Host-induced silencing of essential genes in *Puccinia triticina* through transgenic expression of RNAi sequences reduces severity of leaf rust infection in wheat

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Received 15 February 2017; revised 18 August 2017; accepted 20 September 2017.

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**Keywords:** *Puccinia triticina*, wheat leaf rust, host-induced gene silencing, RNAi, transgenic wheat, rust resistance.

**Summary**

Leaf rust, caused by the pathogenic fungus *Puccinia triticina* (*Pt*), is one of the most serious biotic threats to sustainable wheat production worldwide. This obligate biotrophic pathogen is prevalent worldwide and is known for rapid adaptive evolution to overcome resistant wheat varieties. Novel disease control approaches are therefore required to minimize the yield losses caused by *Pt*. Having shown previously the potential of host-delivered RNA interference (HD-RNAi) in functional screening of *Pt* genes involved in pathogenesis, we here evaluated the use of this technology in transgenic wheat plants as a method to achieve protection against wheat leaf rust (*WLR*) infection. Stable expression of hairpin RNAi constructs with sequence homology to *Pt* MAP-kinase (*PIMAPK1*) or a cyclophilin (*PCYC1*) encoding gene in susceptible wheat plants showed efficient silencing of the corresponding genes in the interacting fungus resulting in disease resistance throughout the *T₂* generation. Inhibition of *Pt* proliferation in transgenic lines by *in planta*-induced RNAi was associated with significant reduction in target fungal transcript abundance and reduced fungal biomass accumulation in highly resistant plants. Disease protection was correlated with the presence of siRNA molecules specific to targeted fungal genes in the transgenic lines harbouring the complementary HD-RNAi construct. This work demonstrates that generating transgenic wheat plants expressing RNAi-inducing transgenes to silence essential genes in rust fungi can provide effective disease resistance, thus opening an alternative way for developing rust-resistant crops.

**Introduction**

Rust fungi, belonging to the genus *Puccinia*, are among the most economically destructive biotrophic phytopathogenic fungi infecting cereal crops. Members of this genus pose a serious threat to global wheat production which is a major staple food for mankind in many parts of the world. Wheat is a host to three important rust pathogen species: *Puccinia graminis* f. sp. *tritici* (*Pt*), *Puccinia striformis* f. sp. *tritici* (*Pst*) and *Puccinia triticina* (*Pt*), which are the causal agents of stem rust, stripe rust and leaf rust, respectively (Bolton et al., 2008; Chen et al., 2014; Singh et al., 2011). Of the three rust diseases, wheat leaf rust (*WLR*, caused by *Pt*) is comparatively more prevalent occurring regularly wherever wheat is grown (Kolmer, 2005). Because of its adaptation to diverse climatic conditions and widespread occurrence, leaf rust results in greater total annual losses worldwide than stem and stripe rusts (Huerta-Espino et al., 2011). Under severe epidemic conditions, *Pt* can inflict yield losses ranging from 10% to 70% (Herrera-Foessel et al., 2006). Fungicides are usually applied for management of rust diseases, but financial costs and adverse environmental impacts are associated with chemical inputs. In addition, a major concern with excessive application of antimicrobial compounds is a high risk of reduced sensitivity or development of resistant pathogen populations.

Genetic resistance remains the most economical, effective and ecologically sustainable approach to minimize the extent of crop damage from leaf rust disease (Draz et al., 2015). Current disease control measures against *Pt* rely predominantly on breeding and development of resistant cultivars. So far, more than 70 leaf rust resistance (*R*) genes have been reported, the majority of which confers protection to a particular genetic variant of pathogen species mediated by gene-for-gene-type interactions (Ellis et al., 2014; Flor, 1971; Singla et al., 2017). However, resistance alleles deployed into elite cultivars followed by monoculture farming instigate enormous selection pressures on rust pathogens. This often leads to new virulent races of the fungus, evolved to avoid recognition by these alleles and thereby rendering the resistant variety ineffective in a relatively short time (McCallum et al., 2016; Webb and Fellers, 2006). Further, finding and introgressing durable resistance genes from wild donor species without any undesirable trait drag remain a challenge to breeders. It is expected that rapid evolutionary dynamics of rust pathogens combined with anthropogenic climate changes will accelerate disease outbreaks in cultivated wheat worldwide (Helfer, 2014). Therefore, a continuous search for developing alternative biotechnological solutions is required to alleviate the negative impact of rust pathogens in wheat.

*Pt* is a highly specialized macrocyclic, heteroecious fungus having a complex life cycle involving up to five different spore stages on two taxonomically different host plants with species in Triticeae being the primary hosts (Bolton et al., 2008). The fungus reproduces on wheat by the formation of asexual dikaryotic urediniospores, and under favourable conditions, this phase can lead to multiple infection cycles thereby causing crop failure within weeks. *Pt* infection in wheat occurs via urediniospore germ...
tubes that differentiate to form appressoria over leaf stomata to penetrate into substomatal cavities. Subsequently, the plant tissue is colonized with an intercellular mycelium, breaching plant cell walls and invaginating the plasma membrane to form haustoria. This intimate connection surrounded by a zone of extrahaustorial matrix marks the basis of molecular crosstalk between the host and fungal cells (Garnica et al., 2014). Through haustoria, the fungus acquires nutrients from the host and there is evidence to suggest that these structures also play a crucial role in the delivery of virulence effectors to suppress host defence responses (Petre et al., 2015; Struck, 2015). The asexual cycle is completed within 7–10 days when sporogenous uredinia are formed containing thousands of infective propagules that are easily wind-dispersed over long distances to establish new infections.

