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Host-induced gene silencing of wheat leaf rust fungus *Puccinia triticina* pathogenicity genes mediated by the *Barley stripe mosaic virus*

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Abstract Rust fungi are devastating plant pathogens and several *Puccinia* species have a large economic impact on wheat production worldwide. Disease protection, mostly offered by introgressed host-resistance genes, is often race-specific and rapidly overcome by newly-emerging virulent strains. Extensive new genomic resources have identified vital pathogenicity genes but their study is hampered because of the biotrophic life styles of rust fungi. In cereals, *Barley stripe mosaic virus* (BSMV)-induced RNAi has emerged as a useful tool to study loss-of-function phenotypes of candidate genes. Expression of pathogen-derived gene fragments in this system can be used to obtain *in planta*-generated silencing of corresponding genes inside biotrophic pathogens, a technique termed host-induced gene silencing (HIGS). Here we test the effectiveness of BSMV-mediated HIGS in the wheat leaf rust fungus *Puccinia triticina* (*Pt*) by targeting three predicted pathogenicity genes, a MAPK, a cyclophilin, and a calcineurin regulatory subunit. Inoculation of BSMV RNAi constructs generated fungal gene-specific siRNA molecules in systemic leaves of wheat plant. Subsequent *Pt* inoculation resulted in a suppressed disease phenotype and a reduction in endogenous transcript levels of the targeted fungal genes indicating translocation of siRNA molecules from host to fungal cells. Efficiency of this host-generated trans-specific RNAi was enhanced by using BSMV silencing vectors defective in coat protein coupled with introducing fungal gene sequences simultaneously in sense and antisense orientation. The disease suppression indicated the likely involvement of these fungal genes in pathogenicity. This study demonstrates that BSMV-mediated *in planta*-generated RNAi is an effective strategy for functional genomics in rust fungi.

Keywords Gene silencing · Host-induced gene silencing · HIGS · Functional genomics · Plant–microbe interaction · *Puccinia triticina* · VIGS · Virulence · Wheat leaf rust

Introduction

RNA-induced gene silencing or RNA interference (RNAi) is a conserved regulatory mechanism of gene expression that has been widely characterized in eukaryotic organisms. The RNA silencing pathway is triggered by the processing of double stranded RNA (dsRNA) into short RNA duplexes of 21–28 nucleotides in length by a series of proteins associated with post-transcriptional gene silencing (PTGS), followed by the guided cleavage or translational repression of complimentary mRNAs by the generated small interfering (siRNA) duplexes (Ruiz-Ferrer and Voinnet 2009). Besides regulatory roles in development, siRNA-mediated RNA silencing also functions as a natural antiviral defence mechanism, a process named virus-induced gene silencing (VIGS).

The RNAi mechanisms have been exploited extensively and have become powerful functional genomics tools to

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silence virtually any gene of interest by introducing target gene sequences into cells or organisms. The subsequent detailed analysis of resulting loss of functions and altered phenotypes represent the most readily interpretable method for experimentally validating the roles respective genes play. In plants, VIGS has been established as a rapid functional analysis assay of plant genes by exploiting viruses to deliver corresponding transgenes (Senthil-Kumar and Mysore 2011). VIGS technology was mainly successful in dicotyledonous plants until the *Barley stripe mosaic virus* (BSMV) genome was modified as a suitable vector for cereals (Holzberg et al. 2002; Scofield et al. 2005; Tai et al. 2005; Renner et al. 2009; Pacak et al. 2010; Van Eck et al. 2010; Yuan et al. 2011). BSMV is a type member of the *Hordeivirus* genus with a positive-sense, single strand RNA genome consisting of three segments, designated as α, β and γ (Jackson et al. 2009). The β component of BSMV encodes the coat protein (CP) that is dispensable for systemic movement of virus and deletion of CP has been shown to enhance the efficiency of BSMV-mediated gene silencing, referred to as BSMV-VIGS (Holzberg et al. 2002). The BSMV-VIGS system has been successfully applied to investigate roles of host resistance genes against several plant fungal pathogens including *Puccinia triticina* (Scofield et al. 2005; Loutre et al. 2009), *Blumeria graminis* (Hein et al. 2005) and *Pyrenophora tritici-repentis* (Tai and Bragg 2007). However, RNA silencing tools have not yet been used extensively to study the function of genes in rust fungi.

Rust fungal pathogens are a major threat to crop yields and result in huge economic losses globally. Leaf rust, caused by *P. triticina* (*Pt*) Eriks., is the most common and important disease of wheat worldwide (Bolton et al. 2008; Kolmer et al. 2009). The infection process of wheat plants by *Pt* involves a high degree of morphological and physiological differentiation allowing the fungus to penetrate and grow within the host, modify host cell structure, reprogram host metabolism and reproduce prolifically (Bolton et al. 2008; Kolmer et al. 2009). Rust fungi exploit the host plant for nutrients by forming a typical biotrophic interaction in which specialized feeding structures called haustoria are developed inside host cells (Szabo and Bushnell 2001; Bolton et al. 2008; Voegele and Mendgen 2011). Haustoria are also implicated in the translocation of macromolecules, notably effector proteins from the pathogen into host cells; this has been reported for Oomycete pathogens but a similar function for rust fungal haustoria had been inferred earlier (Kemen et al. 2005; Catanzariti et al. 2006; Whisson et al. 2007; Rafiqi et al. 2012).

