

TECHNICAL ADVANCE / RESOURCE

Endogenous silencing of *Puccinia triticina* pathogenicity genes through *in planta*-expressed sequences leads to the suppression of rust diseases on wheat

Vinay Panwar¹, Brent McCallum² and Guus Bakkeren^{1,*}¹Pacific Agri-Food Research Center, Agriculture and Agri-Food Canada, Summerland, BC V0H 1Z0, Canada, and²Cereal Research Centre, Agriculture and Agri-Food Canada, Winnipeg, MB R3T 2M9, Canada

Received 7 August 2012; revised 3 October 2012; accepted 8 October 2012.

*For Correspondence (e-mail Guus.Bakkeren@agr.gc.ca).

SUMMARY

Rust fungi are destructive plant pathogens. The draft genomes of several wheat-infecting species have been released and potential pathogenicity genes identified through comparative analyses to fungal pathogens that are amenable to genetic manipulation. Functional gene analysis tools are needed to understand the infection process of these obligate parasites and to confirm whether predicted pathogenicity genes could become targets for disease control. We have modified an *Agrobacterium tumefaciens*-mediated *in planta*-induced transient gene silencing (PITGS) assay for use in *Triticum* spp. (wheat), and used this assay to target predicted wheat leaf rust fungus, *Puccinia triticina* (*Pt*) pathogenicity genes, a MAP kinase (*PtMAPK1*), a cyclophilin (*PtCYC1*) and calcineurin B (*PtCNB*), to analyze their roles in disease. Agroinfiltration effectively delivered hairpin silencing constructs in wheat, leading to the generation of fungal gene-specific siRNA molecules in infiltrated leaves, and resulting in up to 70% reduction in transcription of the endogenous target genes in superinfected *Pt*. *In vivo* silencing caused severe disease suppression, compromising fungal growth and sporulation, as viewed by confocal microscopy and measured by reductions in fungal biomass and emergence of uredinia. Interestingly, using the same gene constructs, suppression of infection by *Puccinia graminis* and *Puccinia striiformis* was also achieved. Our results show that *A. tumefaciens*-mediated PITGS can be used as a reverse-genetics tool to discover gene function in rust fungi. This proof-of-concept study indicates that the targeted fungal transcripts might be important in pathogenesis, and could potentially be used as promising targets for developing RNA interference-based resistance against rust fungi.

Keywords: basidiomycete, fungal resistance, pathogen, RNA interference, *Triticum aestivum*, wheat leaf rust, technical advance.

INTRODUCTION

Cereal rust fungi are amongst the most devastating plant pathogens and their continuously increasing prevalence presents major constraints to *Triticum* spp. (wheat) production, posing a serious threat to global food security. Conventional breeding strategies to reduce yield losses relying on natural, race-specific host resistance genes have limited durability, presumably because of the rapid evolution of these pathogens. This demands a continuous search to identify effective sources of resistance. Three types of rust fungi are known to infect wheat: stem (black) rust (*Puccinia graminis* Pers. f. sp. *tritici*, *Pgt*), leaf (brown) rust (*Puccinia triticina* Eriks, *Pt*) and stripe (yellow) rust

(*Puccinia striiformis* Westend f. sp. *tritici*, *Pst*). These obligate biotrophic fungi are heteroecious, with a sexual cycle restricted to an alternate host, but the major phase of the life cycle occurs on graminaceous hosts (Bushnell and Roelfs, 1984). The different rust species infect their host in a similar fashion. Urediniospores land on leaves and, depending on various chemical and physical cues from the host surface (Hoch *et al.*, 1987; Uppalapati *et al.*, 2012), initiate an intricate series of developmental stages and structures essential for establishing a successful parasitic relationship with the host. After penetration into the substomatal cavity, the fungus attaches to a mesophyll cell

and breaches the cell wall, thereby invaginating the host plasma membrane to form a specialized feeding structure: the haustorium. This haustorium is surrounded by an extrahaustorial matrix (EHM) that represents the interface for signal exchange as well as nutrient uptake (Voegelé and Mendgen, 2003). Within 8–10 days following infection, new urediniospores are produced in uredinia that can be wind-disseminated over long distances and might result in epidemics.

Rust fungus genomic resources are being generated at a fast pace (e.g. Duplessis *et al.*, 2011; Xu *et al.*, 2011), allowing the prediction of many genes. However, because of the lack of functional genomic tools for rust fungi the biological functions of these genes remain elusive. Although procedures to genetically transform rust fungi exist (Webb *et al.*, 2006; Lawrence *et al.*, 2010), they are not routine or easily adaptable for large-scale analysis of gene functions. RNA interference (RNAi) has become a powerful tool to study functions of target genes in a transient way in organisms refractory to genetic manipulation (Hellens *et al.*, 2005). In dicot plants, transient expression of genes from constructs delivered by *Agrobacterium tumefaciens* (Agroinfiltration), is well established and commonly used for gene-silencing approaches to rapidly create gene knock-down phenotypes (Johansen and Carrington, 2001). In monocot plants, gene-silencing methods make use of viral systems (Holzberg *et al.*, 2002), but *Agrobacterium*-mediated delivery of silencing constructs has not been very successful, even though monocots were shown to be susceptible to genetic transformation by this organism (e.g. Grimsley *et al.*, 1987; Hensel *et al.*, 2009).

RNAi mechanisms seem to be present in most fungi studied, although some ascomycetes such as *Saccharomyces cerevisiae* (Scannell *et al.*, 2007) and some *Candida* species (Nakayashiki *et al.*, 2006), as well as basidiomycete fungi such as the corn smut *Ustilago maydis* (Laurie *et al.*, 2008), and some *Cryptococcus* species (D'Souza *et al.*, 2011) do not possess such genome defense mechanisms. It is unknown whether *Pt* has a functional silencing system, although genes coding for the necessary components of the silencing machinery seem to be present in its genome (<http://www.broadinstitute.org>). It was reported that organisms that live within and/or develop intimate contact with their host, such as nematodes (Huang *et al.*, 2006), insects (Baum *et al.*, 2007; Mao *et al.*, 2007) and parasitic plants (Tomilov *et al.*, 2008), are sensitive to RNA silencing molecules generated in the host, but are targeted to endogenous genes in the respective parasites. Recently, using the *Barley stripe mosaic virus* (BSMV), the production of RNAi molecules in barley targeted at several genes in the powdery mildew fungus, *Blumeria graminis*, seemed to affect fungal development in the host (Nowara *et al.*, 2010). Using the same system, effector

genes were targeted in the wheat stripe rust fungus *Pst*, and although a reduction in steady-state levels of corresponding mRNAs was reported, indicating the existence of a functional RNA silencing mechanism, no effect on disease suppression was observed (Yin *et al.*, 2011).