The availability of many cereal rust fungus genome sequences, along with the integration of comparative analyses, has contributed valuable insight into genome organization and annotation of genes involved in pathogenesis (Cantu et al., 2011; Cuomo et al., 2017; Duplessis et al., 2011; Wu et al., 2017; Zheng et al., 2013). However, attempts to translate this knowledge to decipher pathogen biology are hampered because these biotrophic fungi are not amenable to many of the traditional molecular genetic approaches, mainly due to difficulties in culturing these species outside of their host. In the postgenomics era, RNA interference (RNAi) has emerged as a powerful tool for de novo discovery of gene function. This technique, termed post-transcriptional gene silencing (PTGS) in plants, involves the endonucleolytic cleavage of double-stranded RNA (dsRNA) into small, ~20–23 nucleotide (nt)-long, small interfering RNA (siRNA) duplexes by the Dicer-like (DCL) enzymes which along with their associated proteins control the expression of genetic information (Baulcombe, 2004; Wilson and Doudna, 2013). An RNAi-based approach referred to as host-induced gene silencing (HGSI) or host-delivered RNAi (HD-RNAi) in which small RNAs are produced by the host plant to target parasite transcripts has provided a promising strategy for improving plant resistance against pathogens, including fungi and oomycetes (Andrade et al., 2015; Govindarajulu et al., 2015; Huang et al., 2006; Jahan et al., 2015; Koch and Kogel, 2014; Koch et al., 2013; Nowara et al., 2010; Panwar et al., 2013a, 2016; Song and Thomma, 2016; Yin et al., 2010). When targeted against crucial pathogenicity genes, HD-RNAi can potentially be developed as a genetic method to curb pathogen virulence for pesticide-free disease control in crop plants.

Recently, functional genomics assays based on HGI methodology were developed for rust fungi, facilitating the characterization of some rust fungi genes required for virulence (Panwar et al., 2013a,b; Yin et al., 2014; Zhang et al., 2012). These studies harnessed transient gene silencing approaches, either delivering the HD-RNAi constructs through Barley stripe mosaic virus (BSMV) or Agrobacterium tumefaciens-mediated transfer. Applying these methods, we reported that Pt Map-kinase (PtMAPK1), cyclophilin (PtCYC1) and calcineurin B (PtCNB1) encoding genes were required for full pathogenicity on the wheat host (Panwar et al., 2013a,b). Here, we test the efficacy of stably integrated HD-RNAi constructs in transgenic wheat plants to achieve resistance against WLR disease by silencing the 
PtMAPK1 or PtCYC1 genes in Pt. We show that transgenic expression of HIGS cassettes targeting Pt genes provides a viable means to reduce WLR infection in otherwise highly susceptible wheat plants, providing an attractive and precise genetic engineering strategy for combating aggressive plant pathogens.

Results

Generation and analysis of transgenic wheat lines for Pt resistance

The HD-RNAi constructs (Pt-HGSI) or hp-PtCYC1-RNAi designed to express the 520-nt and 501-nt long 3′-end coding segments of the fungal PtMAPK1 and PtCYC1 gene, respectively, were generated and selfed to obtain the T1 seeds. Integration of the PtMAPK1 or PtCYC1 gene segment in T1 lines was verified by polymerase chain reaction (PCR), and expression of the dsRNA was confirmed by reverse-transcription PCR (RT-PCR), using Pt gene-specific primers (Figure S1, Table S1). No visible physiological or growth alterations were observed in the transgenic wheat lines expressing the respective HD-RNAi construct when compared with the nontransformed control plants of the same genotype, confirming no unintended RNAi effect in the host.

To examine whether the presence of hp-PtMAPK1-RNAi or hp-PtCYC1-RNAi constructs in wheat lines affected the susceptibility to infection by Pt, 2-week-old transgenic T1 plants along with nontransformed control plants were challenged with fungal urediniospores and visually observed for WLR development. Analysis of disease phenotype identified six hp-PtMAPK1-RNAi and seven hp-PtCYC1-RNAi lines that were variably effective in restricting the magnitude of WLR symptoms as compared to controls. Segregation analysis revealed that the fungal transgenes segregated in a Mendelian ratio in these lines, indicating that integration of functional inserts occurred at one (3:1 ratio) or two (15:1 ratio) genetic loci (Table S2). The disease response of Pt-inoculated wheat plants in terms of infection type (IT) was scored at 12 days postinfection (dpi) using the 0–4 scale (Dakouri et al., 2013) to reflect uredinia size and abundance. Resistance to Pt in T1 plants expressing the respective HD-RNAi constructs was characterized by the appearance of fewer and smaller uredinia with repressed sporulation, corresponding to low infection types varying from 1 to 3. In contrast, Pt-challenged control wheat plants consistently exhibited vigorously sporulating uredinia resembling high ITs, indicative of highly susceptible reactions (Figure 1). Interestingly, the suppression of infection by Pt in transgenic wheat plants with low ITs (IT < 2) was also notable for delayed appearance of symptoms as compared to controls. WLR symptoms in control plants became apparent at 5–6 dpi which eventually progressed to large and more distinct uredinia, whereas in transgenic T1 plants displaying a low IT, appearance of disease symptoms was delayed by 1–3 days, compared to controls, with markedly reduced uredinia size and density (Figure S2). Taken together, a quantitative reduction of WLR disease symptoms was observed in transgenic wheat lines carrying a HIGS construct targeting the fungal PtCYC1 or PtMAPK1 gene.
This marked reduction in fungal load in T1 lines suggested that PtCYC1 and PtMAPK1 as compared to controls (Figures 1b and 2a). Similarly, fungal biomass was reduced by as much as 79% reductions by up to 77% in comparison with controls (Figures 1a). MAPK1 constructs were the most effective in restricting fungal development, showing biomass reductions in leaves challenged with Pt by over 77% (Figure 2a). Hence, the lines shown compared to control. Photographs were taken at 12 dpi.

