria and is highly pathogenic (Lamari *et al*., 1995), containing 8-10 copies of the wild-type *ToxB* gene (Strelkov *et al*., 2006). The isolates were propagated in 9 cm-diameter Petri dishes on V8-potato dextrose agar (V8-PDA) (150 ml V8 juice, 3 g $CaCO_3$, 10 g Bacto agar, 10 g Difco PDA, and 850 ml sterile deionized water). To produce mycelium in liquid culture, five plugs (1-cm in diameter) were aseptically excised from the margins of colonies actively growing on V8-PDA plates, and used to inoculate a 250 ml Erlenmeyer flask

filled with 100 ml of Fries' medium (Dhingra and Sinclair, 1986). The flasks were incubated in darkness at room temperature without shaking for 10 or 20 days. Mycelial mats were collected by vacuum filtration of the liquid culture through Whatman No 1 filter paper, washed with sterilized distilled water (sdH_2O), immediately frozen in liquid nitrogen, lyophilized and stored at -20°C until further use.

To induce sporulation, mycelial plugs (8 mm in diameter) produced as above were transferred to the center of fresh V8-PDA plates, and allowed to grow in darkness at room temperature until the colony reached about 5 cm in diameter. The colonies were then flooded with sdH_2O and flattened with a flame-sterilized test tube bottom. The water was decanted and the plates were incubated for 18 h under light at room temperature, followed by 24 h in darkness at 15° C to induce sporulation (Lamari and Bernier, 1989). After the completion of one light-dark cycle, conidia formed on the plate were harvested by flooding the plate with 10 ml sdH_2O and gently dislodging the spores with a sterilized wire loop. The conidial suspension was filtered through two layers of sterilized cheesecloth, and the spore concentration was quantified with a haemocytometer and adjusted to 3,000 conidia/ml with sdH_2O.

2.2.2 Plant material, inoculation, and microscopic analysis

Two wheat (*Triticum aestivum* L.) genotypes, 'Erik' and 6B662, were used as hosts for *P. tritici-repentis*. The cultivar Erik is insensitive to Ptr ToxB and

resistant to both races 4 and 5, whereas 6B662 is Ptr ToxB-sensitive and thus susceptible to race 5 but resistant to race 4.

Wheat seeds were sown in 15 cm diameter plastic pots filled with Sunshine Mix (Sungro Horticulture Canada Ltd., Seba Beach, AB) at a density of eight seeds per pot. The pots were then placed in a growth chamber with a 16 h photoperiod (180 μmol/m^2/s) at 20/18°C (day/night) to allow germination and seedling development. Seedlings were inoculated at the 2- to 3-leaf stage (ca. 13 days old) with a suspension of 3000 conidia per ml (to which 10 drops of Tween 20 [polyoxyethylene sorbitol monolaurate] per L were added), using a Kontes TLC glass sprayer (Krackeler Scientific, USA) connected to a laboratory air line. The leaves were sprayed until run-off. Negative controls were inoculated with sdH_2O containing the same concentration of Tween 20 but no fungal conidia. After inoculation, the plants were first placed in a misting chamber for 24 h and then transferred to a growth chamber and incubated for 5 days.

Leaf tissue (ca. 1 cm sections) from the inoculated fresh leaves was sampled at 3, 6, 12, 24, and 48 h after inoculation, and stored in lactophenol blue solution with 75% (v/v) ethanol (Sigma–Aldrich, USA). For microscopic analysis, the leaf samples were boiled for 3 minutes on a heating block, washed with distilled water, and then mounted on a glass slide with a drop of glycerol for microscopic inspection in a Zeiss Ax10 microscope (Carl Zeiss, NY, USA). Thirty randomly selected conidia per sample were examined for

germination, number of germ tubes and number of appressoria formed per conidium.

2.2.3 Extraction of genomic DNA and total RNA

Genomic DNA was extracted from about 100 mg of 10 day-old lyophilized mycelium using cetyltrimethylammonium bromide (CTAB) according to the method of Moreno *et al,* (2008) and stored at -20°C for further use.

Total RNA was isolated from approximately 50 mg of 10 day-old flash frozen mycelium using an RNeasy Plant Mini Kit (Qiagen, Maryland, USA) following the manufacturer's instructions. RNA samples were further treated with RNase-Free DNase (Qiagen) to remove DNA contaminants. The total RNA concentration was quantified with a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and RNA integrity was confirmed by running 200 to 300 ng of each sample on 1% (w/v) agarose gels.

2.2.4 Primer design, Polymerase Chain Reaction (PCR) conditions, electrophoresis, and DNA sequencing

A protein Blast (blastp) search of the National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using the peptide sequence KELLAGFAAGEIDKLAETKG of the hypothetical protein BC1G_05503 (Cao *et al.*, 2009), yielded a strong match with a signal transduction protein (STP) (Accession no. XM_001941371, E-value = 4×10^{-7})

from race 1 of *P. tritici-repentis*. The cDNA sequence of this signal transduction protein (STP) was then used to design the primers FHT5f and FHT5r (Table 1) with Primer5 software, in order to amplify the *stp* gene encoding the hypothetical protein from race 4 isolate 90-2 and race 5 isolate Alg3-24. Similarly, a Blast search with the peptide sequence KLFIGGLAWHTDENALRE of the hypothetical protein BC1G_05706 (Cao *et al.*, 2009) matched a conserved hypothetical protein (Accession no. XP_001938308.1, E-value = 1×10^{-5}), which was designated UNP (for 'unknown protein') in this study. A Blast search with the exo-1,3-β-glucanase (GLU) peptide sequence RAVGFNWGSEKI (Cao *et al.*, 2009) matched a 1,3-β -glucosidase precursor (Accession no. XP_001934893.1, E-value = 203) from race 1 of *P. tritici repentis*, and an exo-1,3-β-glucanase (Accession no. AAF65310.1, E-value = 9×10^{-3}) from *Cochliobolus carbonum*. The primers Unpf1 and Unpr1 were designed as above to amplify the *unp* gene encoding the conserved hypothetical protein UNP, and the primers Gluf4 and Glur4 were designed to amplify the *glu* gene encoding the exo-1,3-β-glucanase, from isolates Alg3-24 and 90-2 (Table 1). The primer sequences and the gene accession numbers on which the primers were based are summarized in Table 1. All of the primer pairs were designed to include both partial upstream and downstream regions of the target gene in order to obtain the entire gene sequence encoding for each protein.

PCR amplification of the *stp* and *glu* genes was conducted in a 50 µl volume consisting of 300 nM of each forward and reverse primer, 0.3 mM of each dNTP, 25 ng of DNA template, 5 µl of 10 × pfx50 DNA polymerase buffer, and 2 U of pfx50 DNA polymerase (Invitrogen, CA, USA). For amplification of the *unp* gene, which has a high GC content, an additional 2.5 µl of dimethyl sulfoxide (DMSO) was added to the reaction mixture in order to obtain better amplification. The amplification cycle consisted of an initial denaturation step at 94^{o}C for 2 min; followed by 35 cycles of 94 ^{o}C for 20 s, 58 ^{o}C for 30 s, 68 ^{o}C for 30 s (68 ^{o}C for 90 s for the *glu* gene amplification); and a final extension at 68 ^{o}C for 5 min. The resultant PCR product amplified by each set of primers was resolved on 1% (w/v) agarose gels and the amplicon bands were excised from the agarose gel and further purified with a Qiaquick Gel Extraction Kit (Qiagen, Maryland, USA). Purified PCR products were submitted to the Molecular Biology Service Unit of the University of Alberta, Edmonton, AB, for sequencing on an ABI PRISM 3730 DNA Analyzer (Applied Biosystems).

2.2.5 Construction of cloning, expression, and gene silencing vectors

To clone the *stp*, *unp*, and *glu* genes in the GATEWAY cloning vector pDONR221 (Invitrogen, CA, USA), a partial TEV protease cleavage site was included in the forward primers 7f1, 10f1, and 9f1 (Table 2) to facilitate removal of tags that facilitate protein purification (His-tags) when necessary.

To enable subsequent insertion of the amplicons to the pDONR221 vector through the site-specific recombination system of bacteriophage lambda (Invitrogen, CA, USA), the attB1 and attB2 adapter sequences were included in some primers. Briefly, the TEV protease cleavage site and attB1 adapter sequence were included in the forward primer 7f2. The reverse primers 7r, 10r, and 9r contained the attB2 adapter and partial *P. tritici-repentis* DNA sequences (Table 2).

PCR amplifications were conducted in a 50 µl reaction volume consisting of 300 nM of the forward primer 7f2, 30 nM of another forward primer (i.e., 7f1, 10f1, or 9f1 depending on the gene amplified), 300 nM of the reverse primer (i.e., 7r, 10r, or 9r), 0.3 mM of each dNTP, 25 ng of cDNA template, 5 µl of 10× pfx50 buffer, and 2 U of pfx50 DNA polymerase (Invitrogen, CA, USA). An additional 2.5 µl of dimethyl sulfoxide (DMSO) was added to the reaction mixtures for construction of pDONR-*unp*. The *stp* gene was amplified from the cDNA of race 4, and the *unp* and *glu* genes were amplified using the cDNA of race 5. The amplification cycle consisted of an initial denaturation step at 94^{o}C for 5 min; followed by 35 cycles of 94 ^{o}C for 30 s, 57 ^{o}C for 30 s, 68 ^{o}C for 60 s; and a final extension at 68 ^{o}C for 5 min. Amplicons were resolved on 1% (w/v) agarose gels and further purified with a Qiaquick Gel Extraction Kit (Qiagen) as above.