Here we show that *A. tumefaciens* wheat leaf infiltration can be used to deliver T-DNA from which hairpin RNA (hpRNA) structures are expressed, which subsequently trigger transient gene silencing. When targeted to *Pt* genes, small interfering RNA (siRNA) molecules generated in wheat can silence these genes inside superinfecting rust fungi. This novel strategy is easier to use than inoculations with a virus or the generation of stable transgenics. We demonstrate here in a 'proof of concept' the feasibility of this reverse-genetic approach to study the likely roles of selected *Pt* genes, an MAP kinase (*PtMAPK1*), a cyclophilin (*PtCYC1*) and calcineurin B (*PtCNB*), the functional orthologs of which are known to be involved in pathogenicity or virulence in other pathosystems (Hu *et al.*, 2007a; Cervantes-Chávez *et al.*, 2011). PITGS specifically reduced the transcript levels of the target genes in the fungus, resulting in decreased fungal development and sporulation. In addition, the same silencing constructs were sufficient to suppress disease development of *Pgt* and *Pst* also on wheat, indicating the potential for developing possibly durable resistance at the genetic level against these important plant pathogens.

RESULTS AND DISCUSSION

Development of an *Agrobacterium*-mediated transient RNAi assay for wheat

The generation and assessment of transgenic wheat lines is a time-consuming process, limiting the large-scale study of pathogen genes. The development of high-throughput transient RNAi approaches based on T-DNA delivery by *A. tumefaciens* has provided an alternative rapid tool for functional genomic studies (Hellens *et al.*, 2005), although the technology has not been extensively studied and applied in monocots, especially in cereals. To optimize the production of RNA silencing molecules in wheat by this approach, we first tested the phenotypic response caused by silencing of the wheat *phytoene desaturase* (*TaPDS*) gene. The *PDS* gene has been used as a visual marker in many studies on gene silencing, and suppression of this gene by RNAi results in easily recognizable photobleaching symptoms (Scofield and Nelson, 2009). A binary vector, pRNAi-*TaPDS*, transcribing the *TaPDS* gene sequence in an hpRNA conformation from the *Zea mays* (maize) ubiquitin promoter (Figure 1), was moved into three different *Agrobacterium* strains, COR308, LBA4404 and GV3101, to test for optimal T-DNA delivery and expression. Infiltration of these *Agrobacterium* strains resulted in efficient endogenous *TaPDS*

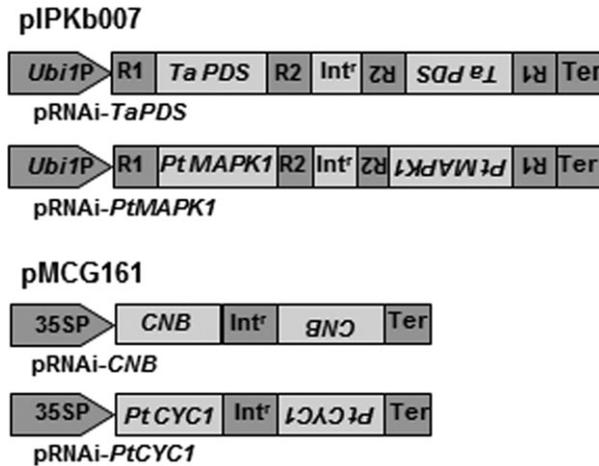


Figure 1. Constructs used in the *Agrobacterium*-mediated *in planta*-induced transient gene silencing (PITGS) assays: 185-bp *TaPDS* and 520-bp *PtMAPK1* gene fragments were recombined in the cereal-specific binary expression vector pIPKb007 (Himmelbach *et al.*, 2007), allowing expression of hpRNAi molecules from the *Zea mays* ubiquitin promoter (*Ubi1P*) and terminator (*Ter*) sequences. R1 and R2, *attB1* and *attB2* recombination site of the GateWay™ system (Invitrogen), respectively. The 463-bp *CNB* and 501-bp *PtCYC1* gene fragments were cloned into binary vector pMCG161 (ChromDB), allowing their expression in hpRNAi form from the cauliflower mosaic virus 35S promoter (35SP) and octopine synthase terminator (*Ter*) sequences. *Int'*, intron. These constructs produce a constitutive supply of gene-specific silencing molecules in host cells that have acquired T-DNA.

silencing in the wheat cv. Thatcher. At 7–10 days post inoculation (dpi), bleached sectors were observed in infiltrated leaves (Figure 2a). The areas lacking pigmentation did not have sharp boundaries and non-responsive cells were apparent. Leaves infiltrated with *Agrobacterium* alone showed no *TaPDS* silencing phenotype, and were indistinguishable from leaf areas infiltrated with buffer. The *Agrobacterium* strains COR308 and LBA4404 produced more apparent *TaPDS*-silencing symptoms compared with strain GV3101. This could indicate that strain GV3101 has a low efficiency in the transfer of T-DNA to cereals (Chen *et al.*, 2010). As the silencing symptoms induced by *Agrobacterium* strains COR308 and LBA4404 appeared similar, strain COR308 was employed for further analysis and optimization of the transient RNAi assay.

The concentration of *Agrobacterium* cells is reported to have a significant effect on the outcome of infiltration (Bhaskar *et al.*, 2009). We therefore standardized the infiltration assay by testing various cell concentrations. Bacterial suspensions with an OD₆₀₀ of 0.5–0.7 resulted in a maximum level of *TaPDS*-related silencing symptoms. A low concentration of bacteria (OD₆₀₀ < 0.4) resulted in a poorly developed or almost negligible photobleaching phenotype, whereas high concentrations (OD₆₀₀ > 1.0) often caused leaf senescence, starting with the production of necrotic spots around the infiltration zone.

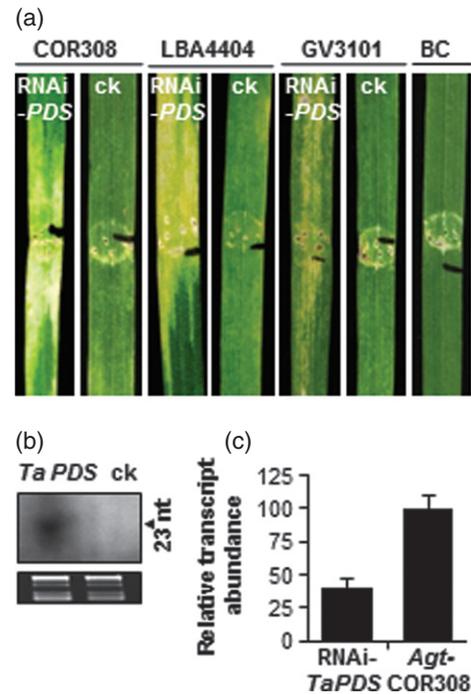


Figure 2. *Agrobacterium*-mediated *in planta*-induced transient gene silencing (PITGS) assay targeting the *phytoene desaturase* (*TaPDS*) gene in wheat cv. Thatcher.