Transgenic wheat lines expressing the HD-RNAi constructs impede Pt development

We next explored whether suppression of Pt development in transgenic wheat T1 lines was associated with impairment of fungal growth inside leaf tissue by measuring the fungal biomass accumulation in leaves challenged with Pt. Determination of fungal biomass showed that the ratio of fungal-to-plant DNA was clearly, and quantitatively, reduced in transgenic lines, compared to nontransformed control (NTC) plants. The HIGS-derived reduction in fungal biomass was more than 50%. The rationale behind such classification was to ensure accurate and unbiased disease resistance pattern detection in transgenic lines. Transgenic plants were considered as highly resistant (HR) to Pt if the HIGS-derived reduction in fungal biomass was more than 75%. HR, highly resistant, fungal biomass reduction by more than 75%; R, resistant, fungal biomass reduction by 50%–75%; MR, moderately resistant, reduction in fungal biomass by 25%–50%; S, susceptible, reduction in fungal biomass by <25% as compared to control plants.

| Disease intensity based on reduction in fungal biomass | n | S | MR | R | HR |
|--------------------------------------------------------|---|---|----|---|----|
| **HD-RNAi construct and line number**                  |---|---|----|---|----|
| hp-PtMAPK1RNAi                                         |   |   |    |   |    |
| MAPK1-2159                                             | 15| 8 | 5  | 2 | –  |
| MAPK1-2162                                             | 12| 7 | 4  | 1 | –  |
| MAPK1-2163                                             | 12| 8 | 2  | 1 | 1  |
| MAPK1-2166                                             | 23| 9 | 9  | 5 | –  |
| MAPK1-2169                                             | 18| 9 | 4  | 4 | 1  |
| MAPK1-2170                                             | 16| 7 | 6  | 3 | –  |
| Fielder-NTC                                            | 15| 15| –  | – | –  |
| hp-PtCYC1RNAi                                          |   |   |    |   |    |
| CYC1-2216                                              | 14| 6 | 5  | 3 | –  |
| CYC1-2224                                              | 15| 10| 2  | 2 | 1  |
| CYC1-2226                                              | 11| 4 | 2  | – | –  |
| CYC1-2227                                              | 11| 3 | 5  | 3 | –  |
| CYC1-2228                                              | 14| 9 | 2  | 3 | –  |
| CYC1-2236                                              | 15| 9 | 3  | 2 | 1  |
| CYC1-2248                                              | 13| 6 | 4  | 3 | –  |
| Fielder-NTC                                            | 15| 15| –  | – | –  |

Generally, resistance to rust pathogens is not an apparent case of immunity versus susceptibility but, more often, is perceived as a quantitative difference in fungal growth between more or less resistant and susceptible plants which makes visual disease scoring systems sporadically prone to variability and error. To circumvent this possibility, we grouped transgenic T1 HD-RNAi plants into four different resistance types based on the quantification of fungal biomass in Pt-infected tissue by qPCR. The rationale behind such classification was to ensure accurate and unbiased disease resistance pattern detection in transgenic lines. Transgenic plants were considered as highly resistant (HR) to Pt if the HIGS-derived reduction in fungal biomass was more than 75% relative to control plants, reduction in fungal biomass by 50%–75% was designated as a resistant (R) phenotype, 25%–50% reduction in fungal biomass was classified as moderately resistant (MR), whereas plants in which fungal biomass accumulation was suppressed by <25% as compared to controls were categorized as susceptible (S). Overall, we observed a close relationship between WLR disease severity and the amount of fungal biomass detected in Pt-challenged wheat plants. For example, wheat plants with visibly high disease symptoms (high ITs) also showed higher fungal biomass in infected leaves than those suppressing fungal development (low ITs) that had less fungal biomass (Figure 2a).

HD-RNAi results in reduction of targeted gene transcript levels in Pt infecting transgenic wheat lines

In order to substantiate the hypothesis that protection to WLR infection in hp-PtMAPK1RNAi or hp-PtCYC1RNAi lines was...
caused by in planta-derived silencing of the target PtMAPK1 or PtCYC1 genes, we quantified the transcript levels of the respective genes in Pt infecting transgenic T1 and nontransformed control (NTC) plants following challenge with Pt. Representative leaves are shown from transgenic plants of selected lines classified into highly resistant (HR), resistant (R), moderately resistant (MR) and susceptible (S) categories based on per cent fungal biomass reduction (%) in infected tissue compared to controls, along with the scored infection type (IT). Photographs were taken at 12 dpi, the same time point when fungal biomass ratios were analysed by qPCR. (b) Quantitation of fungal PtMAPK1 or PtCYC1 transcript abundance in Pt-inoculated leaves of transgenic T1 plants of lines MAPK1-2169 and CYC1-2236 representing different resistance types (as presented in a) by RT-qPCR at 5 dpi. The expression levels of PtMAPK1 or PtCYC1 were normalized to the levels of the Pt succinate dehydrogenase gene and are reported as a percentage, relative to that of the NTC plants (set at 100%). A correlation was observed between HIGS-derived relative reduction in PtMAPK1 and PtCYC1 transcript levels and per cent fungal biomass reduction (%) in a. Bars represent mean values ± SDs of two independent experiments. Values with different letters indicate statistically significant differences (Student’s t-test; P < 0.05).