Purified amplicons containing the attP1 and attP2 adapter sequences were inserted between the attP1 and attP2 recombination sites of the GATEWAY

cloning vector pDONR221 through the BP reaction and the plasmid vectors transformed into library efficiency DH5α *Escherichia coli* cells (Invitrogen) as per the manufacturer's instructions. Bacterial colonies formed on Luria-Bertani (LB) (Sambrook and Russell, 2001) agar plates supplemented with kanamycin (50 μg/ml) were selected for further multiplication in LB broth containing kanamycin (50 μg/ml). The plasmid constructs were then harvested from saturated bacterial cultures grown overnight (37^{o}C) and further purified with a Qiaprep Spin Miniprep Kit (Qiagen). The identities of the inserted sequences in the plasmids were verified by sequencing at the Molecular Biology Service Unit of the University of Alberta, as above. After verification, the inserts were subcloned into the GATEWAY expression vector pDEST17 (Invitrogen) through the LR reaction, according to the manufacturer's instructions, in order to generate the expression vectors pDEST17- *stp*, pDEST17- *unp*, and pDEST17- *glu.* Subsequently, the bacterial colonies were cultured overnight at 37^{o}C on LB agar plates containing ampicillin (100 μg/ml) and the plasmids were purified and sequenced again to confirm that they contained the correct gene sequences. The plasmids were further transformed into One Shot *E. coli*_BL21-AI Chemically Competent Cells (Invitrogen) for heterologous protein expression.

The plasmid pSilent-1 (Nakayashiki *et al*., 2005), obtained from the Fungal Genetics Stock Centre (Kansas, USA), was used to construct the RNA silencing vectors for silencing of the three proteins. The primers StXho and

STHind were used to amplify a 347-bp sense PCR fragment from the *stp* gene (Table 3), whilst the primers StStu and StApa were used for amplification of the antisense fragment. For amplification of the sense and antisense fragments of the *unp* (462 bp) and *glu* (788 bp) genes, the following four sets of primers were used (Table 3): UnpXho and UnpHind (*unp* sense fragment); UnpBgl and UnpApa (*unp* sense fragment); GluXho and GluHind (*glu* sense fragment); and GluApa and GluStu (*glu* antisense fragment). The amplified sense and antisense fragments were inserted into the corresponding multiple cloning sites to generate the RNA silencing vectors pSilent1-*stp*, pSilent1-*unp* and pSilent1-*glu*.

2.2.6 Heterologous protein expression and purification

For heterologous expression of the *stp*, *unp* and *glu* genes in *E. coli*, the transformed *E. coli* cells containing the expression vectors for each of the three genes were first grown overnight at 37 ^{o}C with gentle agitation at 250 rpm in 3 ml of LB medium (Sambrook and Russell, 2001) containing 100 μg/ml ampicillin. A 0.5 ml aliquot of each culture was then transferred to a 250 ml Erlenmeyer flask containing 50 ml of LB medium and 100 μg/ml ampicillin, and incubated at 37 ^{o}C with shaking at 250 rpm until the optical density (OD) at 600 nm reached about 0.6. L-arabinose was added to the culture to a final concentration of 0.2% (w/v) to induce the fusion protein expression. After addition of the L-arabinose to the cultures, the bacterial cells

were allowed to grow at 30 ^{o}C for 4 h with shaking at 250 rpm to maximize protein expression. The bacterial cells were collected 4 h after induction by centrifugation of a 100 ml volume of culture at 11,200 × *g* for 10 min at 4 ^{o}C. The cell pellets were re-suspended in 10 ml of lysis buffer (10 mmol/l PBS, Ph 7.4; 500 μg/ml lysozyme; 1 mmol/l PMSF; 20 mmol/l β-mercaptoethanol (β-ME); 10 μg/ml DNase Ⅰ; 10 μg/ml RNase A; 1% (v/v) Triton X-100) and then incubated on ice for 30 min with occasional shaking. The cells were sonicated with 15 × 10 s pulses and a 10 s interval, without sonication between each pulse to prevent warming of the suspension, on an ice-water bath with a sonic dismembrator (Fisher scientific, USA) at 25% amplitude. The sonicated cell mixture was then centrifuged for 10 min at 11,200 × *g* at 4 °C, with both the supernatant and pellet collected. The pellet was re-suspended in 10 ml denaturing lysis buffer (100 mM NaH_2PO_4; 10 mM Tris-HCl; 8 mol/l Urea; 20 mM β-ME; pH 8.0). Aliquots (20 μl) of the supernatant and the re-suspended pellet fraction were mixed with 5× sample buffer (250mM Tris pH 6.8; 10% SDS (w/v); 50% glycerol (v/v); 0.5% bromophenol blue (w/v); 5% β-mercaptoethanol (v/v) and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below, in order to assess expression of the target proteins.

After confirmation of protein expression, the harvested proteins were further purified with a Poly-Prep Chromatography Column (Bio-Rad) containing nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen),

according to the manufacturer's instructions. For the expressed STP protein, only the supernatant was used for purification purposes because of its high solubility. The heterologously expressed UNP protein and exo-1,3-β-glucanase showed little solubility, and therefore the insoluble fractions of these two proteins were used for protein purification with the Ni-NTA agarose resin column.

2.2.7 Protein quantification and electrophoresis

Protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Thermo scientific, Rockford, IL, USA) as a standard. SDS-PAGE was carried out in a mini-Protean cell (Bio-Rad, Hercules, CA, USA) with a Tris-glycine buffer system (100 mm Tris; 300 mm glycine). The proteins were separated on a 5% stacking and 12% separating gel with a thickness of 0.75 mm. After electrophoresis, gel images were exported to a computer and analyzed with GeneSnap (SynGene, MD, USA).

2.2.8 Fungal transformation

For fungal protoplast isolation, mycelial plugs of race 5 isolate Alg3-24 were transferred to V8-PDA plates and conidia produced and harvested as described above. The protoplasts were isolated from fresh conidia using the method of Aboukhaddour *et al*., (2009). For the transformation, 1×10^7 protoplasts in 100 µl of STC buffer (1.2 M Sorbitol, 10 mM Tris, 50 mM

$CaCl_2$ pH 7.5) were mixed with 2 μl of 100 mM spermidine and 4 μg of the silencing plasmid (Table 3). After incubation on ice for 1 h, the suspension was gently mixed with 1 ml of 40% PEG 4000 solution (40% PEG 4000 w/v, 50 mM Tris, 50mM $CaCl_2$, pH 8) and incubated at room temperature for 20 min. The suspension was then mixed with 4 ml of warm liquid regeneration medium (1.2 M sorbitol, 1% yeast, 0.1% casein hydrolysate, 1.5% agar), and the mixture was poured onto a 9 cm-diameter Petri dish filled with approximately 20 ml of solid regeneration medium. After incubation in darkness for 24 h at room temperature, approximately 5 ml of warm regeneration medium supplemented with 100 μg/ml of hygromycin B was overlaid onto the regeneration medium. The plates were then incubated in darkness for about one week at room temperature to allow mycelial regeneration. The hygromycin B-resistant colonies that appeared on the culture medium were transferred to V8-PDA plates containing 200 μg/ml of hygromycin B for a second round of screening. Hygromycin B-resistant colonies identified in the second round of screening were regarded as successful transformants that contained the silencing plasmids for the *stp*, *unp*, and *glu* genes.

2.2.9 Characterization of the fungal transformants

The growth of the fungal transformants and the non-transformed wild-type isolate Alg3-24 was measured on Petri dishes filled with V8-PDA and in liquid

Fries' medium. Briefly, fungal plugs (0.8 cm in diameter) were excised from the margins of a monoconidial colony growing on V8-PDA and were transferred to the centre of fresh V8-PDA plates supplemented with or without 200 μg/ml of hygromycin B. The plates were incubated in darkness at room temperature and colony diameter was measured in two directions at right angles to each other after 5 days. The average colony diameter was determined and used to assess fungal growth. To measure mycelial growth in the liquid Fries' medium, five plugs (1 cm in diameter) were inoculated into 100 ml of Fries' medium in a 250 ml Erlenmeyer flask in the presence or absence of 200 μg/ml hygromycin B. The flasks were incubated in darkness for 20 days at room temperature without agitation. The mycelial mats were vacuum-filtered through Whatman No.1 filter paper, washed with distilled water, and lyophilized. The mass of the lyophilized mycelium was recorded.

The ability of the fungal transformants to produce conidia was assessed by induction of sporulation of both the transformants and the non-transformed wild-type isolate Alg3-24 under the same light-dark cycle as above. After the completion of one light-dark cycle, conidia formed on the plate were harvested in 100 ml sdH_2O and filtered through two layers of sterilized cheesecloth. The conidial concentration in the suspension was measured with a haemacytometer and expressed as number of conidia per square millimeter of the V8-PDA culture.

2.2.10 Quantitative-PCR (q-PCR)

For q-PCR analysis, the housekeeping gene encoding β-actin was selected as an endogenous control and amplified with the primers ActqF1 (5'-CTACGAGCTTCCCGACGGT-3') and ACTqR1 (5'-TCTGGAGCACGGAAACGC-3') (Amaike *et al*., 2008). The primers to amplify the *glu* gene were designed using Primer Express 3.0 (Applied Biosystems, USA) based on the sequence from race 5 isolate Alg3-24, and included the forward primer GluqF7 (5'- CGTGGGCAGAAGGACATTG-3') and the reverse primer GluqR7 (5'GCCTGCTTGCTTGATCTTGTT-3'). The primers for *glu* were designed to amplify a product 135 bp in size near the 5' end of the gene. Approximately 1 μg of total RNA was used for synthesis of the first strand cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). The q-PCR was conducted on a StepOnePlus Realtime PCR System (Applied Biosystems, USA) in a reaction mixture consisting of 7.5 μl of×2 SYBR Green Master Mix (Molecular Biology Service Unit, University of Alberta), 1.25 μl each of 5 μM forward and reverse primer, 300 ng of the cDNA template, and sdH_2O up to 15 μl . The amplification conditions consisted of an initial denaturation step of 95 ^oC for 10 min, followed by 40 cycles of 15 s at 95 ^oC and 1 min at 60 ^oC. After completion of the PCR amplification, a melting curve analysis was run to evaluate the amplification specificity. Primer efficiency was verified using standard curves generated from the purified PCR product of the *glu* and actin genes. Expression of the target gene

relative to the actin gene was calculated via the delta–delta method, with relative expression = $2^{-(\Delta Ct \text{ sample}-\Delta Ct \text{ control})}$ (Livak and Schmitten, 2001). All q-PCR amplifications were performed in triplicate for each of three biological replicates.