Genetic resistance is the most economical and sustainable way to control plant diseases and extensive wheat breeding programs have been moderately successful in maintaining resistance against the onslaught of ever-changing rust fungal isolates, including *Pt* races (Kolmer 1996; Bolton et al. 2008). The challenges presented by rust fungi therefore demand a continuing search for innovative strategies for developing durable and effective resistance. One promising approach is to select fungal genes identified as likely being crucial for pathogenicity and target these "Achilles heels" to develop resistance in host plants. The concept of expressing dsRNA in host plants targeting essential genes in organisms feeding on them has emerged as a possible alternative strategy to combat parasites not easily amenable to genetic transformation (Huang et al. 2006; Baum et al. 2007; Tomilov et al. 2008). Using the BSMV-VIGS approach, Nowara et al. (2010) showed in a proof-of-concept study that, when targeting pathogenicity genes in the barley powdery mildew fungus, *B. graminis*, such host-induced gene silencing (which they termed 'HIGS') resulted in disease suppression. This approach was emulated to identify possible effector gene function in the wheat stripe rust fungus, *P. striiformis* f. sp. *tritici*, but no effect on disease development could be seen (Yin et al. 2011).

The extensive progress being made in genome data mining is leading to the identification of many genes predicted to be important in pathogenesis. For rust fungi however, being obligate parasites, effective and rapid bioassays are needed to test the role of such predicted genes vital for disease development. Recently, we reported a transient RNAi approach based on *Agrobacterium tumefaciens*-mediated infiltration (agroinfiltration), resulting in the delivery of fungal gene sequences capable of forming self-complementary RNA in wheat plants, as a functional genomics screening system for rust fungi (Panwar et al. 2013). We chose as targets two genes that had been implicated in pathogenicity in other pathosystems, a cyclophilin and the regulatory B subunit of calcineurin (Wang and Heitman 2005; Stie and Fox 2007; Cervantes-Chávez et al. 2011), and a MAP kinase, shown indirectly to function in pathogenicity in a heterologous system (Hu et al. 2007a). A limitation of the agroinfiltration assay was that RNAi of fungal genes was effective only within the leaf segment expressing target gene silencing constructs. In this study, we investigate the feasibility of using the BSMV-based HIGS approach for analysing gene function in the leaf rust fungus by evaluating the same *Pt* target genes. Our results show that ectopic expression of target *Pt* gene fragments in wheat using BSMV constructs results in the generation of complementary siRNA molecules in systemic leaves which trigger RNA silencing of the corresponding genes in colonizing fungi, resulting in disease suppression. We also show that the efficiency of HIGS improves when introducing in wheat a BSMV vector defective in CP function and simultaneously introducing sense and antisense forms of the candidate fungal gene fragments.

Materials and methods

Vector construction

The genome of BSMV consists of three RNA fragments, α, β, and γ. The constructs used in this study were described by Holzberg et al. (2002) with the γ component carrying a 185 bp-fragment from the barley *phytoene desaturase* (*PDS*) gene in either sense or antisense orientation (Fig. 1). The barley *PDS* fragment revealed only 5 bp changes to the closest available *Triticum aestivum* homolog available in GenBank (BT009315.1). A 452 bp 3′-end sequence of *PtMAPK1* (Hu et al. 2007a; GenBank accession #68303937) was amplified using gene specific primers PtMAPK1-F and PtMAPK1-R harboring PacI and NotI restriction sites, respectively, at their extremities (Table 1). The PCR amplified product was digested with NotI and PacI and directionally cloned in sense or antisense orientation, replacing the *PDS* gene inserts in the γRNA component to generate pγ:PtMAPK1s and pγ:PtMAPK1as, respectively. A *Pt* EST assembly, PtContig6674 (TR24, GenBank accession #BU672663; Hu et al. 2007b; Xu et al. 2011), encoded a protein with homology to a cyclophilin. A 435-bp 3′-part of this gene, *PtCYC1*, was amplified using primers PtCYC1-F and PtCYC1-R (Table 1) and cloned into the PacI-and NotI-digested BSMVγ multiple cloning site to generate constructs pγ:PtCYC1s and pγ:PtCYC1as, respectively. A third candidate target was derived from an EST sequence (PT0315.I05.C21.ptt, GenBank accession annotated as calcineurin B regulatory subunit, CNB. This EST clone was used to generate a 422-bp 3′-end fragment using PCR and primers CNB-F and CNB-R (Table 1). Similarly as for *PtMAPK1* and *PtCYC1* gene fragments, this PCR product was subsequently cloned in sense and antisense form to generate pγ:CNBs and pγ:CNBas, respectively. Incidentally, when the stem, leaf and stripe rust fungal genomes became available (Broad Institute, http://www.broadinstitute.org/; Cantu et al. 2011), this EST sequence was found to be more related to ascomycete *CNB* genes, revealing 83 % nucleotide and 96 % amino acid identities with a homolog in *Aspergillus kawachii* (Panwar et al. 2013). Nevertheless, over the length of the fragment expressed in the BSMV construct, it revealed 67 % nucleotide identities with the homolog in *Pt* (Fig. S2). All recombinant γ vectors were verified by restriction enzyme digestion and sequencing.

Biological materials, growth condition and fungal inoculation

P. triticina Eriks race 1 (BBBD) was maintained and grown on susceptible wheat (*T. aestivum*) cv. Thatcher (RL 6101). Fungal inoculations were done as described previously (Panwar et al. 2013). Briefly, 10 day-old wheat plants were spray-inoculated with freshly-collected urediniospores