Figure 2. HD-RNAi of PtMAPK1 and PtCYC1 genes in Pt infecting transgenic wheat plants. (a) Disease symptoms on transgenic wheat T1 plants expressing the hp-PtMAPK1RNAi (line 2169) or hp-PtCYC1RNAi (line 2236) construct and nontransformed control (NTC) plants following challenge with Pt. Representative leaves are shown from transgenic plants of selected lines classified into highly resistant (HR), resistant (R), moderately resistant (MR) and susceptible (S) categories based on per cent fungal biomass reduction (%) in infected tissue compared to controls, along with the scored infection type (IT). Photographs were taken at 12 dpi, the same time point when fungal biomass ratios were analysed by qPCR. (b) Quantitation of fungal PtMAPK1 or PtCYC1 transcript abundance in Pt-inoculated leaves of transgenic T1 plants of lines MAPK1-2169 and CYC1-2236 representing different resistance types (as presented in a) by RT-qPCR at 5 dpi. The expression levels of PtMAPK1 or PtCYC1 were normalized to the levels of the Pt succinate dehydrogenase gene and are reported as a percentage, relative to that of the NTC plants (set at 100%). A correlation was observed between HIGS-derived relative reduction in PtMAPK1 and PtCYC1 transcript levels and per cent fungal biomass reduction (%) in a. Bars represent mean values ± SDs of two independent experiments. Values with different letters indicate statistically significant differences (Student’s t-test; P < 0.05).

Next, we asked whether there was an association between HIGS-derived reduction of PtCYC1 or PtMAPK1 transcript levels in Pt and the growth penalty imposed on the fungus in transgenic wheat HD-RNAi lines. Two lines, MAPK1-2169 and CYC1-2236, one from each hp-PtMAPK1RNAi and hp-PtCYC1RNAi transgenic events, were selected, and the relative abundance of fungal PtCYC1 or PtMAPK1 transcripts was measured in transgenic plants representing the range of four different resistance types (as presented in a) by RT-qPCR at 5 dpi. The analysis showed a close relationship between the endogenous PtMAPK1 and PtCYC1 transcript levels and per cent fungal biomass accumulation within the infected host tissues (Figure 2b). The highest reduction in fungal PtMAPK1 or PtCYC1 transcript abundance by HD-RNAi was observed in transgenic plants that also displayed the lowest fungal biomass accumulation (rated as HR) followed by resistant and moderately resistant plants. These results indicate that the
effectiveness of HD-RNAi to thwart WLR infection in transgenic wheat plants was associated with the silencing efficiency of the targeted genes in the colonizing fungus.

Resistance to \( Pt \) in transgenic wheat persists in the advanced generation

To investigate the impact of HD-RNAi on suppression of \( Pt \) infection in advanced generations, three transgenic wheat lines for each of the hp-\( PtMAPK1 \)RNAi (MAPK1-2163, MAPK1-2169 and MAPK1-2166) and hp-\( PtCYC1 \)RNAi (CYC1-2236, CYC1-2248 and CYC1-2224) construct that showed enhanced disease protection were selected and selfed to obtain T2 progeny. PCR and RT-PCR screening revealed the stable inheritance and expression of respective HD-RNAi constructs in transgenic wheat T2 plants (Figure S3). Resistance to \( Pt \) was assessed by inoculating 2-week-old transgenic and control wheat plants with fungal urediniospores. Disease phenotype analyses showed that WLR development was radically altered in transgenic wheat T2 lines, as indicated by fewer pustules of much smaller size often surrounded by chlorotic or necrotic halos. In contrast, leaves of \( Pt \)-infected control plants were completely overgrown by large sporulating uredinia (Figure 4). The disease lesions evaluated at 12 dpi were mainly scored as low IT (IT 1–3) in the T2 plants, reflecting restricted \( Pt \) proliferation, which was substantially less severe than the highly susceptible phenotype (IT4) that was consistently observed in control plants. Also, a delay in both the onset and progression of WLR symptoms was seen in transgenic T2 plants with low ITs (IT < 2) as compared to control plants (Figure S4). These results suggested that HIGS of \( PtMAPK1 \) or \( PtCYC1 \) genes was effective in limiting the growth of pathogenic \( Pt \) in transgenic wheat T2 lines.

![Figure 3](image)

*Figure 3* HIGS-derived silencing of targeted \( Pt \) genes in transgenic wheat T1 lines. RT-qPCR analyses show significant reduction in transcript abundance of fungal \( PtMAPK1 \) or \( PtCYC1 \) genes in selective transgenic wheat T1 lines expressing the respective hp-\( PtMAPK1 \)RNAi (a) and hp-\( PtCYC1 \)RNAi (b) construct as compared to nontransformed control (NTC) plants. cDNA was generated from total RNA isolated from \( Pt \)-challenged leaves at 5 dpi. The expression levels were normalized to the level of the endogenous \( Pt \) reference succinate dehydrogenase gene with control set at 100%. No HD-RNAi effect is seen on the other, sequence-unrelated target fungal gene. Bars represent mean values ± SDs of three independent sample collections, each using leaves pooled from an average 20–25 infection sites of 4–7 plants. *Statistically significant difference with controls, at \( P < 0.05 \) (Student’s t-test).

![Figure 4](image)

*Figure 4* Leaf rust disease resistance in transgenic wheat T2 lines. \( Pt \)-inoculated leaves of representative transgenic wheat T2 lines expressing the hp-\( PtMAPK1 \)RNAi (a) or hp-\( PtCYC1 \)RNAi (b) construct show suppressed disease symptoms with low infection types (IT), compared to nontransformed control (NTC) plants. Photographs were taken at 12 dpi.
To quantify the inhibitory effect of HD-RNAi on fungal development, the amount of fungal DNA in Pt-inoculated leaf samples of transgenic T2 and control wheat plants was measured by qPCR. As expected, transgenic T2 lines accumulated substantially reduced fungal biomass averaging between 44% and 64%, compared to control plants (Figure 5a). RT-qPCR analysis confirmed that in planta inhibition of fungal growth in transgenic T2 lines was due to silencing of targeted genes in Pt. Silencing induced by HD-RNAi significantly reduced PtMAPK1 and PtCYC1 relative transcript levels by approximately 44%–58% in Pt colonizing the corresponding transgenic wheat T2 line, compared to the normal expression levels detected in fungi infecting control plants (Figure 5b). Microscopic examination of Pt development in infected tissue revealed normal mycelial morphology with extensive colonization of host cells in control plants, whereas hyphal growth was significantly compromised in transgenic lines (Figure 5c). This suggests that depletion of target fungal gene transcripts by HD-RNAi imposed a growth arrest on invading Pt in its transgenic host. Taken together, these results showed that HD-RNAi constructs targeting the PtMAPK1 or PtCYC1 gene in Pt were able to confer genetically stable resistance to WLR infection in transgenic wheat.