2.2.11 Western blotting

Western blotting analysis was carried out to investigate the effect of silencing of the *stp* and *unp* genes on the expression of the corresponding STP and UNP proteins. Polyclonal antibodies specific for UNP and STP were raised in rabbits at the Biosciences Animal Service Unit, University of Alberta, and the antiserum stored as a stock at -20 ^{o}C. For Western blotting analysis, protein samples were extracted from 20-day-old mycelium of the *stp* and *unp* transformants according to the method of Yajima and Kav (2006), and subjected to SDS-PAGE using the Tris-Glycine buffer system described above. Briefly, approximately 20 μg of total protein was loaded on to the polyacrylamide gel, followed by electrophoresis at 150 V until the dye front reached the bottom of the gel. The proteins were then transferred from the SDS-PAGE gel to Polyvinylidene Fluoride (PVDF) membranes (Bio-Rad) using a trans-blot SD semi-dry transfer cell (Bio-Rad) at 15 V for 20 min. Following transfer, the membranes were incubated in a blocking buffer solution consisting of 5% w/v non-fat dry milk in TBS buffer (50 mM Tris, 150 mM NaCl, pH7.5) overnight at 4^{o}C with agitation. The membranes were

then rinsed four times (10 min per wash) in TTBS (0.05%v/v Tween-20 in TBS) and incubated for 1 h with the polyclonal antibodies raised against STP or UNP, diluted 1/3000 in antibody buffer (1% w/v non-fat milk in TTBS). The membranes were rinsed four times (10 min per rinse) with TTBS and incubated for 1 h in antibody buffer containing horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, CA, USA) (1/3000 dilution). The membranes were further washed 4× in TTBS and once in TBS, and stained with Peroxidase Substrate Kit TMB (Vector Laboratories Inc, Burlingame, CA). Images were scanned with a GS-800 Calibrated Densitometer and analyzed with Quantity One software (Bio-Rad).

2.2.12 Glucanase activity assay

As a result of the difficulty in purifying heterologously expressed exo-1,3-β-glucanase, it was not possible to raise polyclonal antibodies against this protein. Thus, a native enzyme assay was employed to evaluate the effect of silencing the *glu* gene on mixed glucanase activity (Izgü *et al*. 2006). Aliquots (100 ml) from 20 day-old culture filtrates of race 4 isolate 90-2, the wild-type race 5 isolate Alg3-24 and the transformants, produced in Fries' medium as described above, were lyophilized and re-dissolved in 5 ml of 50 mM sodium acetate buffer (pH 4.6) and centrifuged at 11,200 × *g* for 10 min. The supernatant was collected and dialyzed overnight against water at 4 ^{o}C in 1,000-molecular mass cut-off dialysis tubing. The dialyzed samples were then

lyophilized and re-dissolved in 50 mM sodium acetate buffer (pH 4.6). Glucanase activity was determined by measuring the reducing sugars produced from the degradation of laminarin from *Laminaria digitata* (Sigma-Aldrich, USA), usually used as a substrate for 1,3-β-glucanase, as described by Izgü *et al*. (2006). Briefly, 3 µg of total protein was mixed with 250 µl of 50 mM sodium acetate buffer (pH 4.5) containing 0.25% laminarin, and incubated at 30 °C for 1h. The reaction was stopped by boiling for 5 min in a water bath. Glucose formation was measured with a Glucose HK Kit (Sigma-Aldrich) according to the manufacturer's instructions. One unit of 1,3-β-glucanase was defined as the amount of enzyme that liberated 1 mg of reducing sugars per min under the conditions described above. Mixed glucanase activity in the silenced strains was expressed relative to that of the wild-type isolate Alg3-24, in which the activity contained in 1 µg of total protein was defined as 100% (1.00).

2.2.13 Statistical analysis

Data were analyzed for statistical significance using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute, Cary, NC). For the data set on the number of germ tubes and appressoria produced per conidium, a square root transformation (0.5 was added to the raw data set because it contained zeros) was used to restore homogeneity of variance before subjecting it to statistical comparison. Otherwise, raw data

were analyzed without transformation if the assumptions of analysis of variance were not violated. All of the data were subjected to one-way analysis of variance when appropriate, and means were separated by Fisher's least significant difference (LSD) at the 5% or 1% level.

2.3 Results

2.3.1 Gene isolation

PCR analysis using each of three sets of the primers FHT5f/FHT5r, Unpf1/Unpr1, and Glur4/Gluf4, enabled amplification and sequencing of the *stp*, *unp*, and *glu* genes, respectively, from isolates representing races 4 and 5 of *P. tritici-repentis.*

A genomic DNA fragment 494 bp in size and corresponding to the *stp* gene was sequenced from isolates representing races 4 and 5. The *stp* sequence from the pathogenic race 5 isolate Alg3-24 was identical to that of the pathogenic race 1 isolate Pt-1C-BFP, for which the entire genome sequence is available (Broad Institute, www.broadinstitute.org); the sequence from the race 4 isolate shared 99.8% homology to the sequences from races 1 and 5 (Fig. 1). The ORF of STP from races 1, 4, and 5 was 393 bp in size, with the deduced amino acid sequences sharing 99.2% homology. The encoded protein was 130 amino acids (aa) long in all three races, with a predicted molecular mass of 15.0 kDa. Annotation of the nucleotide sequence of the *stp* gene with SignalP (http://www.cbs.dtu.dk/services/SignalP/) indicated an absence of a

signal peptide. A blast search of the STP amino acid sequence from the race 5 isolate revealed more than 78% identity with a CipC-like antibiotic response protein from *Stagonospora nodorum* (Accession no. XP_001801331) and 56.2% identity with the CipC-like antibiotic response protein from *Aspergillus fumigatus* (Accession no. XP_753706).

The nucleotide sequences of the *unp* gene from races 1, 4, and 5 were all 774 bp in length with 99.9% homology (Fig. 2). The ORF of UNP was 510 bp in size in races 4 and 5, and 507 bp in size in race 1. The deduced amino acid sequence shared 99.7% homology among the three isolates. In races 4 and 5, *unp* encoded a protein 169 aa in length with a calculated molecular mass of 16.9 kDa, whereas in race 1 it encoded a protein 168 aa in length with a molecular mass of 16.7 kDa. A blast search of the UNP amino acid sequence revealed that it belongs to the superfamily of glycine rich RNA binding proteins (GRPs) (Kim *et al*., 2007).

The ORF of the exo-1,3-β-glucanase was 1266 bp in size in 90-2 and Alg3-24, the same size as in Pt-1C-BFP (Broad Institute), with the sequences from the three isolates sharing 99.9% homology (Fig. 3). The encoded protein was 421 amino acid residues in length with a calculated molecular mass of 46.7 kDa and 99.8% homology among the isolates. A signal peptide 18 amino acid residues in length was identified with SignalP (www.cbs.dtu.dk/services/SignalP).

2.3.2 Heterologous protein expression and purification

All fusion proteins (i.e., STP, UNP, and exo-1,3-β-glucanase) were successfully over-expressed in *E. coli*. STP was expressed in the soluble fraction, whereas most of the over-expressed UNP and exo-1,3-β-glucanase was found in the insoluble fraction. Changing the length of the incubation period and lowering the incubation temperature did not enhance solubility (data not shown). Thus, the soluble fraction was used for purification of the over-expressed STP, and the insoluble fractions were used for purification of over-expressed UNP and exo-1,3-β-glucanase. As the heterologously expressed proteins contained a hexahistidine-tag (His-tag), they were run through a Ni-ATA-agarose column for affinity purification. The expected molecular masses (including the His-tags) were 18.3 kDa, 20.3 kDa and 48.3 kDa for STP, UNP and exo-1,3-β-glucanase, respectively. Over-expressed proteins of the corresponding masses were observed for all three proteins on polyacrylamide gels (Fig. 4) following Ni-ATA agarose purification. However, while single bands were observed for STP and UNP, two bands could be observed for the exo-1,3-β-glucanase, suggesting that this protein was not purified to homogeneity (Fig. 4).

2.3.3 pSilent-1-based gene silencing and analysis of transformants

RNA-silencing of the *stp*, *unp*, and *glu* genes was carried out with the pSilent-1 vector. This vector carries a hygromycin resistance cassette as a

selectable marker and a transcriptional unit for hairpin RNA expression (Nakayashiki *et al*., 2005). DNA fragments of each of the genes were inserted into the pSilent-1 vector as inverted repeats, which were flanked by the cutinase intron spacer from *Magnaporthe oryzae*, to generate the silencing vectors pSilent1-*stp*, pSilent1-*unp*, and pSilent1-*glu*. After transformation, one, two and four hygromycin-resistant transformants were obtained for STP, UNP, and exo-1,3-β-glucanase, respectively.

The extent of *glu* silencing was monitored by q-PCR, since the silencing would lead to the post-transcriptional degradation of the targeted mRNA. Total RNA was extracted from both the wild-type and transformants. The q-PCR results revealed a reduction in the amount of *glu* transcript in all of the transformants. The extent of silencing varied among the transformants, however, and ranged from 42% to 61% of the wild-type (Table 4). Since a single exo-1,3-β-glucanase band could not be obtained to raise antibodies for Western blotting analysis, mixed glucanase activity was assessed instead (Izgü *et al*. 2006); this activity was assessed in 20-day-old culture filtrates, but was not significantly reduced in any transformant relative to the wild-type race 5 isolate Alg3-24. In contrast, mixed glucanase activity in the race 4 isolate was 48% of that in the race 5 isolate (Table 5).

The abundance of STP and UNP proteins was assessed by Western blotting analysis in order to verify the silencing efficiency in the transformants at the protein level. The results revealed that the amount of STP found in 10-day-old

fungal mycelium of the *stp*-silenced transformant was similar to that found in the wild-type isolate (85.6% of wild-type) (Fig. 5). In contrast, while one of the two *unp*-silenced transformants (‘unps’) produced a level of UNP similar to that in the wild-type (91.6% of wild-type), the other (‘unpa’) produced significantly less, based on the intensity of the reacting band (59.8% of wild-type) (Fig. 5). As a consequence of the poor of silencing of the *stp* transformant and the *unp* transformant ‘unps’ at the protein level, these were excluded from further analysis. Interestingly, while STP and UNP appeared to be unique to the mycelium of Alg3-24 as determined by 2-dimensional electrophoresis (Cao *et al*. 2009), Western blotting analysis revealed that both proteins are also present in 90-2 (Fig. 5).