(a) Silencing phenotype caused by different *Agrobacterium* strains carrying a *TaPDS* gene segment. No photobleaching was observed for *Agrobacterium* alone (ck) or buffer controls (BC).

(b) siRNA molecules specific to the *TaPDS* gene were detected in total RNA extracted from pooled leaf material from three different plants 3 days after agroinfiltration with pRNAi-*TaPDS*. An autoradiograph of RNA blot hybridized with a ³²P-labelled gene-specific probe. No signal was detected in the control sample treated with *Agrobacterium* alone (ck). The bottom panel shows ethidium bromide-stained rRNA as the gel loading control; siRNA size indicated.

(c) qRT-PCR measurements of *TaPDS* transcript abundance relative to an endogenous reference gene, *TaEF1*, in leaves infiltrated with pRNAi-*TaPDS* or the control treated with *Agrobacterium* alone (*Agt*-COR308, set at 100%). cDNA generated from total RNA isolated from pooled leaf material from three different plants. Bars represent mean values ± SDs of three independent sample collections. The reduction in the target *TaPDS* gene transcription level in silenced leaves, compared with non-silenced leaves treated with *Agrobacterium* alone, was significant at $P < 0.05$ in a Student's *t*-test.

Detection of low molecular weight RNA (siRNAs) corresponding to the target *TaPDS* gene in leaf tissue agroinfiltrated with the pRNAi-*TaPDS* construct confirmed the degradation of *TaPDS* hpRNA by endogenous dicer-like nucleases (Figure 2b). Subsequent quantitative real-time reverse transcription PCR (qRT-PCR) analysis showed significant reductions (60%) in *TaPDS* gene transcript levels in leaves expressing the silencing construct, compared with controls (Figure 2c). The presence of siRNA molecules and the reduction in *TaPDS* mRNA levels suggest that the observed phenotypic effects are attributable to gene silencing achieved at the post-transcriptional level. RNAi-induced silencing has been reported to be non-cell autonomous

and, once initiated, can spread systemically in a 'source-to-sink' direction (Dunoyer *et al.*, 2010b; Brosnan and Voinet, 2011). However, we did not observe *TaPDS*-related chlorophyll bleaching in distal and newly emerging leaves, and silencing was more or less confined to the infiltration zone. There are indications that silencing signals (siRNA molecules) can move across 10–15 cells through plasmodesmata without the need for amplification (Dunoyer *et al.*, 2010b). However, systemic movement over longer distances requires an amplification of the original signal (Himber *et al.*, 2003), which might be inefficient for the *TaPDS* target. Together, these results provided a benchmark for the application of this transient approach for the functional analysis of fungal genes.

Selection of *P. triticina* target genes

Of prime interest to the host–rust fungus interaction are genes encoding proteins with pathogenicity or virulence functions. The silencing of such genes is likely to result in a quantifiable phenotype, and if successful may offer an effective alternative to combat rust fungi. A previously generated *Pt* expressed sequence tag (EST) database (Xu *et al.*, 2011) provided candidate gene sequences. *PtCYC1* codes for a cyclophilin, a member of a large group of proteins generally possessing peptidylprolyl-*cis/trans*-isomerase (PPIase) activity, and having well-defined functions as folding catalysts and chaperones involved in cell signaling. Cyclophilins play roles in virulence in several pathogens (Wang *et al.*, 2001; Viaud *et al.*, 2002). As a second candidate we used the first identified and characterized rust fungus mitogen-activated protein kinase gene, *PtMAPK1* (Hu *et al.*, 2007a), a member of a family of serine/threonine protein kinases that are conserved in most eukaryotes, and are known to play roles in the phosphorylation of transcription factors required for growth and differentiation processes. We showed earlier that *PtMAPK1* complemented deletion mutants of the homologs *Ubc3/Kpp2* and *Kpp6* in the corn smut fungus *U. maydis*, impaired in mating and pathogenicity (Hu *et al.*, 2007a). In addition, in our database we identified an EST sequence with homology to *CNB*, coding for the calcineurin regulatory subunit. Comparison with the recent public *Pt* genome sequence (<http://www.broadinstitute.org>) indicated that this EST sequence seemed derived from a (possibly contaminating) ascomycete. It shared 65% nucleotide identity positions with the homolog *PtCNB*, and would allow us to test whether diverged sequences could also be used to silence endogenous homologs (Figure S1). *CNB* complexes with the catalytic subunit *CNA* to form calcineurin. In the fungal kingdom, the calcium–calcineurin pathway is involved in morphogenesis, circadian rhythm, cell cycle progression, stress response and virulence (Nguyen *et al.*, 2008; Egan *et al.*, 2009). Previously we showed that the calcineurin subunits are important

virulence factors in the barley smut fungus *Ustilago hordei* (Cervantes-Chávez *et al.*, 2011). These lines of evidence suggested that these three genes might have a similar role in *Pt* parasitism of wheat.

Transient expression of candidate fungal hpRNA is processed by the host silencing machinery

Selected 3' regions of *PtMAPK1*, *PtCYC1* and *PtCNB*-like sequences were amplified and cloned in hpRNA-generating silencing vectors (Figure 1). Small RNA molecules, specific to the *PtMAPK1*, *PtCYC1* or *CNB* sequences, were detected in total RNA isolated from leaves agroinfiltrated with the respective silencing constructs 3 days earlier (Figure 3a). This showed that *Agrobacterium* infection could result in the induction of the transient expression of fungal double-stranded RNAs in wheat cells, which subsequently acted as substrates for plant nucleases catalyzing their cleavage into corresponding siRNA molecules. Various quantities of these molecules were detected in different plants, and similar variation was seen for the three constructs, indicating that both the *35S* and maize ubiquitin promoters resulted in significant expression.