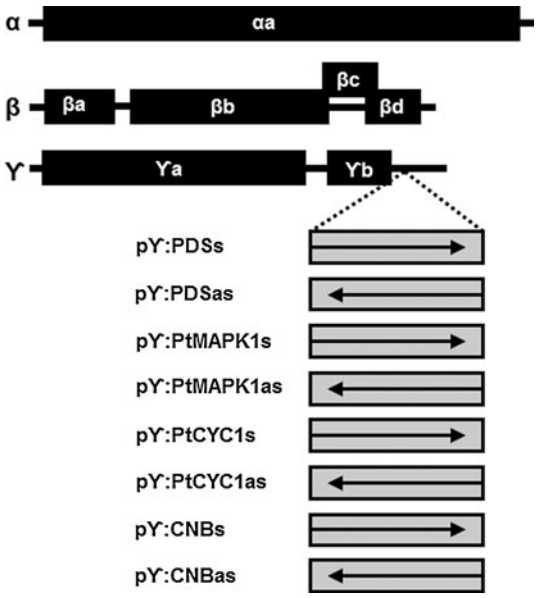


Fig. 1 Schematic representation of the *Barley stripe mosaic virus* (BSMV) genome organization and modified γ RNAs used for target gene insertion. Genomic organization of α-, β- and γRNAs were as described previously (Holzberg et al. 2002). The multiple cloning site is positioned downstream of γb gene. The *βa* gene encodes the coat protein (CP). *Arrows* indicate the orientation of the gene fragments inserted: s, sense orientation; as, antisense orientation. Not drawn to scale

mixed with light mineral oil (Soltrol 130 isoparaffin, Chevron Phillips Chemical, Borger, TX, USA) and left overnight in the dark under high humidity, at 20 °C, which is optimal for spore germination. Twenty mg of urediniospores suspended in 2 ml of Soltrol was used to inoculate a batch of eighteen 10 × 10-cm pots containing 4–5 wheat plants each.

In vitro transcription and BSMV inoculation

The plasmids corresponding to the three components of BSMV were linearized with MluI (α component), BSSHII (γ component) or SpeI (β component) and used as template for in vitro transcription using the mMessage mMachine transcription kit (Ambion), following the manufacturers protocol. The concentration of RNA derived from in vitro transcription reactions was adjusted to 1 μg/μl and 2.5 μl of each of the three viral RNA components were combined and mixed with 45 μl of FES buffer (77 mM glycine, 60 mM K_2HPO_4, 22 mM $Na_4P_2O_7{\cdot}10H_2O$, 1 % (w/v) bentonite and 1 % (w/v) Celite). For mixed inoculations, using the sense and antisense form of the target gene, the recombinant γRNA transcripts were mixed in equimolar ratio. The mixture was applied to the first fully expended leaf of 7–8 days old wheat plants by rub inoculation with a gloved finger. To minimize damage and improve virus infection, the inoculated plants were misted with water from a distance (without forming droplets on the plants)

Table 1 Primers used in this study

Gene	Primer sequence
PtMAPK1-F	ATATTAATTAAAGCCATCGACGTCTGGTC
PtMAPK1-R	TATGCGGCCGCAGCTGTTGATTTCCTCAA
CNB-F	ATATTAATTAACGCTTCATGAAGCTTGAT
CNB-R	TATGCGGCCGCTCATACTGAGGCTCACGT
PtCYC1-F	ATATTAATTAATTGGTCGCATTGAATTCA
PtCYC1-R	TATGCGGCCGCAGTCAGCGATCTTGACGG
PtMAPK1-QPCR-F	TTGAAGCCATCGAACCTTTT
PtMAPK1-QPCR-R	TGGCTTTGGTGTATTGCTTG
PtCYC1-QPCR-F	GGTGAAGTTGTCAAGGGCTTC
PtCYC1-QPCR-R	GTATGACTCGCTTCTGGATGG
BSMV-QPCR-γF	GGAACAACCCCGTGTAATTG
BSMV-QPCR-γR	GACCACGTTCAACTCCCACT
Ta_EF1-QPCR-F	GGTGATGCTGGCATAGTGAA
Ta_EF1-QPCR-R	GATGACACCAACAGCCACAG
TaPDS-QPCR-F	CGGAGAAGGTGAAGTTTGCT
TaPDS-QPCR-R	TGCATGGATAACTCGTCAGG
PtRTP1-QPCR-F	CGGAAGAATAGCCGGAAAATG
PtRTP1-QPCR-R	CTTAGACATCTCGATGTCTCG
Pt succinate dehydrogenase-QPCR-F	GGTTCCAGCGATAGATCGAG
Pt succinate dehydrogenase-QPCR-R	CAACTACGACCAGCCACTCA
PtCNB-QPCR-F	AAAGTCTTCAAACTTCACGGG
PtCNB-QPCR-R	GGAAATCTACCGTTCCACCA

and kept overnight in the dark covered with a plastic bag before transferring them in controlled environmental chambers at 20 °C with 16 h of light and 8 h darkness. In our studies, more than 95 % of inoculated wheat plants developed virus symptoms. For each experiment, all inoculated plants were of the same age and only those displaying virus symptoms were included in analyses.

DNA and RNA extraction

Plant genomic DNA was extracted from pooled leaves using the CTAB method (Allen et al. 2006). For *in planta* fungal biomass quantification, epiphytic fungal mycelium was wiped off the leaf surfaces using wet cotton pads prior to genomic DNA extraction. Fungal genomic DNA was isolated from *Pt* urediniospores germinated in petri dish over water supplemented with 10^{-7} M nonanol (nonyl alcohol; Sigma-Aldrich, Oakville, ON, Canada) as described (Hu et al. 2007b). The resulting mesh of germinated tubes was collected and ground to a fine powder in liquid nitrogen from which total fungal genomic DNA was isolated using a Plant DNA Extraction kit (Qiagen) as recommended by the manufacturer. Total RNA was isolated using Trizol reagent (Invitrogen) as described by the manufacturer.

Uredinia count

The fungal disease phenotype was quantified by counting the number of uredinia 10 days after rust inoculation using photographed leaves and ImageJ Software (National Institutes of Health, Bethesda, Maryland, USA). Only those lesions that were sporulating were counted. To avoid bias among leaf samples, leaves from ten treated plants from two different experiments were randomly selected. Interpretation of results was based upon values in silenced plants compared to those of controls.