Production of siRNAs in transgenic wheat

We examined whether the silencing of the targeted PtMAPK1 and PtCYC1 genes in Pt by HD-RNAi was associated with the presence of homologous siRNAs in transgenic wheat plants. Northern blot analysis of total RNA extracted from transgenic T2 wheat lines expressing the respective hp-PtMAPK1RNAi or hp-PtCYC1RNAi construct prior to Pt inoculation revealed the presence of corresponding siRNA molecules (Figure 6). No detectable hybridization signals were observed in nontransformed control plants. The findings showed that hp-PtMAPK1RNAi or hp-PtCYC1RNAi sequences expressed in transgenic wheat plants were processed by the host silencing machinery into siRNA molecules, and these probably are translocated to fungal cells during infection to act upon the target endogenous mRNAs in a nucleotide-specific manner thereby inhibiting their function in the fungus.

![Figure 5](image-url)  
**Figure 5** Quantitative protection from Pt infection in transgenic wheat T2 lines carrying HD-RNAi constructs. (a) Fungal biomass measurements determined by qPCR in Pt-challenged leaves of hp-PtMAPK1RNAi or hp-PtCYC1RNAi expressing transgenic wheat lines and nontransformed control (NTC) plants at 12 dpi. (b) Relative transcript abundance of target PtMAPK1 or PtCYC1 genes analysed by RT-qPCR in Pt-challenged transgenic wheat T2 lines expressing the corresponding hp-PtMAPK1RNAi or hp-PtCYC1RNAi constructs and NTC plants. cDNA was generated from total RNA isolated at 5 dpi. The expression levels were normalized to the level of the endogenous Pt reference succinate dehydrogenase gene, with control set at 100%. Bars represent mean values ± SDs of three independent sample collections, each using leaves pooled from an average 20–25 infection sites of 4–7 plants. *Statistically significant difference with controls, at P < 0.05 (Student’s t-test). (c) Confocal microscopy of Pt infection in wheat leaves at 5 dpi, showing restricted mycelial development in transgenic samples expressing the respective HD-RNAi construct as indicated, compared to the control plant. Bars: 70 μm.
molecules in transgenic wheat T2 lines expressing the respective hp-oligonucleotide marker size. NTC, nontransformed control. Hybridized with target fungal gene-specific DIG-labelled probes. Ethidium bromide-stained rRNA serve as loading controls (LC). The arrow indicates oligonucleotide marker size. NTC, nontransformed control.

Figure 6 sRNA detection in transgenic wheat lines. RNA blots showing the presence of PtMAPK1 (a) and PtCYC1 (b) sequence-specific siRNA molecules in transgenic wheat T2 lines expressing the respective hp-PtMAPK1RNAi or hp-PtCYC1RNAi construct. Total RNA was extracted from wheat leaf tissue prior to fungal inoculations. RNA blots were hybridized with target fungal gene-specific DIG-labelled probes. Ethidium bromide-stained rRNA serve as loading controls (LC). The arrow indicates oligonucleotide marker size. NTC, nontransformed control.

Discussion

In this study, we have shown that developing transgenic wheat plants stably expressing HD-RNAi constructs designed to target Pt genes that are crucial for fungal pathogenicity is an effective strategy to obtain enhanced resistance against the destructive WLR disease. The constitutive expression of HD-RNAi constructs in transgenic wheat lines induced efficient silencing of the targeted PtMAPK1 or PtCYC1 genes in the interacting Pt resulting in subsequent impaired fungal development and a reduction in disease severity and progression in otherwise highly susceptible wheat plants. The engineered resistance trait was heritable and stable in the T2 generation. Suppression of WLR development was correlated with the presence of siRNA molecules specific to the fungal PtMAPK1 or PtCYC1 genes in the transgenic wheat lines expressing the complementary HD-RNAi construct. The in vivo reduction in PtMAPK1 and PtCYC1 transcript levels in Pt feeding on transgenic wheat leaves expressing the homologous siRNA molecules indicated the manifestation of PTGS by HIGS.

The MAP-kinase and cyclophilin-encoding genes were selected for the transgenic HD-RNAi approach because they are implicated in playing fundamental roles in the regulation of various physiological and pathogenesis processes in eukaryotes (Wang and Heitman, 2005). In addition, we demonstrated previously by transient HIGS assays that PtMAPK1 and PtCYC1 genes are important for pathogenicity in Pt (Panwar et al., 2013a,b). Cyclophilins have been reported to act as virulence determinants in diverse plant pathogenic fungi such as Magnaporthe grisea (Viaud et al., 2002), Botrytis cinerea (Viaud et al., 2003) and Cryphonectria parasitica (Chen et al., 2011). MAP-kinases from phytopathogenic fungi have also been extensively studied and shown to regulate cell wall biogenesis, morphogenesis, patho- genic development and disease (Martinez-Soto and Ruiz-Herrera, 2015; Zhao et al., 2007). Earlier, it was reported that the PtMAPK1 gene could complement an Ustilago maydis MAPK (kpp2 kpp6) double deletion mutant to restore mating and full virulence (Hu et al., 2007a). Similarly, Fus3/Kss1-type MAPK from Pst, when expressed in ascomycete Fusarium graminearum and M. oryzae, complemented the corresponding kss1 and pmk1 mutants to partially restore vegetative growth and ability to infect plants (Guo et al., 2011). Consistent with the well-defined functions of MAP-kinases and cyclophilins in ensuring pathogen ability to cause disease, we have shown that silencing their homologs in Pt by transgenic HD-RNAi significantly protected host wheat plants from the disease.