2.3.4 Phenotypic analysis of the transformants

All of the transformants were able to grow on V8-PDA medium, and with the exception of the *glu*-silenced transformant C1, all produced colonies similar in morphology and color to the wild-type race 5 isolate Alg3-24 (Fig. 6). C1 also exhibited the lowest amount of growth on V8-PDA and liquid Fries’ medium (Table 6). Growth of C2 was also significantly ($P<0.01$) lower than the wild-type both on V8-PDA and Fries’ medium. In contrast, the growth of the *glu*-silenced transformants C3 and C4 did not differ significantly from the wild-type on V8-PDA, but they both produced significantly less biomass on the Fries’ medium ($P<0.01$) (Table 6). Sporulation was similar to the wild-type

in all of the transformants with the exception of C1, in which it was significantly lower (Table 6). This transformant (C1) also produced conidium-like structures that lacked the septa associated with 'normal' conidia of *P. tritici-repentis*, and which were not observed in any other strains of the fungus (Fig. 7). Growth and sporulation of the *unp*-silenced transformant unpa were not significantly different from the wild-type on V8-PDA ($P<0.01$), but growth was lower in the liquid Fries's medium.

2.3.5 Conidial germination and appressorium formation

The conidial germination rate of the *glu*-silenced transformant C1 at 3 h after inoculation was 63% and 83% on the susceptible and resistant host genotypes, respectively, which was significantly lower than the rates observed for the wild-type isolate Alg3-24 and the other transformants (which ranged between 90% and 100%). However, at 12 h after inoculation, the germination rate of C1, the wild-type isolate and all of the other transformants approached 100% (data not shown). The transformant C1 produced an average of between 0.80 to 1.17 germ tubes per conidium from 3 to 48 h after inoculation, which was significantly ($P<0.01$) lower than the 1.73 to 2.87 germ tubes per conidium produced by the wild-type isolate and the other transformants (Table 7). Similarly, the number of appressoria per conidium produced by the transformant C1 was significantly ($P<0.01$) lower than that produced by the wild-type Alg3-24 and the other transformants at most of the time-points examined (Table 8). Germination rates and appressorium formation in the

unp-silenced transformant unpa were similar to the wild-type (data not shown). Both of these parameters (germination rate and the number of appressoria produced per conidium) were generally similar for each fungal strain on the resistant and susceptible host genotypes.

2.3.6 Pathogenicity of transformants

Inoculation of all the transformants and the wild-type race 5 isolate Alg3-24 of *P. tritici-repentis* onto wheat line 6B662 and cv. Erik resulted in the development of typical chlorosis symptoms on the former and no chlorosis on the latter (Fig. 8). However, symptom severity on 6B662, measured as the percent diseased leaf area (DLA), was significantly ($P<0.01$) lower in the transformants C1, C3 and C4 than in the wild-type (Fig. 8). The DLA was 16.9%, 32.5% and 33.5% for C1, C3 and C4, respectively, while it was 53.7% for the wild-type; the DLA for C2 was 52.4%, which was significantly higher than for C1, but not significantly different from the wild-type or C3 and C4. The symptom severity caused by the *unp*-transformant unpa did not significantly differ from the wild-type (Fig 8). All transformants induced the formation of small necrotic flecks on the wheat cv. Erik, representing a typical resistance reaction. However, the number of flecks caused by C1 was very small.

2.4 Discussion

The silencing of genes encoding proteins previously reported to be differentially abundant in pathogenic and non-pathogenic isolates of *P. tritici-repentis* (Cao *et al.*, 2009) enabled an assessment of their role, if any, in fungal pathogenicity or virulence. While Cao *et al*. (2009) had found that STP and UNP appeared to be unique to the proteome of the pathogenic race 5 isolate Alg3-24, and absent from the proteome of the non-pathogenic race 4 isolate 90-2, this does not seem to be the case, as both proteins were identified via Western blotting analysis in the mycelium of 90-2 (Fig. 5). This discrepancy is likely a reflection of the protein detection techniques employed in the two studies; Cao *et al.* (2009) used Colloidal blue staining to analyze protein abundance, whereas in the current study Western blotting with antisera raised against the specific proteins was utilized, representing a much more sensitive technique.

In the case of STP, the proteins appeared to be of a different mass in races 4 and 5, with a reacting band of approximately 17 kDa in the case of race 5 and 15 kDa in the case of race 4 (Fig. 5). Since the proteins were predicted to share 99.2% homology and be 15 kDa in mass based on the sequence analysis, this finding would suggest post-translational modification or some other processing of STP in race 5, which would affect protein mobility on an SDS-PAGE gel (Gutierrez *et al.*, 1989). Indeed, a glucosaminoglycan attachment site was predicted in the form of the protein from the race 5 isolate Alg3-24, which was absent from the race 4 protein (http://elm.eu.org/).

Despite the occurrence of STP in both races 4 and 5, a blast search of the genome database of the race 1 isolate Pt-1C-BFP (www.broadinstitute.org) did not reveal any matching proteins in this race of *P. tritici-repentis* other than STP. Unfortunately, the function of STP could not be assessed in this study because the abundance of the protein in the *stp*-transformant was similar to that in the non-transformed wild-type (Fig. 5), suggesting that the *stp* gene was not successfully silenced. A blast search of the STP amino acid sequence from race 5 of *P. tritici-repentis* revealed that it has 56.2% identity with a CipC-like antibiotic response protein from *Aspergillus fumigatus* (Accession no. XP_753706, E-value= 6×10^{-33}). CipC-like protein was first identified as a concanamycin-induced protein in *A. nidulans*, when this fungus was cultured on yeast nitrogen base medium (Melin *et al*., 2002). It has also been identified in many other fungi, but its function is still largely unknown. Tan *et al*. (2008) found that a CipC-like protein was significantly more abundant in wheat leaves inoculated with *Stagonospora nodorum* than in uninoculated leaves, although disruption of the gene had no effect on the pathogenicity or phenotype of *S. nodorum*.

The *unp* gene appeared to be successfully silenced in one of the transformants, unpa, based on Western blotting analysis to estimate protein quantity (Fig. 5). However, the phenotype, growth rate, sporulation rate, and virulence of this transformant were not significantly different from the wild-type. A Blast search revealed that the UNP protein belongs to the

superfamily of glycine-rich RNA binding proteins (GRPs) (Kim *et al*., 2007). However, the function(s) of most of the proteins sharing high identity with UNP in other fungi remains unknown. The most similar protein to UNP with a known function is Crp2 from *Metarhizium anisopliae* (Accession no. ACL52165), which shares an amino acid sequence identity of 38.7% with *unp* (E-value = 5×10^{-33}) and is reported to contribute to cold and freezing tolerance in *M. anisopliae* (Fang and St Leger, 2010).

Silencing of the *glu* gene encoding GLU (exo-1,3-β-glucanase) allowed the generation of four transformants (C1, C2, C3, and C4). Reduced quantities of the corresponding transcript were observed in all of these transformants, based on q-PCR analysis (Table 4). However, the enzyme activity assay conducted revealed that mixed glucanase activity did not decrease in the transformants, which was not in accordance with the q-PCR results. In the current study, laminarin was used as the substrate in the mixed glucanase activity assay. Laminarin is a β-(1→3)-linked d-glucan with occasional β-(1→6)-linked branches (Read *et al*., 1996). It is possible that other glucanases, such as exo-1,6-β-glucanase or endo-1,3-β-glucanase, could have confounded the results, since the enzyme assay was not specific for exo-1,3-β-glucanase activity. Martin *et al* (2007) also found that more than one form of exo-1,3-β-glucanase are present in many fungi. A Blast search of the exo-1,3-β-glucanase amino acid sequence revealed the presence of at least five other *P. tritici-repentis* hypothetical 1,3-β-glucanases, which shared

49.4%, 41.3%, 32.5%, 26.1%, or 18.1% identity with the GLU silenced in the current study. However, caution should be employed when identifying proteins based solely on homology since exo-1,3-β-glucanase is one of many glycosyl hydrolases (Davies and Henrissat., 1995) and additional research would be needed to conclusively establish the identity of the protein encoded by *glu*.

One of the *glu*-silenced transformants, C1, exhibited a different growth morphology relative with the wild-type isolate (Fig. 6). This transformant also grew more slowly than the wild-type and was a poor sporulator (Table 6). Gene knock-out studies with β-glucanase resulted in decreased growth rates in *Cochliobolus carbonum* (Kim *et al*., 2001; Martin *et al*., 2007). Hosford (1971) found that continuous subculture of a dark colony of *P. tritici-repentis* can result in a change of colony color from dark to white, along with slower colony growth. Hunger and Brown (1987) also reported the same phenomenon, but noted that white colonies did not always grow more slowly or sporulate more poorly than dark colonies. In the current study, the reduced growth and white color of the C1 colonies likely resulted from the success of gene silencing rather than continuous subculturing of the fungus, since C1 was not subcultured more than any of the other silenced strains or the wild-type isolate (which maintained a 'normal' colony phenotype, growth and sporulation rates). It seems probable that since the *glu* gene was strongly silenced in C1 (Table 4), the impact of this silencing was strongest in this strain. In the other strains, in which GLU silencing was not as strong, the activity of 1,3-β-glucanases other

than the exo-1,3-β-glucanase encoded by *glu* may have been sufficient to compensate for the loss of GLU activity. Nonetheless, additional research may be needed to rule out the possibility that silencing of *glu* in C1 had any other unexpected impact on this strain.