Staining and confocal microscopy

Leaf samples were collected 5 days after fungal inoculation. Samples were fixed, cleared and fungal structures stained with Uvitex-2B as described (Moldenhauer et al. 2006) with some modifications. Briefly, leaf tissues were washed with 50 % (v/v) ethanol, rinsed in ddH_2O and incubated at 90 °C in 0.5 M sodium hydroxide for 30 min. Leaves were then rinsed in ddH_2O and soaked in 0.1 M Tris–HCl buffer (pH 5.8) for 30 min. Subsequently, samples were stained in Uvitex 2B (Polysciences Inc., Warrington, PA) solution (0.1 % (w/v) in 0.1 M Tris–HCl, pH 5.8) for 5 min. After several washes in ddH_2O, specimens were treated with 0.1 % (w/v) Acridine Orange (Sigma, Aldrich) for 2 min to stain plant cell walls. Excessive dye was removed by a thorough wash in ddH_2O. Samples were then rinsed twice with 25 % (v/v) glycerol and finally stored in 50 % (v/v) glycerol until observation. Fluorescence microscopy was carried out using a Leica SP2-AOBS laser scanning confocal microscope. Fluorescence of Uvitex 2B and Acridine Orange was detected by excitation at 405 and 514 nm and scanning with filter settings at 411–485 and 550–560 nm, respectively.

Quantitative real-time PCR analysis

Total RNA was treated with DNase I using the TURBO DNA-Free kit following instructions by the manufacturer (Ambion) to remove DNA contamination prior to cDNA synthesis. First strand cDNA was synthesized from 1 μg of total RNA using SuperscriptIII enzyme (Invitrogen) according to manufacturer's instructions. No-reverse transcriptase controls were included to check for contaminating gDNA. The quantity and purity of RNA was analyzed after each step by 1.5 % formaldehyde gel electrophoresis as well as optical density reading using a Nanodrop spectrometer (Thermo Fisher Scientific). All qPCR analyses were carried out on a CFX96TM real-time

PCR machine (Bio-Rad). Specific primers for each gene were designed using the Primer 3.0 program and are shown in Table 1. PCR reactions were performed in a final volume of 10 µl containing SsoFast EvaGreen Supermix (Bio-Rad), 1 µl of template (10 × diluted) and 200 nM of each primer. Thermal cycling parameters were: 98 °C for 2 min, followed by 39 cycles of 95 °C for 10 s and 60 °C for 30 s. Three technical replicates were performed on each sample. The specificity of the PCR reaction was determined by melting curve analysis between 65 and 95 °C. Experiments included a non-template control. Expression levels of gene transcripts were quantified by the qPCR analysis software version 2.2 (Bio-Rad). The wheat *EF1* gene (GenBank accession #M90077.1; Table 1) and the succinate dehydrogenase gene from *Pt*, previously selected among several candidates (Song et al. 2011), were used as respective endogenous reference genes for normalization. RT-qPCR primers specific to the *PtCNB* gene transcripts were designed to distinguish them from the *CNB* sequences derived from the EST sequence (Table 1). Quantitation of the amount of γ molecules in wheat leaves was performed on cDNA synthesized on the RNA isolated from third systemically infected leaves with BSMV silencing vectors. For this, qPCR primers specific to γ component were used. To quantify fungal biomass, the relative ratio of the single copy gene *PtRTP1* (Rust transferred protein; Song et al. 2011), and the wheat *EF1* gene was assessed using gene-specific primers (Table 1). The relative amounts of PCR product of *PtRTP1* and *TaEF1* in mixed/infected samples were calculated using generated gene-specific standard curves to quantify the *Pt* and wheat gDNA, respectively.

Short interfering RNA detection

Small RNA detection was carried out using total RNA extracted (following procedures as described above) from wheat plants 10 days post inoculation with BSMV vectors or buffer. From different samples analyzed, a similar amount of RNA (10 µg) was separated on a 15 % polyacrylamide-7 M urea gel and transferred to neutral Hybond NX membrane (Amersham) using a semi-dry transfer unit (Bio-Rad). Equal loading of small RNAs was estimated by ethidium bromide staining of predominant RNAs in the fraction. The transferred molecules were chemically cross-linked to the membrane using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC: Sigma), essentially as described (Pall and Hamilton 2008). Oligonucleotide primers were used as molecular weight marker. For probe preparation, PCR-amplified target gene fragments were labelled with [α^{32}P]-dCTP using a random primer DNA labelling kit (Amersham). RNA blot hybridization was carried out using ULTRAhyb Hybridization buffer (Ambion) at 38 °C for 18 h. Membranes were then exposed to HyperfilmTM MP (Amersham).

Results

Selection of a suitable wheat cultivar and optimization of BSMV-VIGS assay

The efficiency of VIGS depends on the ability of host plants to sustain virus infection without having any detrimental effect on the assayed phenotype. In order to establish BSMV-induced gene silencing in a wheat host to target fungal genes, we first screened among cultivars available to us for one that provided a suitable genetic background to condition susceptibility to *Pt* and tolerate virus infection to elicit a significant VIGS response, similar to tests performed by Bennypaul et al. (2012) on a different set of cultivars. Several cultivars susceptible to *Pt* were tested, including Morocco, Little Club, Thatcher, Katepwa and Neepawa. We screened these cultivars visually for symptoms produced upon BSMV infection and the ability of this system to induce VIGS by targeting the endogenous phytoene desaturase gene (*PDS*). The *PDS* gene is involved in the carotenoid biosynthesis pathway and is a common phenotypic marker used in gene silencing studies (Holzberg et al. 2002; Bruun-Rasmussen et al. 2007). *PDS* silencing results in reduced levels of photoprotective carotenoids leading to rapid destruction of chlorophyll by photo-oxidation which subsequently results in a photobleached leaf phenotype. The first leaf of 7–8 day-old wheat plants of these cultivars was mechanically rub-inoculated with a mixture of in vitro transcripts from plasmids containing the BSMV αRNA, βRNA and γRNA carrying either no insert (BSMV:00) or a *PDS* gene segment cloned in antisense form (BSMV:PDSas; Fig. 1). All infected plants produced systemic mosaic viral symptoms characteristic of BSMV infection when no insert was present, or additional easily discernible photobleached patterns, when a *PDS* gene fragment was present, in upper non-inoculated leaves by 7–10 days post infection (dpi). A variation in virus and *PDS* gene silencing symptoms was observed among different cultivars (Fig. S1). BSMV symptoms were more pronounced and severe in cultivars Little Club, Katepwa and Neepawa, resulting in plant stunting, necrosis and curling of leaves. A similar virus infection phenotype was also observed in BSMV:PDSas-inoculated plants of these cultivars that displayed in addition weak photobleaching symptoms which were mostly restricted to the vicinity of veins and did not spread across the whole leaf. In comparison, virus infection was less severe in cultivars Thatcher and Morocco and did not seem to affect growth patterns of the plant. These two cultivars also displayed strong and more uniformly distributed photobleaching symptoms across the upper non-inoculated leaves; although, green streaks of non-photobleached tissues remained. Both Thatcher and Morocco are susceptible