An obligatory requirement for HIGS is the mobility of silencing signals from host plant species into the invading pathogen. The reduction in endogenous transcript abundance of PtMAPK1 and PtCYC1 genes in Pt by the respective HD-RNAi construct was associated with the presence of complementary siRNAs in the transgenic wheat plants. This indicated that these could be silencing molecules that initiated in vivo RNAi of the corresponding PtMAPK1 and PtCYC1 genes in the colonizing fungus. Recently, siRNAs have been reported to move across interacting organisms, encompassing cross-species silencing and communication between host and pathogens or parasites (Knip et al., 2014; Weiberg et al., 2015). A correlation between HIGS in phytopathogenic fungi and the presence of pathogen gene-specific siRNAs in host plants was also observed in other diverse interactions, suggesting that there is a successful translocation of siRNAs from host plants to parasitic fungi (Andrade et al., 2015; Ghag et al., 2014; Govindaraju et al., 2015; Nowara et al., 2010; Tinoco et al., 2010). In the wheat-rust pathosystem, the HD-RNAi signals may traverse from host to fungus via the haustorial interphase (Panwar et al., 2013a) utilizing the mechanism of vesicle-mediated (exosomes) transport (Knip et al., 2014). Despite the progress being made, several aspects of the trans-kingdom RNAi are still poorly understood and more extensive research is required to validate the nature of silencing signals (whether siRNA or precursor dsRNA; e.g., Koch et al., 2016) and their route of delivery during plant–pathogen interactions.

Inhibition of WLR infection in transgenic wheat lines demonstrated that the degree of silencing achieved by HIGS of the target PtMAPK1 and PtCYC1 genes was sufficient to attenuate fungal pathogenicity. Although the severity of disease was significantly compromised in transgenic wheat leaves, we did not observe complete protection against Pt as few pustules continued to develop from initial infection sites. This can be attributed to partial silencing of the PtMAPK1 or PtCYC1 gene as seen in incomplete knockdown of the target fungal transcripts (Figure 3 and 5b); it is possible that the presence of residual transcripts was sufficient to confer some level of virulence. While RNAi can inhibit transcript expression, it normally does not completely eliminate gene function (Eamens et al., 2008). The fact that we observed variability from R to S in the resistance response within transgenic lines in the T1 generation (Fig. 2 and Table 1) is most likely due to segregation of the transgene locus or loci in this generation. Gene expression levels would be expected to vary between plants where the transgene locus is homozygous vs heterozygous which could explain why some plants are scored as R and some as MR, and segregants lacking the transgene will be scored as S. Other factors possibly affecting HIGS efficiency could be the abundance and turnover rate of target mRNAs. Nevertheless, we observed that the efficacy of HD-RNAi constructs in reducing fungal growth in transgenic wheat lines correlated well with the reduced levels of the corresponding PtMAPK1 and PtCYC1 gene transcripts in the invading fungus. A similar cause–effect relationship between HIGS-derived depletion of pathogen mRNA levels and impairment of host infection competency was also reported in different fungal–plant pathosystems (Andrade et al., 2015; Koch et al., 2013). These results indicate that, when exploring HIGS to generate effective plant disease resistance, the extent of target gene silencing in the
pathogen is likely to be important. However, the choice of effective candidate genes is arguably the most crucial parameter for the success of this technology (Panwar et al., 2016).

The WLR disease protection in the transgenic wheat lines was marked by reduced uredinia density and size and by a general delay in symptom appearance in infected host tissue. This supports the notion that the HD-RNAi effect against Pt was quantitative in nature. From a breeding point of view, partial resistance or their actions have often proved to be ephemeral size in the field. In contrast, rust disease control measures that reduce sporulation would decrease fungal population in the field. Indeed, in our transgenic plants, reduced sporulation would decrease fungal population size in the field. In contrast, rust disease control measures that rely on exploiting major resistance genes that recognize pathogen effectors or their actions have often proved to be ephemeral owing to rapid adaptations by these fungi to escape recognition in resistant plants (Dangl et al., 2013; Stokstad, 2007). In this regard, targeting pathogen genes that are indisputable for morphogenesis and/or pathogenesis by HD-RNAi can presumably provide long-lasting protection because modifying or deleting such essential genes would likely result in major consequences to the pathogen. To increase the rate of functional constraints facing the pathogen upon infection, an effective HIGS design can be silencing multiple genes that are required for pathogenicity rather than relying on single gene targets. In a recent study, simultaneous silencing of three cytochrome P450 lanosterol C-14α-demethylase (CYP51) genes in F. graminearum by HD-RNAi was shown to completely restrict fungal infection in transgenic Arabidopsis and barley (Koch et al., 2013), although multiple targets may not always be more effective (Chen et al., 2016). Therefore, one approach would be crossing the hp-PtMAPKTRNAi and hp-PtCYC1TRNAi lines to investigate whether or not a cumulative effect would result in more Pt-resistant lines.