In vivo silencing of fungal target genes

Host cell-produced fungal siRNAs are postulated to navigate across haustorial membranes and matrix to induce degradation of cognate mRNAs in fungi (Nowara *et al.*, 2010; Yin *et al.*, 2011). qRT-PCR analysis of total RNA isolated from silenced leaves, 5 days after superinfection with *Pt* urediniospores, revealed specific reductions in transcript levels of the fungal target genes. The endogenous transcript levels of the *PtCYC1* and *PtMAPK1* genes showed an approximate 63 and 70% downregulation, respectively, in plants agroinfiltrated with the corresponding RNAi constructs, compared with controls (Figure 3b). Interestingly, the endogenous *PtCNB* gene transcript levels were also reduced by 59%, although this was caused by a *CNB* sequence sharing 65% nucleotide identity with the *PtCNB* homolog. There are, however, several stretches of identical nucleotides along the target sequence, the longest match being 14 bases and part of a 32-bp homology, with only two mismatches, and it has been shown that as few as between eight and sixteen nucleotides of contiguous homology between an siRNA and target mRNA can attenuate gene activity (Jackson *et al.*, 2006; Haley *et al.*, 2010). The silencing induced by each respective RNAi construct was specific to the corresponding fungal gene targeted for PITGS, and was not found to influence the expression of other, unrelated genes such as the other two fungal genes tested (Figure 3b). The silencing induced against *TaPDS* was also not observed to influence the expression of candidate fungal genes (Figure 3b). The reduction in the relative abundance of fungal gene transcripts strongly suggested that the translocation of silencing molecules from host cell

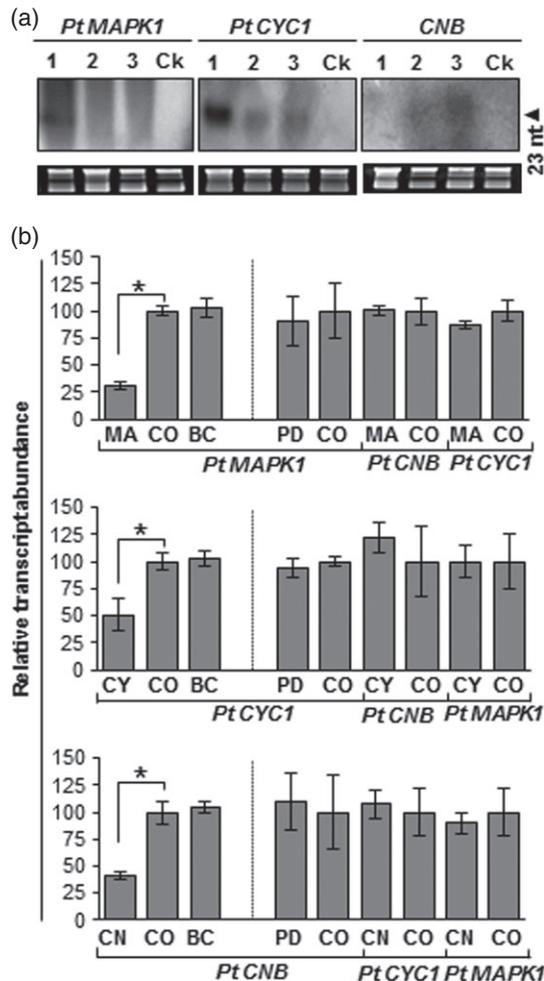


Figure 3. Host-generated silencing RNAs and *in planta*-induced transient gene silencing (PITGS) effect on transcription of *Pt* target genes.

(a) siRNA molecules specific to the *PtMAPK1*, *PtCYC1* and *PtCNB*-like sequences were detected in total RNA extracted from pooled leaf material from three different plants, 3 days after agroinfiltration with the respective silencing vectors. Autoradiograph of RNA blots hybridized with ^{32}P -labeled gene-specific probes. Bottom panels, rRNA gel loading controls. Lanes 1–3, different individual samples; Ck, control treated with *Agrobacterium* alone. siRNA size indicated.

(b) Transcript abundance of the *PtCYC1*, *PtMAPK1* and *PtCNB* genes, as indicated under each panel, quantified by qRT-PCR 5 days after superinfection with *Pt* on plants previously infiltrated with the respective RNAi constructs (MA, RNAi-*PtMAPK1*; PD, RNAi-*TaPDS*; CY, RNAi-*PtCYC1*; CN, RNAi-*CNB*), (BC, buffer control) or treated with *Agrobacterium* alone (CO, *Agt-COR308*). Transcript abundance was measured relative to a fungal endogenous reference gene (Experimental procedures), and the CO control was set at 100%. Transcript reduction is specific to the targeted gene. cDNA was generated from total RNA isolated from pooled leaf material from three different plants. Values represent means \pm SDs of three independent sample collections. The asterisks indicate that the reduction in the target gene transcription levels in silenced leaves, compared with in non-silenced leaves (CO), was significant at $P < 0.05$ in a Student's *t*-test.

to fungal cytoplasm occurred. The induction of *in vivo* silencing of the *Pt* target genes, in conjunction with the detection of gene-specific siRNA molecules in the wheat host, indicate RNAi as the underlying mechanism.

PITGS of candidate *Pt* pathogenicity genes compromises fungal development

To study whether the *PtMAPK1*, *PtCYC1* and *PtCNB* genes have any roles in pathogenicity or virulence, we investigated the possible effect of gene silencing on fungal development in wheat host leaves superinfected with *Pt* urediniospores after agroinfiltration with the corresponding RNAi constructs. Disease incidence was first assessed by a physiological parameter. A quantitative measurement was made of the number of sporulating uredinia developing on a defined surface area of the infiltrated leaves at 10 days after fungal inoculations (Experimental procedures). The plants infiltrated with either of the three silencing constructs exhibited much reduced *Pt* disease symptoms, as indicated by significantly lower (51–68%) pustule densities compared with those infiltrated with *Agrobacterium* alone, which in turn were as susceptible to *Pt* as buffer-inoculated control plants; silencing *per se*, induced by the pRNAi-*TaPDS* construct, also did not affect sporulation (Figure 4a,b). This indicated a clear PITGS effect on urediniospore production. The suppression was, however, local (mainly confined to the infiltration zone), and no systemic silencing effect on disease development was observed in upper, non-agroinfiltrated leaves.

To test whether the observed reduction in sporulation was correlated with mycelial growth in host tissue, fungal biomass measurements were made in the infected leaves. Total genomic DNA was extracted from agroinfiltrated leaves superinfected with *Pt*, and the relative levels of single-copy *Pt* and wheat genes were quantified by qRT-PCR. At 5 and 10 days after *Pt* inoculation on leaves previously agroinfiltrated with silencing constructs, fungal biomass was significantly reduced by 60–65 and 59–69%, respectively, compared with controls infiltrated with *Agrobacterium* alone or buffer, or silenced control plants (Figure 4c). This result indicated that fungal development was impaired, probably as a result of the virulence penalty imposed by the silencing of the targeted fungal genes.