to *Pt* race 1 used in this study and since they showed more efficient *PDS* silencing and less pronounced BSMV symptoms than the other cultivars tested, we chose cultivar Thatcher for all subsequent experiments.

A variant of the BSMV silencing vectors, where the *βa* gene encoding for the coat protein is deleted (Fig. 1), was reported to enhance the efficiency of VIGS in barley (Holzberg et al. 2002). To test whether removal of the *βa* component would increase the efficiency of endogenous *PDS* gene silencing in wheat cv. Thatcher plants as well, we inoculated them with viral vectors with (BSMV:PDSas) or without (BSMVΔ:PDSas) the CP gene. Thatcher plants inoculated with BSMV variants lacking the *PDS* gene fragment or with buffer (FES) were included as controls. Phenotypically, wheat plants treated with BSMVΔ:PDSas showed more extensive *PDS* silencing, observed as white patches on upper non-inoculated leaves with more profound photobleaching symptoms developing on third systemic leaves, compared to those inoculated with BSMV:PDSas. No photobleaching was observed in BSMV control or buffer inoculated plants (Fig. 2a). In some cases, Thatcher plants inoculated with the BSMV CP defective mutants showed milder stunting and leaf tip necrosis at later infection stages, but overall, virus symptoms and growth phenotypes were not significantly different from plants inoculated with BSMV having the CP gene. Thus, the silencing effect seemed stronger when using the ΔCP vector. Though BSMV-VIGS can spread throughout the plant, the nature of this type of silencing is mostly transient with the third systemic leaf reported to show the most pronounced silencing (Bruun-Rasmussen et al. 2007). Therefore, we chose the third systemic leaf for VIGS assays in all experiments.

RNA based silencing mechanisms require the production of small RNAs that are often generated as a result of gene regulation and are a natural defence mechanism of most organisms against any foreign genetic material such as viruses or transposable elements (Mlotshwa et al. 2008). Detection of siRNA molecules specific to the *PDS* gene in upper, non-inoculated leaves indicated that the BSMV constructs triggered in the plant the production of molecules known to cause silencing (Fig. 2b). The BSMV CP deletion vectors appeared to produce a higher level of siRNA molecules compared to CP+ constructs. To verify that the observed photobleaching phenotypes and *PDS* sequence-specific siRNA molecules correlated with altered *PDS* gene transcript levels, quantitative reverse-transcription PCR (RT-qPCR) was carried out to determine the abundance of *PDS* transcripts in silenced and control plants. Inoculation with the different variants of BSMV-PDS silencing vectors showed indeed a significant reduction in endogenous *PDS* transcript levels compared to plants treated with BSMV alone (Fig. 2c). As anticipated from the siRNA levels, the BSMVΔ:PDSas vector caused a small but significant further reduction in *PDS* transcript levels compared to the CP+ construct. Viral genome accumulation, as measured by RT-qPCR using a region away from the insert and the subgenomic γRNA, was not significantly higher in wheat plants infected with the ΔCP vectors compared to those treated with CP+ constructs (Fig. 2d). Overall, the results indicated that BSMV silencing vectors defective in the CP gene enhanced silencing in wheat cv. Thatcher. We hypothesized that these variants might also increase the efficiency of HIGS when targeting pathogen genes.

To see the effect of transgene orientation on gene silencing, Thatcher plants were also inoculated with a BSMV ΔCP silencing vector having the *PDS* fragment cloned in the sense orientation (BSMVΔ:PDSs). The *PDS* silencing phenotype generated by the BSMVΔ:PDSs construct was not noticeably different from that induced by the antisense *PDS* gene construct (BSMVΔ:PDSas) and also the siRNA levels and subsequent transcript abundance analysis by RT-qPCR showed no significant difference (Fig. 2). This result suggested that silencing efficiency was independent of the orientation of the target gene. With the conditions for the BSMV-based gene silencing assay established, several fungal *Pt* genes were targeted.

Expression of target fungal gene fragments using BSMV-VIGS in wheat reduces *Pt* disease symptoms and mycelial growth

In our previous study (Panwar et al. 2013), we had selected three candidate pathogenicity genes from an EST database, representing transcribed sequences from wheat infected with *Pt* (Hu et al. 2007b; Xu et al. 2011). To validate our assays, pathogen genes that are necessary for disease development are of interest because disruption or loss-of-function of such pathogenicity determining genes might result in the reduction or complete loss of disease symptoms; a change in pathogenicity or virulence is likely relatively easy to score. Targeting these genes would allow us to assess whether the BSMV silencing system is also suitable for functional analysis of *Pt* genes. The three candidate genes chosen were *PtCYC1*, coding for a cyclophilin (cyclophilins are proteins with defined functions as folding catalysts and chaperones involved in cell signaling), a MAP Kinase gene, *PtMAPK1* (a family member of serine/threonine protein kinases with known functions in the phosphorylation of transcription factors regulating cell growth and differentiation), and the regulatory subunit of calcineurin, *CNB* (relaying calcium signals and responding to environmental stress by regulating morphogenesis and cell cycle; see "Materials and Methods"). These genes had been implicated in pathogenicity in other pathosystems