Earlier, transient HD-RNAi assays were utilized for analysing gene function in the wheat rust pathogens (Panwar et al., 2013a, b; Yin et al., 2015; Zhang et al., 2012). A major limitation of transient RNAi techniques, despite being useful in fast forward functional genomic platforms, is that they elicit uneven and short-lived silencing responses (Bhaskar et al., 2009; Holzberg et al., 2002) which are not suitable for sustainable applications of HIGS in agriculture. Stable HD-RNAi on the other hand should offer many advantages such as the establishment of more uniform gene silencing and the transmission of disease resistance traits to next generations. Production of transgenic crop plants will therefore provide a genetic resource to harvest the full potential of HIGS for engineering long-lasting resistance against rust diseases. However, a drawback of transforming a monocotyledonous species, such as wheat, is that it is an arduously lengthy and random process, making it practically unsuitable for screening large number of genes to identify efficient HIGS targets. Therefore, a rational approach would be to first validate the function of potential candidate genes identified in the pathogen by transient HD-RNAi approaches and those showing the ability to deter parasitism in the host can then be tested for the development of transgenic disease-resistant varieties.

The ability to express HIGS constructs in transgenic plants for control of plant pathogens offers an attractive strategy for managing pests of significant importance (Knip et al., 2014). However, the effectiveness of HD-RNAi to impart genetically stable resistance against rust pathogens has not been explored. An important issue to investigate in the future is whether in subsequent wheat generations RNA-mediated DNA methylation of the silencing constructs may occur, thereby reducing transgene expression levels. Our study provides proof of concept that HIGS against rust fungi can be made operational in stable transgenic plants and results are in line with, and extend beyond, technical advancements made in recent years over the potential application of this technology for fighting rust infections in cereal crop plants (Panwar et al., 2013a, b; Yin et al., 2014). The lack of homology between the chosen Pt sequences and published wheat genomic sequences ensured that the likelihood of any off-target silencing effect against a host gene was low; indeed, we did not observe any sort of deviation from a normal growth phenotype in the transgenic wheat plants. By the same analogy though not directly tested here, the transgenic wheat hp-PtMAPKTRNAi and hp-PtCYC1TRNAi lines will likely also prove effective against many other leaf (Pt), stem (Pgt) and stripe (Pst) rust isolates as the sequences used in the HD-RNAi constructs have high homology to the corresponding gene sequences in other sequenced isolates and species. Earlier, we showed through extensive molecular studies using the same constructs in the transient system that the suppression of disease symptoms was highly correlated with the silencing of the target genes and reduction in fungal biomass for various different isolates from all three rust species (Panwar et al., 2013a). Due to complexities associated with conducting reverse genetics studies in rust pathogens, to date, the role of only a few fungal genes involved in the infection processes has been identified. Given that HIGS has emerged as a promising method to analyse gene function in otherwise genetically recalcitrant rust fungi, more studies are needed to address the roles of the large repertoires of predicted pathogenicity-related genes available from genome sequencing projects. The use of such virulence-determining or essential genes that might be functionally difficult to overcome by pathogens promises a potential avenue towards sustainable rust disease protection in wheat cultivars.

Experimental procedures

Plant materials, Pt inoculations and disease assessment

Leaf inoculations were performed using Pt isolate CCDS. Urediospores were increased on susceptible wheat cultivar Fielder plants. The inoculum was prepared by suspending urediospores in light mineral oil (Baytol) at a rate of 12 mg/mL. Inoculum was applied to the leaves of 2-week-old wheat plants using an air brush. The inoculated plants were transferred to a dark dew chamber preconditioned at 22 °C and 100% relative humidity. After 18 h of incubation, plants were transferred to the greenhouse and grown under 16:8 h day: night regime at 22 °C. Disease development in terms of infection types (IT) was assessed when rust symptoms were fully expressed at 12 dpi using the scale 0–4 (Dakouri et al., 2013). In short, infection types 0: no flecks or uredinia; IT 1: small uredinia with necrosis; IT 2: small-to-medium uredinia with necrosis: IT 3: moderate-to-large size uredinia with/without chlorosis; IT 4: very large uredinia. The variation within each class is indicated by the use of – (less than average) and + (more than average).

Construction of HD-RNAi vectors

The Pt genes encoding a MAP-kinase (PtMAPK1) and a cyclophilin (PtCYC1) that we earlier characterized to have an essential role in pathogenicity were used in this study (Panwar et al., 2013a). Binary vectors for wheat transformation were constructed as described earlier (Panwar et al., 2013a). Briefly, a 520-bp
fragment representing the 3'-end coding region of the PtMAPK1 gene (Hu et al., 2007a) GenBank accession #68303937) was PCR-amplified from cDNA of Pt Race 1 isolate BBBD using primers PtMAPK1-F and PtMAPK1-R (Table S1). The amplified segment was cloned into pENTER™ vector using the D-TOPO® Cloning kit (Invitrogen, Carlsbad, CA) and the recombinant entry module recombined in the cereal-specific binary vector pPK007 (Himmelbach et al., 2007) to generate hp-PtMAPK1RNAi. The resulting RNAi vector allowed expression of hpRNA molecules under the control of the Zea mays ubiquitin promoter and terminator sequences. To generate hp-PtCYC1RNAi, a 501-bp 3'-end coding region of PtCYC1 (Hu et al., 2007b) GenBank accession #BU672663) was amplified from the EST sequence (Picontig6674) using primers PtCYC1-F and PtCYC1-R (Table S1). The amplified fragment was sequentially cloned into plasmid pUBL-leX1-RNAi to generate an intron-spliced hpRNA segment that was subsequently cloned into the binary vector pMCG161 (http://www.chromodb.org/rnai/vector_info.html) under the control of the Cauliflower mosaic virus 35S promoter and octopine synthase terminator. All final constructs were verified by sequencing.