The formation of mature haustoria and the development of secondary hyphae are prerequisites for a successful interaction between hosts and biotrophic fungi. Therefore, we further investigated the possible role of the *PtMAPK1*, *PtCYC1* and *PtCNB* genes in host invasion by microscopic assessment of *Pt* development in foliar tissue. Wheat leaves infiltrated with *Agrobacterium* without silencing constructs showed widespread colonization of mesophyll cells by *Pt* 5 days after inoculation, typified by an extensive hyphal network and numerous established haustoria (Figure 5). In contrast, the presence of the various fungal siRNA molecules in the host cells clearly suppressed fungal development in silenced leaves. Primary haustoria development was limited and hyphal growth was severely reduced, remaining mainly confined to the first few host

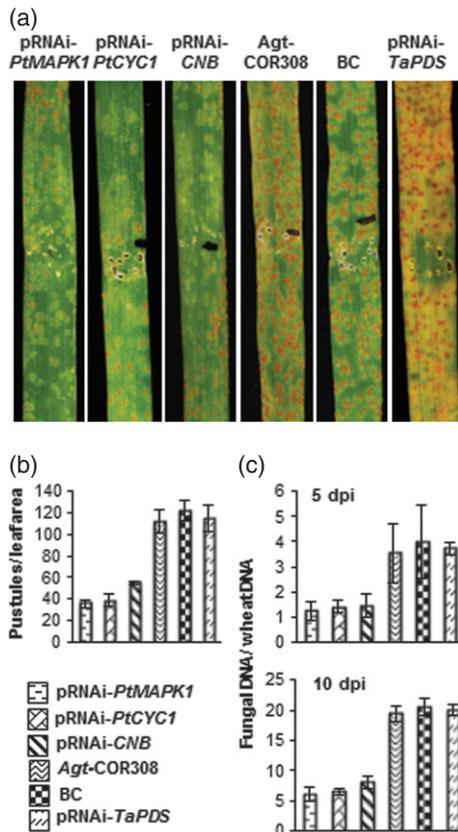


Figure 4. Effect of the presence of gene-specific siRNA molecules in wheat cv. Thatcher on *Pt* infection.

(a) Agroinfiltration with the *PtMAPK1*, *PtCYC1* or *CNB* RNAi constructs resulted in the suppression of *Pt* disease symptoms on leaves, whereas no effect on disease susceptibility was observed on leaves infiltrated with *Agrobacterium* alone (*Agt-COR308*), buffer (BC) or pRNAi-*TaPDS* silenced control plants. Plants photographed 10 days after *Pt* inoculation.

(b) Quantification of pustule density on leaves caused by superinfection with *Pt* after PITGS of the target fungal genes by the respective RNAi construct, compared with controls.

(c) Fungal biomass measurements by qRT-PCR analysis of total genomic DNA, extracted 5 and 10 days after *Pt* inoculation of infiltrated leaves. Ratio of fungal nuclear to wheat nuclear genomes using single-copy genes *PtRTP1* (rust transferred protein 1) and *TaEF1*, respectively (Song *et al.*, 2011). Relative ratio of fungal biomass in leaves silenced with RNAi constructs targeting *Pt* genes compared with control leaves infiltrated with *Agrobacterium* alone. DNA was extracted from pooled leaf material from three different plants. Mean values \pm SDs from three independent sample collections. Differences were significant at $P < 0.05$ (Student's *t*-test).

mesophyll cells (Figure 5). A differential effect on the developmental pattern of the secondary hyphae caused by the presence of the various siRNA molecules was also observed. This was most pronounced when caused by the *PtMAPK1* silencing construct, which seemed to keep the secondary hyphae shorter compared with the growth inhibition imposed by the targeting of the *PtCYC1* and *PtCNB* genes. We reported before that *PtMAPK1* is differentially expressed, with high levels found during early infection and sporulation (Hu *et al.*, 2007a). A similar expression

profile was also observed for a *Pst* homolog (Guo *et al.*, 2011). The cytological observations corroborated these expression studies and further suggested that the MAPK signaling cascade plays an important role during *Pt* development in wheat. The differential effects seen by microscopy suggest that the targeted genes may be regulating different development stages during *Pt* infection, warranting further investigation, but indicating the feasibility of using this approach for the functional analysis of rust fungus genes. The observed suppression of fungal growth inside silenced host tissue correlated well with the measured reduction in fungal biomass and uredinia formation. Overall, these results strengthen the view that the targeted fungal genes might have important roles in pathogenesis.

Sequence comparisons had indicated that effective RNAi targets for these candidate genes should be present in both *Pgt* and *Pst* (Figure S1). Therefore, wheat leaves agroinfiltrated with or without silencing constructs were also challenged with urediniospores from *Pgt* and *Pst* isolates known to infect wheat cv. Thatcher. A reduction in uredinia development of 43–61 and 48–69% in leaves infiltrated with target RNAi constructs, compared with controls treated with *Agrobacterium* alone, was also seen for *Pgt* and *Pst*, respectively, suggesting that these conserved genes might have similar roles in the virulence of the different *Puccinia* species (Figure 6).

Although RNAi is considered to be highly sequence specific, siRNAs can induce ‘off-target’ gene silencing effects (Jackson *et al.*, 2006; Haley *et al.*, 2010; Senthil-Kumar and Mysore, 2011). No obvious phenotypic effects were observed in wheat plants infiltrated with the various silencing constructs. Indeed, only low overall sequence similarity between the 3′ fungal target sequences and a large, currently available collection of wheat sequences could be revealed (Figure S1). However, we cannot completely rule out potential silencing effects by fungal-specific siRNA molecules on transcripts in the wheat genome. Overall though, as all three silencing constructs had a major effect on fungal development in the wheat host, and since the *CNB* sequence had virtually no sequence similarity to its closest homolog in wheat (being extremely fungal-specific), we think that a contribution from a host gene product(s) to the suppression effect is unlikely.

The off-target effect could be more pronounced in the fungus, and this might have contributed to the disease suppression we observed. With more extensive genome sequences now available, we identified possible fungal homologs using the BLASTN algorithm. Depending on the overall nucleotide similarity to the 3′ fungal target regions used, and judged significant and possibly able to trigger silencing effects, six potential *Puccinia* MAPK-related homologs and possibly one cyclophilin-related homolog were identified: the *CNB* sequence seemed to represent a single gene. Therefore, at this point we cannot ascertain

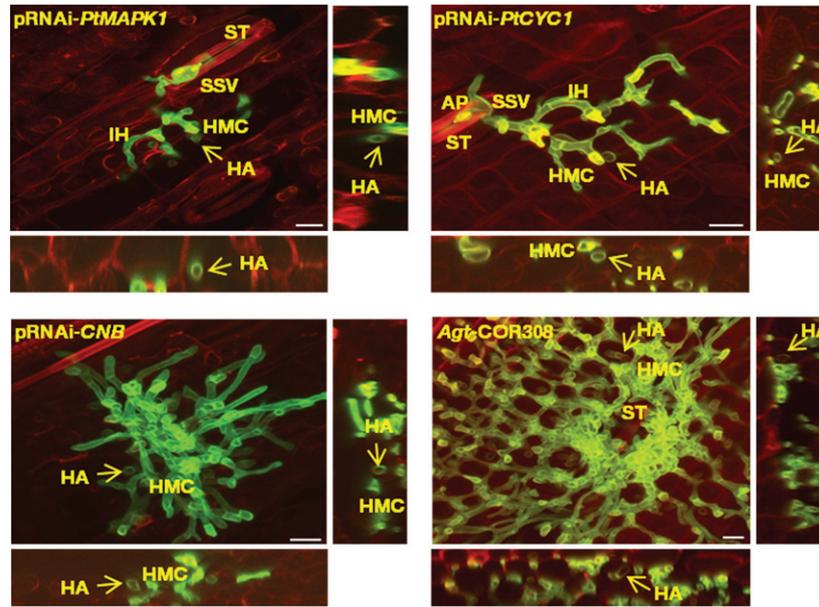


Figure 5. Confocal microscopic visualization of the *in planta*-induced transient gene silencing (PITGS) effect targeting the *PtMAPK1*, *PtCYC1* and *PtCNB* genes on colonization of wheat leaf tissue by *Pt*.