Amaike and coworkers (2008) found a strong correlation between *ToxB* gene expression and appressorium formation, and suggested a possible relationship between the ability of fungal isolates to produce Ptr ToxB and form appressoria. In the current study, the transformant C1 also showed reduced appressorium formation (Table 8), suggesting a reduction in basic parasitic ability. However, while in the earlier study the amount of *ToxB* transcript was not correlated with features that are not exclusively associated with parasitic ability (such the number of germ tubes per conidium) (Amaike *et al.*, 2008), these parameters were affected by the strong silencing of GLU, at least in the transformant C1 (Table 7). This observation, combined with the fact that growth rate (Table 6) and colony morphology (Fig. 6) were affected in C1, would indicate some impact of the silencing of GLU on other biological processes in *P. tritici-repentis*, beyond those related to virulence or pathogenicity.

Fungal β-glucanases have been implicated in morphogenesis, mobilization of β-glucans, cell wall growth and extension, autolysis, and carbon depletion (Martin *et al.*, 2007). Exo-1,3-β-glucanases have also been shown to have an impact on fungal virulence or pathogenicity. In one recent

study, an exo-1,3-β-glucanase mutant of *Colletotrichum higginsianum* exhibited reduced virulence on *Arabidopsis thaliana* (Huser *et al.*, 2009). In another study, the virulence of *A. alternata* isolates causing mouldy-core disease of apples was found to be related to their capacity to produce glucanase (Reuveni *et al.*, 2007). In contrast, mutation analysis of an exo-1,3-β-glucanase encoded by *EXG2* (Accession no. AAF65310) in *Cochlibolus carbonum*, which shares 72.7% identity with the *glu* silenced in this study, did not affect the pathogenicity of *C. carbonum* on maize (Kim *et al*., 2001).

While Ptr ToxB appears to be an important pathogenicity factor for *P. tritici-repentis* (Strelkov *et al.*, 1999; Strelkov and Lamari, 2003), and silencing of the *ToxB* gene in race 5 resulted in a significant loss of virulence (Abboukhaddour *et al.*, 2010), other molecules may also contribute to the biological fitness, basic parasitic ability and/or virulence of this fungus. The results of the current study revealed that silencing of the *glu* gene resulted in a transformant with reduced growth, sporulation and virulence, suggesting that exo-1,3-β-glucanase encoded by *glu* plays a role in the morphology and virulence of *P. tritici-repentis*.

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Table 1. Primers used for PCR amplification of the gene sequences encoding signal transduction protein (STP), unknown protein (UNP) and exo-1,3-β-glucanase (GLU).

Primer	Sequence (5'-3')	GenBank Accession No.
FHT5f	GCAGAGTAAGCATTGTAAGCATT	XM_001941371
FHT5r	ACCTTCAATCAACTCCATCATAA	XM_001941371
Unpf1	CCGTTCAGTGTTCGACCCAGACAT	XM_001938273
Unpr1	ATTCGCCCCTTCCCGTCATACTAA	XM_001938273
Gluf4	ACCCACAGTATCTCATACTACTTC	XM_001934858.1
Glur4	ATCCAACGTCCCATTTAGCTT	XM_001934858.1

Table 2. Primers used for construction of the gene cloning vectors pDONR-*stp*, pDONR-*unp* and pDONR-*glu*.

Primer	Sequence[a] (5'-3')	Vector
7f1	GAGAACCTGTACTTTCAGGGT**ATGGGTTTCTGGGACAACAAG**'	pDONR-*stp*
7f2	*GGGGACAAGTTTGTACAAAAAAGCAGGC*TCGGAGAACCTGTACTTTCAG'	pDONR-*stp*
7r	*GGGGACCACTTTGTACAAGAAAGCTGGGT***CCGTCCGTCAATCTCCCGCCTA**	pDONR-*stp*
10f1	GAGAACCTGTACTTTCAGGGT**ATGTCTAAGCTCTTCATTGGGTAAGTATG**	pDONR-*unp*
7f2	*GGGGACAAGTTTGTACAAAAAAGCAGGC*TCGGAGAACCTGTACTTTCAG	pDONR-*unp*
10r	*GGGGACCACTTTGTACAAGAAAGCTGGGT***CGAGGGTTCGTCAGCGTGTTTA**	pDONR-*unp*
9f1	GAGAACCTGTACTTTCA**GGGTGCCCCAACTGAGAAGCGT**	pDONR-*glu*
7f2	*GGGGACAAGTTTGTACAAAAAAGCAGGC*TCGGAGAACCTGTACTTTCAG	pDONR-*glu*
9r	*GGGGACCACTTTGTACAAGAAAGCTGGGT***CAACGTCCCATTTAGCTTCTAGC**	pDONR-*glu*

[a] Gene-specific primer sequences are noted in bold text, TEV protease cleavage sites are underlined, and attB1 and attB2 adapters are shown in italics.

Table 3. Primers used in construction of the silencing vectors pSilent1-*stp*, pSilent1-*unp*, pSilent1-*glu*.

Primer	Sequence[a](5'-3')	Restriction enzyme	Silencing vector
StXho	CCGCTCGAGTGGGACAACAAGGGAGAGAA	XhoⅠ	pSilent1-*stp*
STHind	CCCAAGCTTTACTGGTTGGGGTCGTATTG	HindⅢ	pSilent1-*stp*
StStu	AAAAGGCCTTACTGGTTGGGGTCGTATTG	StuⅠ	pSilent1-*stp*
StApa	GCGGGGCCCTGGGACAACAAGGGAGAGAA	ApaⅠ	pSilent1-*stp*
UnpXho	CCGCTCGAGCATACCGACGACCAAGCTCT	XhoⅠ	pSilent1-*unp*
UnpHind	CCCAAGCTTACCACCCTGACCACCTTCTT	HindⅢ	pSilent1-*unp*
UnpBgl	GGAAGATCTACCACCCTGACCACCTTCTT	BglⅡ	pSilent1-*unp*
UnpApa	GCGGGGCCCCATACCGACGACCAAGCTCT	ApaⅠ	pSilent1-*glu*
GluXho	CCGCTCGAGTCCCAGAACGGATTCGACAA	XhoⅠ	pSilent1-*glu*
GluHind	CCCAAGCTTTGAGCCGCCGAACTTGTAGT	HindⅢ	pSilent1-*glu*
GluStu	AAAAGGCCTTGAGCCGCCGAACTTGTAGT	StuⅠ	pSilent1-*glu*
GluApa	GCGGGGCCCTCCCAGAACGGATTCGACAA	ApaⅠ	pSilent1-*glu*

[a] Restriction enzyme recognition sites are underlined and were used for cloning of the amplicons into the pSilent-1 vector.

Table 4. Quantitative PCR analysis of the relative expression of the *glu* gene, coding for exo-1,3-β-glucanase, in 10-day-old mycelium of the wild-type race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis* and the *glu*-silenced transformants C1, C2, C3 and C4.

Relative expression[a]				
Alg3-24	C1	C2	C3	C4
1.00 (0.66-1.41)	0.43 (0.42-0.44)	0.42 (0.32-0.72)	0.54 (0.33-1.01)	0.61 (0.50-0.82)

[a] Gene expression data were normalized using an actin endogenous control. Relative expression was calculated against *glu* transcript level in the wild-type isolate Alg3-24 according to the comparative C_T method for relative quantification of gene expression (Livak and Schmitten, 2001), with expression in Alg3-24 designated as 1.00. The range of relative *glu*-expression observed for each fungal strain is indicated in parentheses and represents variation in three biological replicates.

Table 5. Mixed glucanase activity in 20-day-old culture filtrates of the non-pathogenic race 4 isolate 90-2, pathogenic race 5 isolate Alg3-24, and transformants of *Pyrenophora tritici-repentis* (C1, C2, C3 and C4) that were silenced for the *glu* gene, coding for exo-1,3-β-glucanase.

Relative activity[a]					
Alg3-24	90-2	C1	C2	C3	C4
1.00±0.02a	0.48±0.07b	1.00±0.09a	1.05±0.46a	1.12±0.18a	1.48±0.01a

[a] Each value represents the mean of two replications ± standard error. Means followed by the same latter are not significantly based on Fisher's least significant difference at $P<0.01$. Glucanase activity was calculated from the amount of glucose released with laminarin as substrate (Izgü *et al*. 2006). Activity is indicated relative to race 5 isolate Alg3-24, in which the enzyme units in 1 μg of total protein were defined as 100% (1.00).

Table 6. Growth and sporulation of pathogenic race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis* and transformants that were silenced for the *glu* gene, coding for exo-1,3-β-glucanase (C1, C2, C3 and C4), or *unp*, coding for a protein with unknown function (unpa).

Isolate or transformant	Mycelial growth		Sporulation ($\times 10^2/mm^2$)[cd]
	V8-PDA (diameter in mm)[a]	Fries' medium (dry mass in g)[b]	
Alg3-24	5.88±0.03a	0.99±0.00a	1.55±0.11a
C1	4.03±0.02c	0.08±0.00d	0.11±0.00b
C2	5.57±0.03b	0.41±0.00c	1.54±0.12a
C3	5.80±0.08a	0.87±0.03b	1.69±0.08a
C4	5.70±0.03ab	0.86±0.01b	1.41±0.08a
unpa	5.70±0.02ab	0.78±0.04b	1.52±0.22a

[a] Colony diameters were measured after 5 days of incubation on V8-potato dextrose agar (PDA) medium.

[b] Growth on Fries' medium represents the average mass of lyophilized mycelium from 20-day-old cultures.

[c] Sporulation is expressed as the number of conidia produced per square millimeter of the colony grown on V8- PDA culture.

Each value represents the mean of three replicates (cultures) ± standard error. Means followed by the same letter are not significantly different based on Fisher's least significant difference at $P<0.01$.

Table 7. Mean number of germ tubes produced per conidium of *Pyrenophora tritici-repentis* after inoculation of wheat line 6B662 and cv. Erik with wild-type isolate Alg3-24 or transformants silenced for the *glu* gene, coding for exo-1,3-β-glucanase.