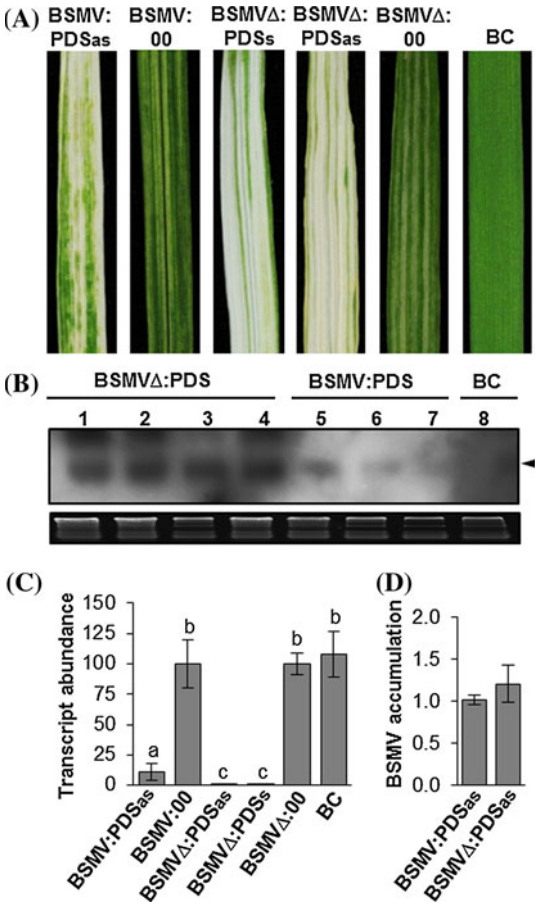


Fig. 2 Silencing of the phytoene desaturase (*PDS*) gene in wheat cv. Thatcher using BSMV-VIGS. **a** *PDS* silencing phenotypes induced by viral vectors with (BSMV) or without (BSMVΔ) the coat protein and expressing the *PDS* gene fragment in sense (PDSs) or antisense (PDSas) orientation. BSMV:00 or BSMVΔ:00, empty vector controls; BC, mock-inoculated buffer control. Plants inoculated with BSMV harboring *PDS* gene insert show severe chlorophyll photobleaching symptoms, indicating *PDS* silencing, in contrast to those treated with empty viral vectors or buffer. Photographs taken 10 days post inoculation. **b** RNA gel blot analysis of *PDS* siRNA accumulation. Small RNAs were readily detected in wheat plants inoculated with BSMV variants carrying the *PDS* gene insert in sense (*lanes 1 and 2*) or antisense orientation (*lanes 3–7*). No signal was detected in buffer-inoculated control (*lane 8*). The RNA blot was hybridized with the radiolabeled *PDS* DNA fragment. Bottom panel, rRNA gel loading control. The *arrow* indicates an oligonucleotide size marker of 23 nucleotides. **c** RT-qPCR analysis of *PDS* transcript abundance in wheat plants inoculated with BSMV variants as described in 2a (y-axis). Given are values relative to an endogenous wheat reference *EF1* gene with the empty vector (BSMV:00) set at 100 %. Values represent mean ± SD (*error bars*) of three independent sample collections and cDNA was generated from total RNA isolated from pooled systemic (third) leaf material from three different plants. Different small letters above the columns indicate values were statistically significant at $P \leq 0.05$ (pairwise *t* test). **d** RT-qPCR analysis of virus accumulation in wheat plants inoculated with BSMV CP variants with *PDS* insert. Values represent mean ± SD (*error bars*) in three independent sample collections

(Xu 2000; Wang and Heitman 2005; Stie and Fox 2007; Cervantes-Chávez et al. 2011) or, in the case of *PtMAPK1*, shown indirectly in a heterologous system to affect pathogenicity (Hu et al. 2007a).

The 3′-end coding sequences of *PtMAPK1, PtCYC1* or the *CNB*-homolog were cloned in a sense or antisense orientation in the BSMV γ component to produce the silencing constructs (Fig. 1; "Materials and methods"). First leaves of 7–8 days-old wheat cv. Thatcher plants were inoculated with the transcripts produced from the BSMV constructs and carrying the candidate fungal gene fragments in antisense conformation. By 10 dpi, when virus symptoms became apparent on upper non-inoculated leaves, plants were challenged with *Pt* urediniospores and observed for leaf rust disease symptoms in the days following the inoculation. The period between virus and fungal inoculation can determine the efficiency of VIGS and though silencing induced by BSMV can significantly persist for a period of time (Hein et al. 2005; Scofield et al. 2005), fungal infection 10 days after initial virus challenge was reported to be optimal for host-generated silencing of endogenous fungal genes (Yin et al. 2011). The fungal disease phenotype displayed a reduction in number and size of lesions, which started appearing as small flecks in plants infected with BSMV vectors targeting fungal genes compared to BSMV controls or mock-inoculated plants (Fig. 3a).

Since the efficiency of BSMV-VIGS was found to be enhanced by the deletion of the *βa* gene encoding the CP (Holzberg et al. 2002; this study), we also inoculated Thatcher plants with the BSMV ΔCP vectors carrying the candidate fungal gene inserts to determine whether these would improve trans-specific silencing efficiency of the targeted *Pt* genes as well. As shown in Fig. 3a, disease symptom severity appeared reduced compared to the same treatments using the CP+ silencing constructs. No difference in *Pt* susceptibility of control plants pre-inoculated with either BSMV variant was observed. To investigate whether the introduction of both sense and antisense forms of the selected fungal gene fragments influenced the endogenous silencing of the corresponding *Pt* genes, wheat plants were also inoculated with in vitro transcripts of BSMV αRNA, βΔβaRNA and mixture of recombinant γRNAs carrying the candidate fungal gene segments in sense or antisense orientation. Upon subsequent infection with *Pt*, rust symptom development was even further reduced using the sense plus antisense sequence combinations compared to the previous two treatments (Fig. 3a).