Plates represent a projection of a 3D Z-series of scans taken from inside wheat cv. Thatcher leaves 5 days after *Pt* inoculation; side panels provide the respective 3D views through the Z-series. Fungal structures stained with Uvitex 2B and acridine orange-stained plant cell walls are visible. Plants agroinfiltrated to deliver hpRNAi constructs, as indicated, show poorly developed fungal mycelium with very few developed haustoria (arrows). This contrasted starkly with the control treated with *Agrobacterium* alone (*Agt-COR308*), where an extensive mycelial network with many formed haustoria inside mesophyll cells could be observed. AP, appressorium; HA, haustorium; HMC, haustorium mother cell; IH, infection hypha; SSV, substomatal vesicle; ST, stoma. Scale bar: 20 μ m.

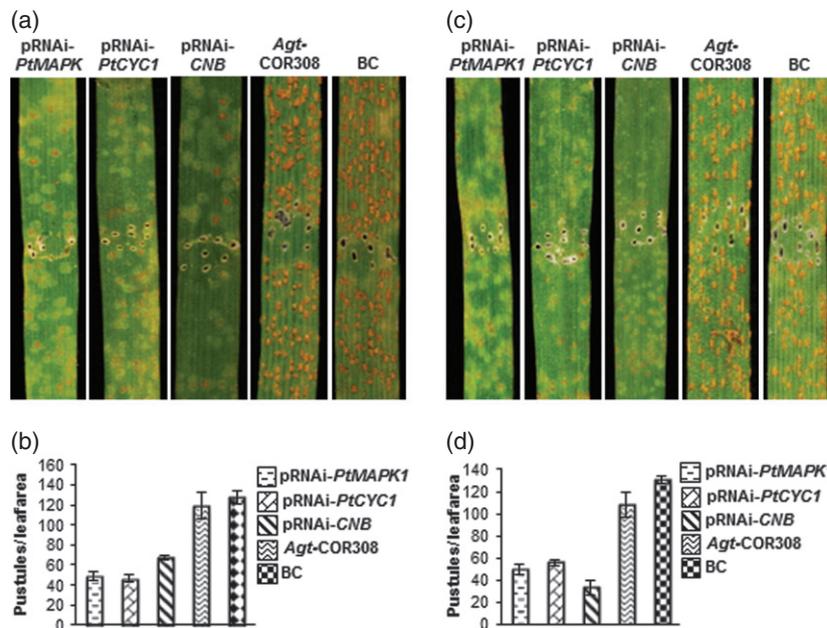


Figure 6. Response caused by the various silencing constructs on *Puccinia graminis* f. sp. *tritici* (*Pgt*, stem rust) and *Puccinia striiformis* f. sp. *tritici* (*Pst*, stripe rust) infection of wheat cv. Thatcher. (a) The generation of *PtMAPK1*, *PtCYC1* or *CNB* gene-specific siRNA molecules in the wheat host, caused by agroinfiltration with the respective hpRNAi constructs, resulted in the suppression of disease symptoms on leaves superinfected with *Pgt* (a) or *Pst* (c), whereas no effect on disease susceptibility was observed on leaves infiltrated with *Agrobacterium* alone (*Agt-COR308*) or buffer control (BC). Plants were photographed 10 days after *Pgt* inoculation. (b) Quantification of pustule density on leaves caused by superinfection with *Pgt* or *Pst* (d) after *in planta*-induced transient gene silencing (PITGS) of the indicated genes, compared with controls. Pustules were counted 10 days after fungal inoculation per area of leaf surface, spanning 3 cm around the infiltration site. The mean value is given, representing data from 30 randomly selected leaves from three experiments. Error bars depict standard errors. Statistical analysis was performed with a Student's *t*-test ($P < 0.05$ versus control).

that the disease suppression observed was solely the result of the silencing of the respective candidate fungal gene, possibly with the exception of the *CNB* gene. Therefore, the use of this tool to assess (virulence and pathogenicity) gene function probably depends on whether related homologs in the genome are expressed at the same time. To achieve crop protection, however, a 'broad-spectrum', multiple fungal gene-specific silencing strategy is preferable. The *CNB*-like sequence, sharing a higher level of identity with ascomycete homologs, efficiently silenced the endogenous *PtCNB* gene. This result illustrates the flexibility of this system, but we have not tested the interesting possibility that infection by other (ascomycete) fungal pathogens of wheat, such as the tan spot fungus *Pyrenophora tritici-repentis*, could be compromised as well.

Movement of silencing signals from host to fungus

The generation of self-complementary RNA, whether through antisense RNA transcription or hairpin formation, triggers a sequence-specific mRNA degradation, leading to gene silencing. RNAi signals are diffusible and can traverse across source-to-sink gradients (Brosnan and Voinnet, 2011). In plants, the transgene silencing signals mainly move short distances from cell to cell, but can also spread over long distances through the vasculature, the latter process being mostly evident through grafting experiments (Palauqui *et al.*, 1997; Dunoyer *et al.*, 2010a). Like other eukaryotes, most fungi are also sensitive to RNAi and recently it was shown that silencing signals can travel from host cell to fungus, presumably through the haustorial interface (Nowara *et al.*, 2010; Yin *et al.*, 2011). Uptake of genetic material, in the form of RNA, has also been demonstrated between host plants and parasites such as insects, nematodes or other parasitic plants feeding on them (Huang *et al.*, 2006; Baum *et al.*, 2007; Mao *et al.*, 2007; Tomilov *et al.*, 2008). However, the mechanism behind such cross-species transfer of silencing molecules remains to be elucidated. Our data show that in the *Puccinia*-wheat pathosystems, the silencing signals are likely to be the siRNA molecules, as these were generated in the host cells (Figures 2 and 3). Preliminary studies suggest that translocation of genetic material from host to infecting fungal cells may occur via the exosomal biogenesis pathway (Casadevall *et al.*, 2009; Meyer *et al.*, 2009; Lu *et al.*, 2012). At sites of fungal penetration, multivesicular compartments, including multivesicular bodies (MVBs) and cell wall-associated paramural bodies, aggregate in host cytoplasm around haustorial complexes, facilitating an anterograde, and probably retrograde, polarized vesicle trafficking across the plant-pathogen cellular interface. These MVBs contain multiple intraluminal vesicles (ILVs), which upon fusion with the plasma membrane are released extracellularly as exosomes into the paramural space (Meyer *et al.*, 2009;