		Time after inoculation (h)				
Isolate	Host	3 [a]	6	12	24	48
Alg3-24	6B662	2.00(1.55 a)	1.93(1.54a)	2.10(1.60a)	2.07(1.59b)	2.40(1.68a)
	Erik	2.03(1.57 a)	1.83(1.50a)	2.20(1.63a)	2.03(1.58b)	2.20(1.63ab)
C1	6B662	0.80(1.09b)	0.97(1.18b)	0.93(1.20b)	1.03(1.21c)	1.13(1.27d)
	Erik	1.00(1.20b)	0.97(1.19b)	1.13(1.26b)	0.80(1.12c)	1.17(1.27d)
C2	6B662	1.93(1.53a)	2.03(1.57a)	2.00(1.56a)	1.93(1.54b)	1.83(1.51bc)
	Erik	1.80(1.49a)	1.93(1.54a)	1.73(1.48b)	2.03(1.58b)	2.03(1.58abc)
C3	6B662	2.13(1.59a)	2.13(1.59a)	2.13(1.59a)	2.33(1.66b)	2.33(1.67a)
	Erik	2.00(1.56a)	1.80(1.49a)	2.20(1.61a)	2.87(1.82a)	2.33(1.67a)
C4	6B662	2.10(1.59a)	2.17(1.62a)	2.07(1.59a)	2.13(1.61b)	2.13(1.61abc)
	Erik	2.20(1.63a)	2.00(1.55a)	2.10(1.58a)	2.20(1.64b)	1.73(1.47c)

[a] The number of germ tubes represents the mean from 30 randomly selected conidia at each time-point after inoculation of 6B662 and cv. Erik. ANOVA was run to compare isolate*host, with 0.5 added to the original data and square root transformed prior to statistical analysis. Transformed data are included in the parentheses. Numbers followed by the same latter are not significantly different based on Fisher's least significant difference at $P<0.01$.

Table 8. Mean number of appressoria produced per conidium of *Pyrenophora tritici-repentis* after inoculation of wheat line 6B662 and cv. Erik with wild-type isolate Alg3-24 or transformants silenced for the *glu* gene, coding for exo-1,3-β-glucanase.

		Time after inoculation (h)				
Isolate	Host	3	6	12	24	48
Alg3-24	6B662	0.93(1.17cde) [a]	1.27(1.30ab)	1.30(1.31ab)	1.47(1.39ab)	1.47(1.39abc)
	Erik	1.00(1.18bcde)	1.10(1.23bc)	1.37(1.35ab)	1.30(1.32bc)	1.40(1.36bcd)
C1	6B662	0.57(0.98e)	0.73(1.08c)	1.00(1.20bc)	0.93(1.17cd)	1.00(1.21d)
	Erik	0.70(1.06de)	0.77(1.10c)	0.77(1.10c)	0.77(1.10d)	1.10(1.24cd)
C2	6B662	1.53(1.38ab)	1.63(1.42a)	1.53(1.40a)	1.67(1.45ab)	1.67(1.45ab)
	Erik	1.40(1.42a)	1.57(1.42a)	1.67(1.46a)	1.87(1.53a)	1.90(1.53a)
C3	6B662	1.17(1.26abcd)	1.43(1.37ab)	1.50(1.39a)	1.57(1.40ab)	1.40(1.36bcd)
	Erik	1.13(1.23abcd)	1.40(1.36ab)	1.57(1.41a)	1.47(1.37ab)	1.50(1.38abc)
C4	6B662	1.47(1.38ab)	1.50(1.38ab)	1.47(1.38a)	1.57(1.42ab)	1.37(1.35bcd)
	Erik	1.43(1.36abc)	1.33(1.33ab)	1.33(1.31ab)	1.77(1.49a)	1.33(1.33bcd)

[a] The number of appressoria represents the mean from 30 randomly selected conidia at each time-point after inoculation of 6B662 and cv. Erik. ANOVA was run to compare isolate*host, with 0.5 was added to the original data and square root transformed prior to statistical analysis. Transformed data are included in the parentheses. Numbers followed by the same latter are not significantly different based on Fisher's least significant difference at ($P<0.01$).

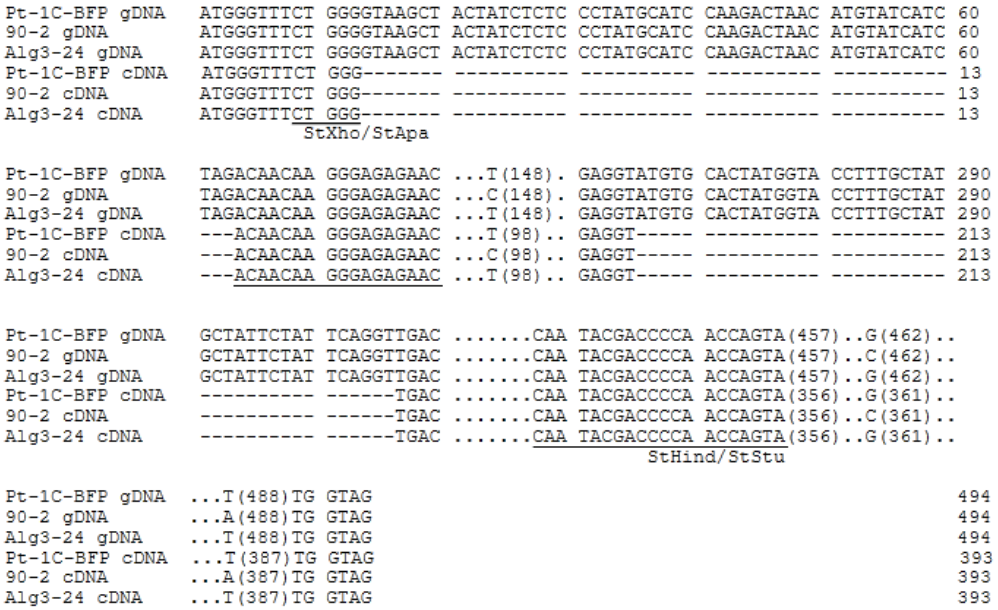

Fig. 1. Alignment of the *stp* gene, coding for STP protein, from isolates 90-2 (race 4) and Alg3-24 (race 5) of *Pyrenophora tritici-repentis*. The *stp* sequence from the isolate Pt-1C-BFP (race 1), for which the entire genome is available (Broad Institute), is also included for comparison. The underlined sections of sequence indicate the location of the primers (StXho/StApa and StHind/StStu) used in silencing vector construction. gDNA = genomic DNA; cDNA = complementary DNA.

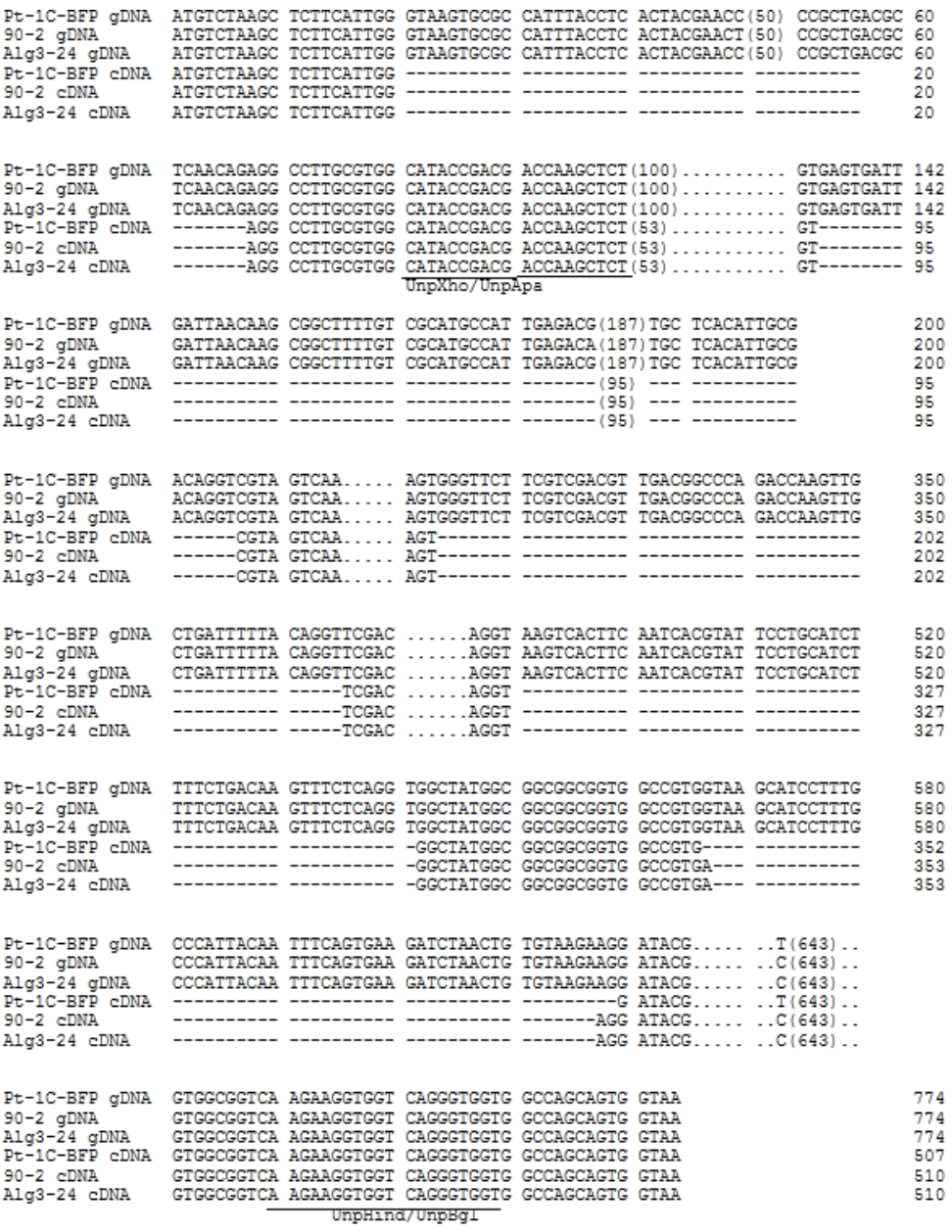

Fig. 2. Alignment of the *unp* gene, coding for UNP protein, from isolates 90-2 (race 4) and Alg3-24 (race 5) of *Pyrenophora tritici-repentis*. The *unp* sequence from the isolate Pt-1C-BFP (race 1), for which the entire genome is available (Broad Institute), is also included for comparison. The underlined sections of sequence indicate the location of the primers (UnpXho/UnpApa and UnpHind/UnpBgl used in silencing vector construction. gDNA = genomic DNA; cDNA = complementary DNA.