To study the effect of *Pt* gene silencing on the observed disease phenotype, we quantified the number of rust uredinia on third systemic leaves 10 days after fungal

infection of the wheat plants. As shown in Fig. 3b, the pustule density was considerably reduced in wheat leaves silenced for any of the three targeted *Pt* genes compared to controls. Uredinia density was slightly lower when the BSMV ΔCP silencing vectors were used whereas no major difference was observed in morphology or number of uredinia developing on systemic leaves of control plants treated with empty BSMV vectors with or without CP. Moreover, in mixed inoculations with recombinant γ components delivering both sense and antisense forms of the target fungal genes, even more disease suppression was obtained (Fig. 3b).

Because the observed reduction in uredinia production was expected to be accompanied by a concomitant decrease in fungal growth, we next analyzed the effect of *in planta*-generated silencing on fungal biomass accumulation inside leaf tissue. The ratio of fungal-to-plant biomass was significantly lower in plants silenced with BSMV vectors targeting the various candidate *Pt* genes compared to non-silenced control plants (Fig. 3c). Following the trend seen for the uredinia density measurements, treatment with ΔCP vectors delivering simultaneously both the sense and antisense forms of the target fungal sequences, seemed the most effective in reducing fungal biomass. These results showed that growth of *Pt* inside the host was impaired, likely due to the silencing of the endogenous *PtMAPK1*, *PtCYC1*, or *PtCNB* genes.

To further study the potential effect of the *in planta*-generated trans-specific silencing on the development of fungal structures inside host cells, randomly chosen sites from third systemic leaves of BSMV-silenced and non-silenced plants, 5 days after infection with *Pt*, were