Lu *et al.*, 2012). Interestingly, exosomes have been shown to contain both mRNA and non-coding small RNAs that can be delivered, and can be functional, in recipient cells, thereby facilitating genetic exchange between cells (Valadi *et al.*, 2007). Transport of RNA by exosomes might require appropriate receptors at the cell surface for attachment, or follow other specialized transfer pathways. MVB-like compartments have been reported in trafficking mechanisms at intercellular channels called gap junctions, nanotubes or even internalization of sections of plasma membrane by neighbouring cells (Gibbins and Voinnet, 2010). It is possible that in our system the siRNA species generated in the host 'silencing donor' are transferred into the 'fungal recipient' through this exocytic/endocytic exchange mechanism at the haustorium interface (Figure 7). During plant infection, biotrophic and hemibiotrophic fungi secrete effector proteins, some of which translocate to the plant apoplast or cytosol where they can alter host responses to condition susceptibility. Recently, it was reported that some effectors secreted by oomycete pathogens, which have infection strategies similar to rust fungi, may enter host cells via receptor-mediated endocytosis (Kale and Tyler, 2011). However, similar effector delivery has not yet been discovered for biotrophic fungi. It is currently unknown whether a bidirectional trafficking across the host-parasite cellular interface can take place, and whether this could include small RNA species. Nutrient uptake has been reported to occur in bean rust fungal haustoria through specific transporters (Voegelé and Mendgen, 2003), and it is possible that siRNAs use these or similar transporters. Alternatively, their sizes may be sufficiently small as to allow other (passive) ways for crossing various membranes and the EHM.

Genomic resources for the agronomically important cereal rust fungi are being generated at a fast pace, yet functional assay systems are lacking to test for genes predicted to be involved in pathogenicity or virulence in these obligate biotrophic pathogens. We made use of an RNAi approach by developing a functional *A. tumefaciens*-mediated PITGS assay for wheat that should allow the rapid testing of many genes. Using this approach we demonstrated the silencing of three endogenous *Pt* genes, suggesting the feasibility of this system to study loss-of-function phenotypes in rust fungi. Gene silencing by transient expression of hpRNA does not require stable genetic transformation, and is consequently a promising technique for functional genomic studies in organisms refractory to genetic manipulation. Our results demonstrate that wheat-expressed fungal double-stranded RNA (dsRNA) can trigger RNAi of corresponding homologous genes in several *Puccinia* species. Furthermore, silencing of *PtMAPK1*, *PtCYC1* and *PtCNB* results in significant disease suppression of three major wheat rust pathogens, showing that these genes and the

Similarly, to generate the *PDS* silencing construct, a 185-bp 3' end of the *TaPDS* gene was amplified from wheat (*T. aestivum* cv. Thatcher) cDNA using primers *TaPDS-F* and *TaPDS-R* (Table S1), recombined directionally in pENTR/D-topo, and its insert subsequently recombined into binary vector pIPKb007 to yield pRNAi-*TaPDS*.

To generate pRNAi-*CNB*, an EST (PT0315.I05.C21.ptt, GenBank accession GR495416), isolated from a *Pt* teliospore cDNA library was used. This sequence was annotated as a calcineurin B regulatory subunit, *CNB*, but later, when the stem, leaf and stripe rust fungal genomes became available (Broad Institute, <http://www.broadinstitute.org>; Cantu *et al.*, 2011), proved to be more closely related to ascomycete *CNB* gene sequences. For example, this EST sequence revealed 83% nucleotide and 96% amino acid identities with a homolog in *Aspergillus kawachii* (Figure S1). A 463-bp 3'-end fragment of this EST clone was amplified by PCR using the forward primer *CNB-F*, containing *SpeI* and *Ascl* restriction sites, and reverse primer *CNB-R* containing *SgfI* and *AvrII* restriction sites (Table 1). The amplified fragment was then sequentially cloned into the *SpeI/Ascl* and *SgfI/AvrII* sites of plasmid pUBleX1-RNAi, containing a fungal intron (Laurie *et al.*, 2008). The 1250-bp *BglII* *CNB*-intron hairpin-containing fragment was made blunt with the Klenow *Poll* fragment, then digested with *SpeI* and subsequently cloned into the *EcoRV*- and *SpeI*-digested binary vector pMCG161 (ChromEDB). This placed the 3'-*CNB* gene sequences behind the 35S promoter-*Adh1* intron sequences for expression as a self-complementary dsRNA form in cereals.

pRNAi-*PtCYC1* was generated as follows: a *Pt* EST assembly, PtContig6674, consisting of ESTs originating from cDNA libraries constructed from several developmental stages (TR24, GenBank accession number BU672663; PT0012a.A01.B7.ptu.chim2, EC395818; PT0055c.B07.BR.ptu, EC399927; PT0121b.F04.BR.pth, EC414784; and PT0191.G22.CPTR.ptm, EC418145 (Hu *et al.*, 2007b; Xu *et al.*, 2011), encoded a protein with homology to a cyclophilin. In the *Pt* draft genome (Broad Institute), PtContig6674 matched 15 sequences with various homology using the BLASTX algorithm (with probability of $e = 0$, PTTG_00425, to $e < 10^{-4}$, PTTG_06503). In the *Pgt* draft genome, homology to at least 13 genes, with significance ranging from $e = 0$ (e.g. PGTG_14910 and PGTG_14936) to as high as $e = 10^{-5}$ (PGTG_10482) was found. PtContig6674 seemed to represent a full-length cDNA, matching the best-predicted gene PTTG_00425 of 985 bp with six introns, coding for a protein of 138 amino acids, and was named *PtCYC1*. PtContig6674 contained two ESTs from the wheat infection stage, strengthening the idea that this cyclophilin might have a function during pathogenicity. Similarly as for *CNB*, a 501-bp 3' part of *PtCYC1* was amplified using primers *PtCYC-F* and *PtCYC-R* (Table S1) and cloned sequentially into the *SpeI/Ascl* and *SgfI/AvrII* sites of pUBleX1-RNAi. The 1350-bp *BglII* *PtCYC1*-intron hairpin-containing fragment was cloned into pMCG161 to generate binary vector pRNAi-*PtCYC1* similar to the *CNB* fragment. All constructs described were verified by sequencing and subsequently transferred into the respective *Agrobacterium* strains: COR308 (Hamilton *et al.*, 1996), LBA4404 (Hoekema *et al.*, 1983) or GV3101 (Koncz and Schell, 1986) by electroporation.