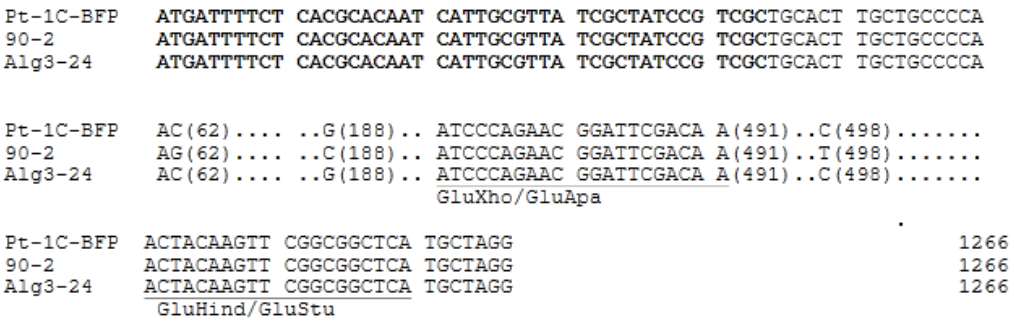

Fig. 3. Alignment of the *glu* open reading frame, coding for exo-1,3-β-glucanase, from isolates 90-2 (race 4) and Alg3-24 (race 5) of *Pyrenophora tritici-repentis*. The open reading frame from the isolate Pt-1C-BFP (race 1), for which the entire genome is available (Broad Institute), is also included for comparison. The region coding for a signal peptide is indicated in bold. The underlined sections of sequence indicate the location of the primers (GluXho/GluApa and GluHind/GluStu) used in silencing vector construction. gDNA = genomic DNA; cDNA = complementary DNA.

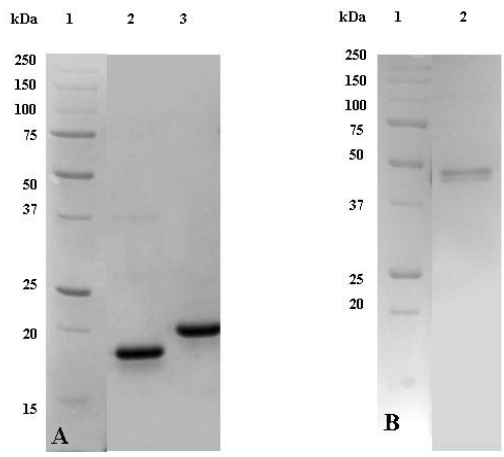

Fig. 4. SDS-PAGE of heterologously expressed STP protein, encoded by the *stp* gene (lane 2, panel A), UNP protein, encoded by the *unp* gene (lane 3, panel A), and exo-1,3-β-glucanase, encoded by the *glu* gene (lane 2, panel B). The hexahistidine (His)-tagged proteins were heterologously expressed in *Escherichia coli* BL21-AI cells and purified by running through a Ni-NTA agarose resin column. Protein molecular mass standards (Precision Plus Protein Unstained Standards, Bio-Rad, USA) were run in lane 1 of panels (A) and (B).

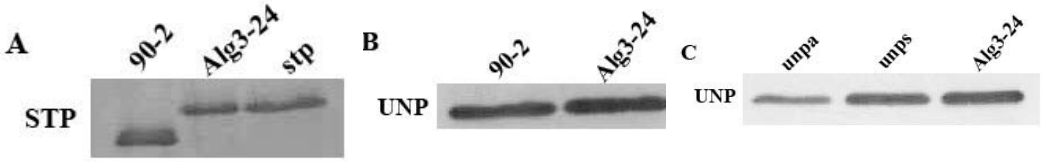

Fig. 5. Western blotting analysis of the STP and UNP proteins, encoded by *stp* and *unp*, respectively, in race 4 isolate 90-2, race 5 isolate Alg3-24, and *stp*- or *unp*-silenced strains of *Pyrenophora tritici-repentis*. (A) Bands reacting with polyclonal antibodies against STP in 10-day-old mycelium of 90-2, Alg3-24 and an *stp*-silenced transformant (stp) (15 μg total protein per lane). (B) Bands reacting with polyclonal antibodies against UNP in 10-day-old mycelium of 90-2 and Alg3-24 (15 μg total protein per lane). (C) Bands reacting with polyclonal antibodies raised against UNP in 10-day-old mycelium of Alg3-24 and two *unp*-silenced transformants (unpa, unps) (15 μg total protein per lane).

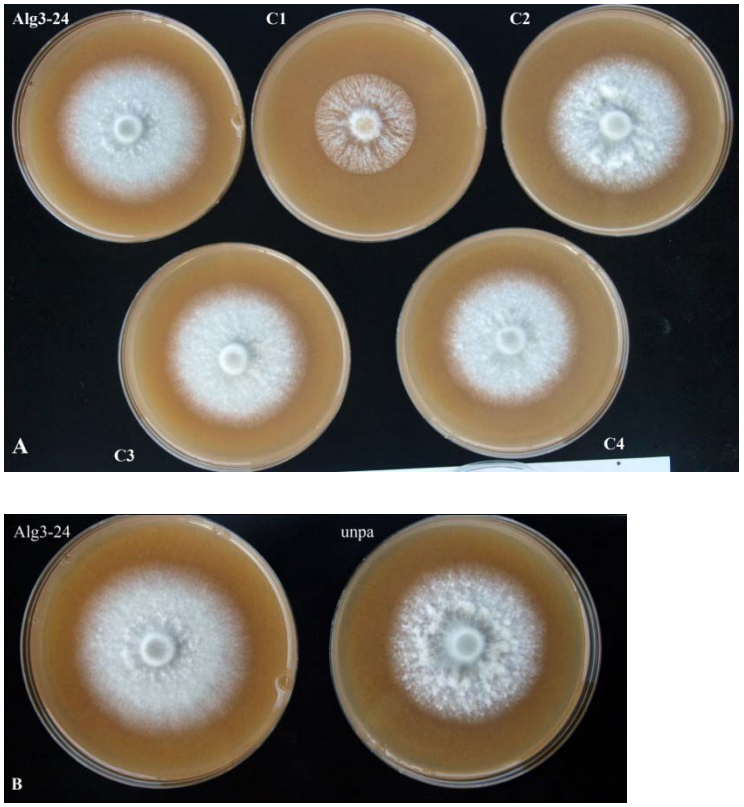

Fig 6. Growth and appearance of colonies of *Pyrenophora tritici-repentis* after five days of incubation on V8-potato dextrose agar in darkness at room temperature. (A) Wild-type race 5 isolate (Alg3-24) and transformants silenced for the *glu* gene, coding for exo-1,3-β-glucanase (C1, C2, C3 and C4). (B) Wild-type race 5 isolate (Alg3-24) and transformant silenced for the *unp* gene, coding for UNP protein (unpa).

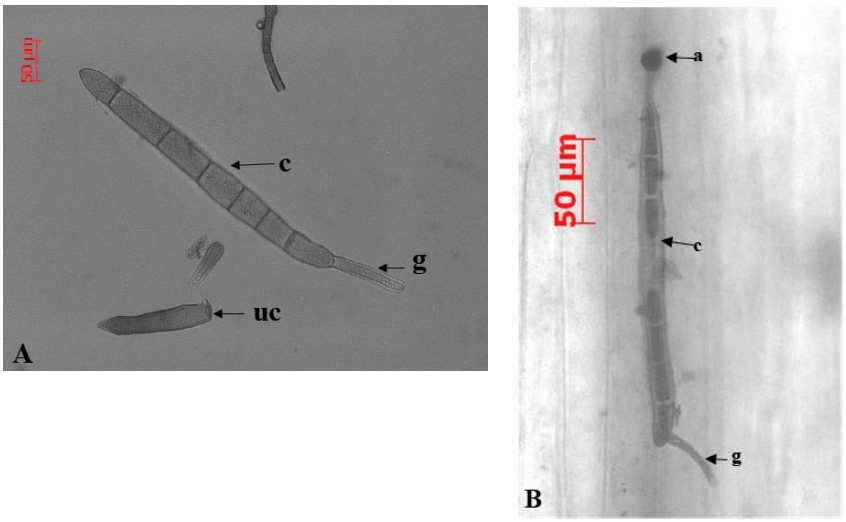

Fig. 7. Typical conidia and conidium-like structures produced by the *glu*-silenced transformant C1 of *Pyrenophora tritici-repentis*. The fungal structures were visualized with a Zeiss Ax10 microscope (Carl Zeiss, NY, USA). (A) Conidium (c), germ tube (g), and conidium-like structure (uc) produced on a sporulating colony that was grown on V8-PDA medium and observed in water. (B) Conidium (c), germ tube (g) and appressorium on the surface of a leaf of the wheat line 6B662 three hours after inoculation by the *glu*-silenced transformant C3 of *Pyrenophora tritici-repentis*.