Genomic DNA extraction

Fungal genomic DNA was extracted from germinating urediniospores as described earlier (Panwar et al., 2013a). Urediniospores were germinated by dusting them over sterile water in dishes for over 8 h using a volatile nonanol solution [1.56 μL nonanol (Sigma-Aldrich, ON, Canada), 1 mL acetone, 19 mL of ddH2O] spotted on filter paper suspended in the lids to stimulate germination. Plant genomic DNA was extracted from leaf tissue using the DNeasy plant Mini Kit (Qiagen, Mississauga, ON, Canada) as described by the manufacturer.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol reagent (Invitrogen) as described by the manufacturer. The quality and concentration of total RNA were assessed by denaturing 1.2% agarose gel and 260/280ABS measurements on a NanoDrop spectrophotometer (ThermoFisher Scientific, Mississauga, ON, Canada). Total RNA extraction and cDNA synthesis were performed with filter setting at 411–485 and 550–560 nm, and scanning was performed using candidate gene-specific standard curves to quantify the Pt and wheat gDNA, respectively.

Confocal microscopy

Microscopic analysis of mycelium growth in Pt-inoculated wheat leaves was carried out using a Leica SP2-AOBS laser scanning confocal microscope (Leica, Mannheim, Germany) at 5 dpi. Fungal structures were stained with Uvitex-2B and Acridine Orange (Sigma-Aldrich) was used to stain plant cell walls followed by hybridization as described previously (Panwar et al., 2013b). The fluorescence of Uvitex-2B and Acridine Orange was detected by excitation at 405 and 514 nm, and scanning was performed with filter setting at 411–485 and 550–560 nm, respectively.

siRNA detection

Small RNA detection was performed using total RNA extracted from wheat leaves prior to Pt inoculation. Equal amounts of RNA (15 μg) from different samples were resolved on denaturing 15% urea-polyacrylamide gels and transferred to Hybond-N+ membranes (Amersham Biosciences, Mississauga, ON, Canada) using a semidy blotter (Bio-Rad). Transferred RNA was then cross-linked to the membrane by UV with an energy output of 1200 J/cm² for 5 min. DIG-labelled DNA probes were synthesized using target fungal gene sequences as template. Prehybridization was performed using DIG Easy Hybridization buffer (Sigma-Aldrich) followed by hybridization at 42 °C overnight. The membranes were washed twice with 1 x SSC, 0.1% SDS for a total of 15 min at 42 °C. Detection of the hybridized probe was performed using the DIG Nucleic Acid Detection Kit (Sigma-Aldrich) following the manufacturer instructions. Photoemissions were captured using a ChemiDoc-IT Imaging System (Bio-Rad).

Acknowledgements

This work was supported by grants from the Western Grains Research Foundation and the Alberta Wheat Commission (Project...
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#A08853) and from the Canadian Wheat Improvement Flagship as part of the Canadian Wheat Alliance. The authors declare no conflict of interest.

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Leaf rust resistance in transgenic wheat by HD-RNAi

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Molecular analysis of transgenic wheat T1 lines. Integration of hp-PtMAPK1RNAi or hp-PtCYC1RNAi construct in transgenic plants analyzed by PCR-amplification of the corresponding PtMAPK1 (a) or PtCYC1 (b) transgenes. Genomic DNA extracted from transgenic and non-transformed control (NTC) plants prior to Pt inoculation was used as template in PCR reactions. Expression of PtMAPK1RNAi or hp-PtCYC1RNAi in the same plants analysed by RT-PCR using primers specific to the corresponding PtMAPK1 (c) or PtCYC1 (d) transgene. cDNA prepared from total RNA extracted from transgenic and control plants prior to Pt inoculations was used as template in RT-PCR reactions. M indicates 1 kb DNA ladder (Invitrogen); plasmid DNA served as positive control (PC). The PCR products were fractionated on a 1% agarose gel. Results from representative plants of each selected line are shown (lanes 1–20).

Figure S2 Delay in progression of symptoms in leaves of hp-PtMAPK1RNAi or hp-PtCYC1RNAi expressing wheat T1 plants. Pt inoculated transgenic leaves are characterized by a slower and more restrictive uredinia development as compared to non-transformed control Fielder plants. Representative leaves from transgenic T1 lines M1APK1-2166 and CYC1-2236 are shown. Photographs were taken at 6 dpi.

Figure S3 Molecular analysis of transgenic wheat T2 lines. Integration of hp-PtMAPK1RNAi or hp-PtCYC1RNAi constructs in transgenic plants analyzed by PCR-amplification of the corresponding PtMAPK1 (a) and PtCYC1 (b) transgenes. Genomic DNA extracted from transgenic and non-transformed control (NTC) plants prior to Pt inoculation was used as template in PCR reactions. Expression of PtMAPK1RNAi or hp-PtCYC1RNAi in the same plants was analysed by RT-PCR using primers specific to the corresponding PtMAPK1 (c) or PtCYC1 (d) transgene. cDNA prepared from total RNA extracted from transgenic and control plants prior to Pt inoculations was used as template in RT-PCR reactions. M indicates 1 kb DNA ladder (Invitrogen); BC, buffer control; plasmid DNA served as positive control (PC). The PCR products were fractionated on a 1% agarose gel. Results from representative plants of each selected line are shown (lanes 1–3).

Figure S4 Delay in progression of symptoms in leaves of hp-PtMAPK1RNAi or hp-PtCYC1RNAi expressing wheat T2 plants. Pt inoculated transgenic leaves are characterized by a slower and more restrictive uredinia development as compared to non-transformed control Fielder plants (NTC). Representative leaves from transgenic T2 lines MAPK1-2166 and CYC1-2236 are shown. Photographs were taken at 6 dpi.

Table S1 Primers used in the research.

Table S2 Segregation analysis of transgenic wheat T1 lines.