Agroinfiltration assay

A single colony of *Agrobacterium* was cultured in 10 ml of YEB medium (per liter, 1 g yeast extract, 5 g beef extract, 5 g peptone, 5 g glucose and 0.493 g MgSO₄·7H₂O, pH 7.2), supplemented with antibiotics as required, and grown overnight at 28°C with continuous shaking at 200 rpm on an orbital shaker. Of this bacterial cul-

ture, 250 µl was transferred to 50 ml fresh YEB with antibiotics as required, 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5.6) and 20 µM acetosyringone, and grown at 28°C to an OD₆₀₀ of 1 for induction. The cells were harvested twice by centrifugation at 1700 g for 5 min at 4°C and the pellet washed with 5 ml of ice-cold sterile distilled water. Cells were then resuspended in infiltration buffer (10 mM MES, pH 5.6, 10 mM MgCl₂, 200 µM acetosyringone) to a final desired concentration. The bacterial suspension was then incubated for 3 h at 20–25°C before infiltrating leaf tissue. As controls, *Agrobacterium* strains without constructs, or infiltration buffer solution, was used in all experiments. The inoculum was infiltrated through the abaxial surface of wheat seedlings (8–9 days old, two-leaf stage) using a 1-ml sterile syringe (without a needle) by applying gentle pressure. A small incision was made at the site of infiltration using a sterile needle to enhance the efficiency of infiltration. Plants were maintained in growth chambers under a 16-h photoperiod with a temperature of about 22°C.

DNA isolation

Fungal genomic DNA was isolated as described by Hu *et al.* (2007b). Plant genomic DNA was isolated using the CTAB method (Allen *et al.*, 2006). For biomass quantification, epiphytic fungal mycelium was wiped off wheat leaf surfaces using wet cotton pads prior to genomic DNA extraction.

RNA extraction and cDNA synthesis

Total RNA was isolated from leaf tissue around infiltrated areas using Trizol reagent (Invitrogen), following the manufacturer's instructions. For qRT-PCR analysis, RNA samples were treated with TURBOTM DNase I (Ambion, now Invitrogen, <http://www.invitrogen.com>). The absence of genomic DNA contamination was subsequently confirmed by the lack of PCR amplification of the RNA samples using *Pt*-specific primers for the *PtRTP1* gene (rust transferred protein homolog, PTTG_03497; Song *et al.*, 2011) and wheat-specific primers for the *TaEF1* gene (GenBank accession M90077.1; Table 1). First-strand cDNA was synthesized from 1 µg of total RNA in a final volume of 20 µl using SuperScriptIII enzyme (Invitrogen) and oligo (dT)₁₈ primers, according to the manufacturer's instructions.

Quantitative real-time PCR

Quantitative real-time PCR was performed on a CFX96TM Real-Time PCR machine (Bio-Rad, <http://www.bio-rad.com>). Specific primers for each gene were designed using PRIMER 3.0, and are listed in Table S1. qRT-PCR was conducted in a 10-µl volume using SsoFast EvaGreen Supermix (Bio-Rad). Thermal cycling parameters were: 98°C for 2 min, followed by 39 cycles of 95°C for 10 sec and 60°C for 30 sec. Three technical replicates were performed on each sample. All products were subjected to melting curve analysis between 65°C and 95°C, to determine the specificity of the PCR reaction. Experiments included a non-template control. Relative levels of gene transcripts were computed by qPCR 2.2 (Bio-Rad) using the comparative quantification method, with the *Pt* succinate dehydrogenase gene as the endogenous reference gene for normalization, previously selected from several candidates (Song *et al.*, 2011). To distinguish the *PtCNB* gene transcripts from the *CNB* sequences derived from the EST, *PtCNB*-specific qRT-PCR primers were designed (Table 1).

To measure changes in fungal biomass, the relative quantification of the single-copy target genes *PtRTP1* and *TaEF1* was

assessed (Song *et al.*, 2011). Total genomic DNA of wheat cv. Thatcher or *Pt* was used to prepare standard curves derived from at least six serial dilutions for each. The correlation coefficients for the analysis of the dilution curves were above 0.99. The relative quantities of PCR product of *PtRTP1* and *TaEF1* in mixed/infected samples were calculated using the gene-specific standard curves to quantify the *Pt* and wheat gDNA, respectively.

siRNA detection

To detect small RNA species, total RNA was extracted from wheat leaves 3 days after agroinfiltration. For each sample, the same quantity of RNA was separated on a 15% polyacrylamide gel and transferred to neutral Hybond NX membrane (Amersham) by electroblotting. The transferred molecules were chemically cross-linked to the membrane as described by Pall and Hamilton (2008). As signal markers, gene-specific oligonucleotides were also loaded on the same gels. For hybridization, the ULTRAhyb Hybridization buffer (Ambion, Burlington, ON, Canada) was used, and to generate probes, PCR-amplified target gene fragments were labeled with [α - 32 P]dCTP using a random primer DNA labelling kit (Amersham, now GE Healthcare Life Sciences, <http://www.gelifesciences.com>). Hybridization was carried out at 38°C for 18 h, after which membranes were washed twice in double-strength SSC (30 mM sodium citrate, 300 mM NaCl) + 0.1% SDS buffer for 15 min, each at 42°C, before exposure to HyperfilmTM MP (Amersham).

Uredinia count

The visual assessment of disease was performed by counting the number of uredinia that appeared 10 days after rust inoculation per cm² of infiltrated leaf surface using IMAGEJ (<http://rsb.info.nih.gov/ij/>). Uredinia were counted within a 3-cm² area around the infiltration point (this was the area where solution could easily be infiltrated in leaves), and the average number per leaf was calculated. Only lesions that were sporulating were counted. To avoid bias among leaf samples, leaves from thirty treated plants from three experiments were randomly selected.

Confocal microscopy

Leaf samples were collected 5 days after fungal inoculation. Fungal structures were stained with Uvitex-2B as described by Moldenhauer *et al.* (2006). Acridine orange (Sigma-Aldrich, <http://www.sigmaaldrich.com>) was used to stain plant cell walls. Fluorescent microscopy was carried out using a SP2-AOBS laser scanning confocal microscope (Leica, <http://www.leica.com>). The fluorescence of Uvitex 2B and Acridine Orange was detected by excitation at 405 and 514 nm, respectively, and scanning with filter settings at 411–485 and 550–560 nm, respectively.

ACKNOWLEDGEMENTS

The authors wish to thank T. Fetch for *Pgt* isolates, M. Weis for help with confocal microscopy and D.L. Joly and H. Sanfaçon for their comments. This research was partially funded through the interagency NSF/USDA-CSREES Microbial Genome Sequencing Program (award 2008-35600-04693 to C. Cuomo, J. Fellers, L. Szabo and G. Bakkeren).

SUPPORTING INFORMATION

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

Figure S1. Nucleotide sequence alignments revealing relatedness.

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