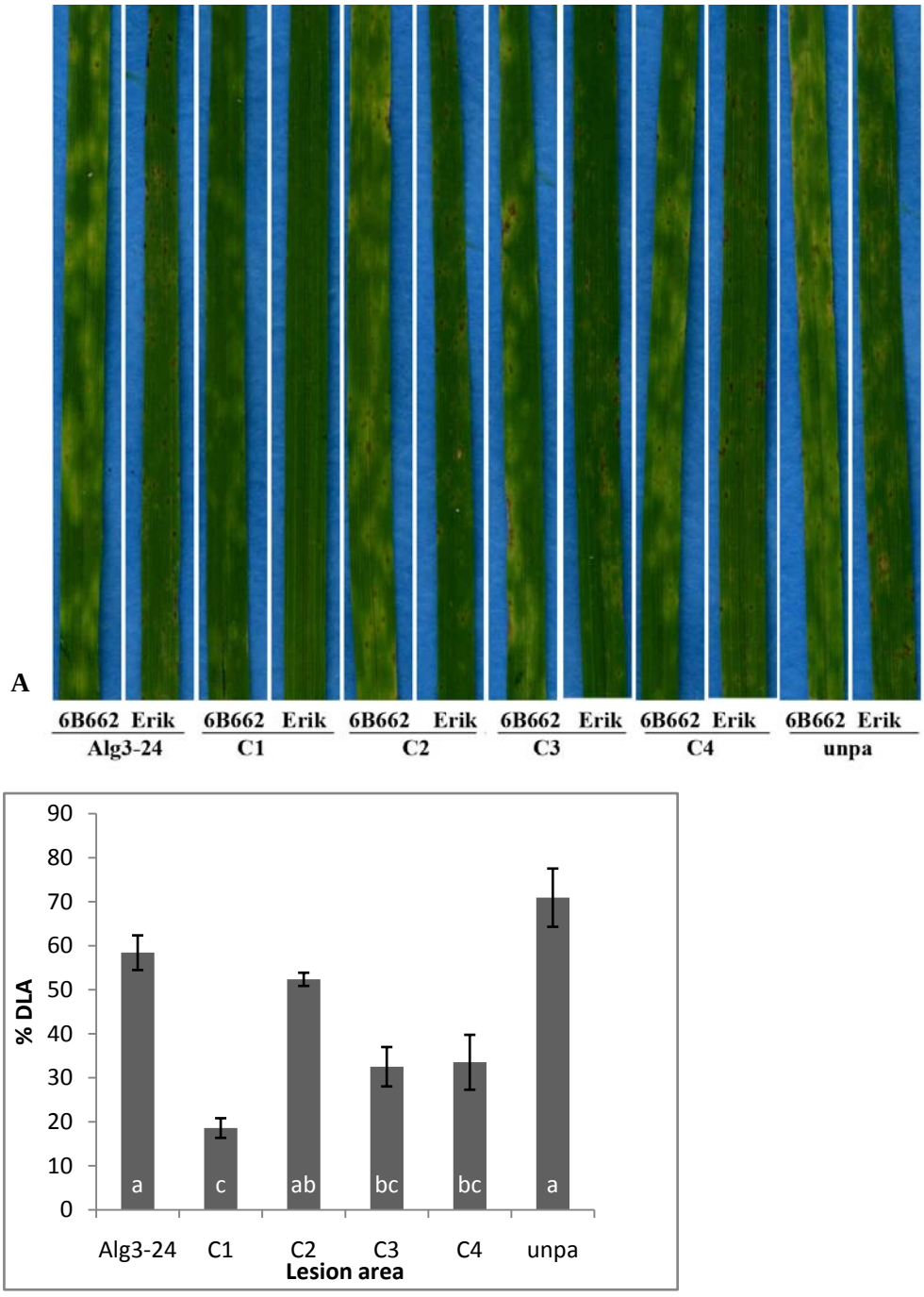

Fig. 8. Symptom development on the susceptible wheat line 6B662 and the resistant cv. Erik 120 h after inoculation with the wild-type race 5 isolate Alg3-24 of *Pyrenophora tritici-repentis* and transformants silenced for the *glu* gene coding for exo-1,3-β-glucanase, or the *unp* gene coding for UNP protein. (A) Foliar symptoms 5days after inoculation with Alg3-24, the *glu*-silenced transformants C1, C2, C3, C4 or the *unp*-silenced transformant unpa. (B)

Diseased leaf area (DLA) as a percentage of the total leaf area after inoculation with Alg3-24, C1, C2, C3, C4 or unpa. Assessments were made on the second leaf 5 days after inoculation with Assess 2.0 Image Analysis Software (APS Press, Minneapolis, MN). Columns with the same letter are not significantly different based on Fisher's least significant difference at $P<0.05$. The bars represent the standard error of the mean.

Chapter 3. General Discussion

Three HSTs, termed Ptr ToxA, Ptr ToxB, and Ptr ToxC, have been reported to date from *Pyrenophora tritici-repentis* (Lamari and Strelkov, 2010). These toxins are responsible for the necrosis and/or chlorosis symptoms that develop on susceptible wheat lines and cultivars after infection by isolates of the fungus (Strelkov and Lamari, 2003). The evidence suggests that these toxins act as pathogenicity (Strelkov and Lamari, 2003) or virulence factors (Friesen *et al*., 2003) for *P. tritici-repentis*. For instance, transformation of an avirulent or non-pathogenic isolate of the fungus with the *ToxA* gene made it pathogenic on a susceptible wheat genotype (Ciuffetti *et al*., 1997). Similarly, infusion of purified Ptr ToxB into toxin-sensitive wheat leaves caused the development of chlorosis symptoms identical to those induced by inoculation with Ptr ToxB-producing isolates of *P. tritici-repentis* (Kim and Strelkov, 2007), while RNA silencing of *ToxB* in a pathogenic race 5 isolate significantly reduced chlorosis symptom severity (Aboukhaddour *et al*., 2010). This evidence, as well as numerous genetic studies, indicate that the interaction between wheat and *P. tritici-repentis* is a mirror image of the classical gene-for-gene model (Lamari *et al*., 2003; Strelkov and Lamari, 2003), with pathogen-produced toxins or effectors interacting in a highly specific manner with targets or receptors in the host. Indeed, most studies on tan spot suggest that the HSTs produced by *P. tritici-repentis* play the most

critical role in the pathogenicity of the fungus (reviewed in Lamari *et al*., 1998, Strelkov and Lamari, 2003, and Lamari and Strelkov, 2010).

However, in addition to the variable production of the Ptr toxins, many other proteins (some of which have also been implicated in fungal virulence) were recently found to be differentially abundant in pathogenic and non-pathogenic isolates of *P. tritici-repentis* (Cao *et al*., 2009). In our study, three of these non-HST proteins were selected for additional characterization, in order to explore any possible contribution to fungal virulence or pathogenicity. Based on a earlier proteomic analysis, two of the targeted proteins (STP, UNP) were thought to be unique to the pathogenic race 5 isolate Alg3-24, since they were apparently absent from the proteome of the non-pathogenic race 4 isolate 90-2 (Cao *et al*., 2009). In the present study we were able to show, through Western blotting analysis, that STP and UNP are in fact produced by both isolates. However, the mass of STP was different in Alg3-24 and 90-2 (Fig. 5), suggesting the existence of different forms of the protein in the two isolates. It is possible that the different forms of STP affect the pathogenicity of *P. tritici-repentis*, but this remains just a hypothesis, since *stp* could not be adequately silenced in order to assess the impact of an STP deficiency. Further studies with transformants in which the *stp* gene is significantly silenced would provide more insights into the function of this protein.

In contrast, the *unp* gene was successfully silenced in one transformant,

named unpa (Fig. 5). However, silencing of this gene did not have a significant impact on any of the parameters examined in this study, including growth rate, phenotype, sporulation, or virulence. A Blast search indicated that the most similar match to UNP is a protein (Crp2) from *Metarihizium anisopliae*, which has been reported to contribute to cold and freezing tolerance in this microorganism (Fang and St. Leger, 2010). Therefore, while silencing of the *unp* gene in our study did not have an impact on the virulence or growth parameters of *P. tritici-repentis*, it is possible that the encoded gene could contribute to abiotic stress tolerance in the tan spot pathogen. Additional experiments, in which the transformed isolate is exposed to various stresses, will be needed to test this hypothesis.

The earlier proteomic analysis of Cao *et al*. (2009) also revealed that the abundance of exo-1,3-β-glucanase in the secretome of the pathogenic isolate Alg3-24 was 6-fold greater than in the secretome of the non-pathogenic isolate 90-2. Multiple exo-1,3-β-glucanases have been reported in many fungi, including *Acremonium blochii*, *Rhizoctonia solani* and *Cochliobolus carbonum* (Kim *et al*. 2001; Martin *et al.*, 2006). These different forms of exo-1,3-β-glucanase may act synergistically to degrade β-glucan. However, the functions of these multiple forms of the enzyme are still unclear (Kim *et al*., 2001; Martin *et al.*, 2006). In the current study, the differentially abundant exo-1,3-β-glucanase encoded by a gene we termed *glu* was targeted for RNA silencing. Although the transformed isolates showed a significant reduction in

the amount of *glu* transcript (Table 4), mixed glucanase activity (Table 5) was not significantly different from the wild-type. As discussed in Chapter 2, this likely resulted from the fact that the enzyme assay was not specific for exo-1,3-β-glucanase. Nonetheless, silencing of the *glu* gene resulted in a transformant (C1) with reduced growth (Table 6), sporulation (Table 6) and virulence (Fig. 8). This suggests that exo-1,3-β-glucanase plays some role in the virulence of *P. tritici-repentis* and/or other biological processes related to normal fungal growth and development.

It would be useful to raise antibodies against purified heterologously expressed exo-1,3-β-glucanase, in order to confirm that the reduction in transcript observed by quantitative PCR is correlated with a reduction in the abundance of the protein. In addition, other experiments may be useful in identifying factors, beyond the Ptr toxins, that might contribute to fungal virulence. For instance, metabolomic profiling of *P. tritici-repentis* isolates may serve to identify secondary metabolites that could have a role in tan spot disease development. For instance, Bouras and Strelkov (2008) found that the anthraquinone mycotoxin catenarin contributed to the development of non-specific necrosis on wheat leaves. It is likely that other such molecules may be produced by the fungus, which could either contribute non-specifically to symptom development, or obscure true toxin-target interactions (Lamari and Strelkov, 2010). Since Ptr ToxC has been reported to be a polar, non-ionic molecule of low molecular mass, metabolomic profiling could eventually lead

to the full characterization of this toxin.

It is clear that the Ptr toxins play a critical role as pathogenicity factors for *P. tritici-repentis*. Nonetheless, the focus on these toxins should not cause us to overlook other factors that could contribute to fungal pathogenicity and/or general parasitic ability. As evidenced by the reports of Bouras and Strelkov (2008), Cao *et al.*, (2009), and the current analysis of exo-1,3-β-glucanase, other metabolites and proteins could be contributing to the fitness of *P. tritici-repentis* as a parasite of wheat. An improved understanding of the role of such factors in fungal virulence could lead to a more comprehensive picture of the wheat-*P. tritici-repentis* interaction, which could ultimately result in improved, knowledge-based management of tan spot of wheat.

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