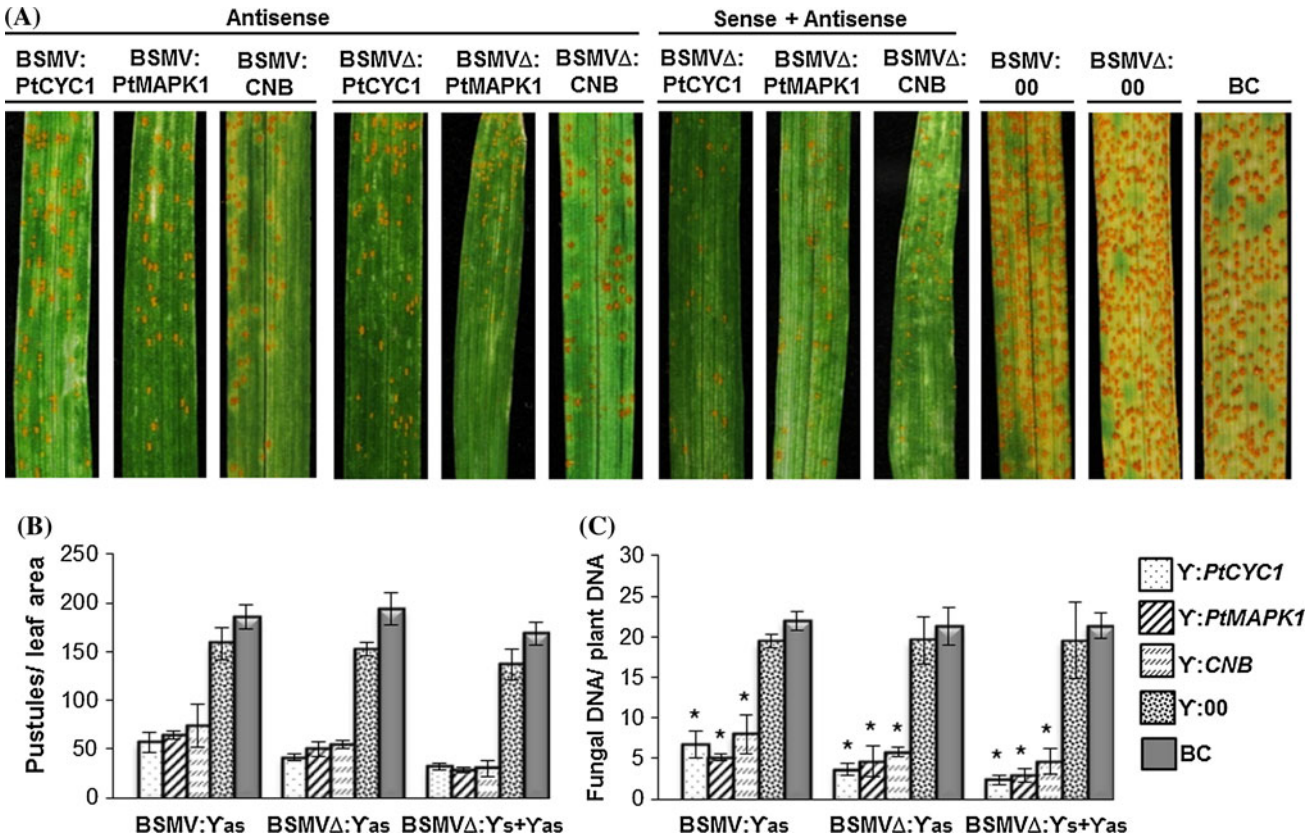


Fig. 3 Effect of BSMV-derived HIGS of targetted *Pt* genes on disease development in wheat. First leaves of 8 days-old wheat cv. Thatcher plants were inoculated with viral vectors with (BSMV) or without (BSMVΔ) coat protein and carrying derivatives of recombinant γ vector expressing target *PtMAPK1*, *PtCYC1* and *CNB*-like gene segments, either in antisense (γ_{as}) or as a mixture of sense plus antisense form ($\gamma_s + \gamma_{as}$). BSMV without insert (γ00) or buffer-inoculated (BC) plants were used as controls. Plants were spray-inoculated with *Pt* uridiniospores 10 days after virus or buffer inoculations. **a** Leaf rust disease phenotype in silenced and non-silenced wheat leaves. Plants inoculated with BSMV vectors habouring candidate *Pt* gene segments as indicated, show disease suppression whereas controls are heavily infected. Photograps taken of 3rd systemic leaves, 10 days after fungal inoculation. **b** Quantification of uredinia density on wheat plants silenced with BSMV vectors carrying target fungal gene segments and controls. Uredinia were counted 10 days after fungal infection; n = 10. **c** qPCR measurement of fungal biomass. Ratio of fungal to wheat nuclear genomes using fungal *PtRTP1* and wheat *TaEF1* genes, respectively, in plants treated with BSMV variants targeting fungal genes compared with controls (significant at $P < 0.05$; *t* test). Genomic DNA extracted from pooled whole systemic (third) leaf material pooled from three different plants 10 days after *Pt* infection. Mean values ± SD (*error bars*) from three independent sample collections

selected and analyzed by confocal microscopy. Control plants treated with BSMV variants or with buffer showed widespread fungal growth. Mycelial morphology appeared normal with extensive colonization and breaching of neighbouring mesophyll cells by secondary hyphae and a prolific formation of haustoria was seen (Fig. 4). In contrast, plants previously inoculated with BSMV silencing vectors in which *Pt* infection was greatly compromised, revealed significant arrest of mycelial growth. In silenced tissues, growth of the fungus remained confined to the first few mesophyll cells in close vicinity of the substomatal cavity, revealing restricted intercellular secondary hyphal growth with few additional haustoria formed. The confocal microscopy results correlated well with the fungal biomass measurements indicating that fungal growth was indeed arrested.

BSMV-VIGS results in the production of target fungal gene-specific siRNA molecules in host cells and reduced transcript abundance of endogenous *Pt* genes

Host-generated silencing directed towards two biotrophic fungal pathogens has been reported to result in the degradation of the endogenous fungal target transcripts (Nowara et al. 2010; Yin et al. 2011). For example, when targeting candidate effector genes in the wheat stripe rust fungus, *P. striiformis* using the BSMV-based HIGS system, a reduction in the fungal transcripts was seen but the silencing signals involved in this interspecies RNA interference were not described (Yin et al. 2011). We therefore investigated whether the observed *Pt* disease suppression was correlated with target fungal gene-specific small RNA molecules generated in wheat. siRNA molecules specific to the fungal gene fragments contained in the BSMV silencing vectors were detected in upper, non-inoculated leaves of the respective silenced plants (Fig. 5a). Introducing target fungal sequences simultaneously in sense and antisense form resulted in the production of substantially more siRNA molecules compared to when the same sequences were present in antisense form alone. The higher levels of siRNA molecules correlated well with the reduced disease symptoms and fungal biomass (Fig. 3).

Next, the effect of the siRNA molecules on the target *Pt* genes was examined by RT-qPCR analysis, 5 days after *Pt* infection. Significant reductions in endogenous transcript abundance were detected for all three corresponding *Pt* genes (Fig. 5b). The effect of different BSMV variants, or mixed inoculation with recombinant γRNA components, on silencing ability was analyzed in detail for the *PtCYC1* gene. As shown in Fig. 5b, the silencing of the *PtCYC1* gene was strongest when sense and antisense fragments of the gene were introduced simultaneously using the BSMV ΔCP mutant as compared to introducing the same sequence in antisense form alone from the BSMV CP vector. These results demonstrate that effectiveness of interspecies silencing can be enhanced by using BSMV vectors defective in the CP and by introducing pathogen gene fragments in a way that enhances siRNA production.

Discussion

The expression of dsRNA targeted at essential genes in pathogens was reported in several crop plants to increase resistance against a number of parasites, including the biotrophic fungus *B. graminis* (Nowara et al. 2010). In this study we show that the BSMV-mediated HIGS assay can be used as a powerful reverse genetic tool to study and identify novel leaf rust fungus *P. triticina* genes for control of this obligate basidiomycete pathogen. We made use of this approach to engineer expression in wheat plants of three *Pt* genes predicted to be important for growth and pathogenesis, a MAP kinase, a cyclophilin and the regulatory B subunit of calcineurin. BSMV-VIGS resulted in the generation of fungal gene-specific siRNA molecules in plants treated with silencing vectors which subsequently triggered RNAi of complementary genes in invading and colonizing *Pt*, ultimately inhibiting the invasion of host cells by the fungus and reducing disease severity. These results indicate successful translocation of silencing signals from host to fungal cells and the ensuing arrest of *Pt* development suggests that the target fungal genes are required components of disease establishment in host plants.

We also found that the efficiency of BSMV-mediated trans-specific silencing of *Pt* genes was greater when using viral vectors defective in CP function and coupled with introducing in the host fungal gene fragments concurrently in sense and antisense orientation (Figs. 3, 5). BSMV CP is hypothesized to be involved in the regulation of the γRNA or subgenomic γRNA and its deletion would hence lead to higher levels of these components, including the inserted sequences (Holzberg et al. 2002). VIGS induced by mixed inoculation of sense and antisense form of fungal transcripts showed higher accumulation of cognate siRNAs in wheat tissue compared to when expressed in antisense form alone (Fig. 5). When present on the same template, such engineered, self-complementary sequences can enhance the effectiveness of silencing vectors by increasing their capability for dsRNA generation, even though such inverted repeat structures in virus-derived vectors are often unstable inside the viral genomes and can cause packaging constraints (Simon and Bujarski 1994; Lacomme et al. 2003; Pacak et al. 2010). In our experiments, a higher production of siRNA molecules in host cells resulted in a stronger inhibition of target gene expression inside *Pt*. This

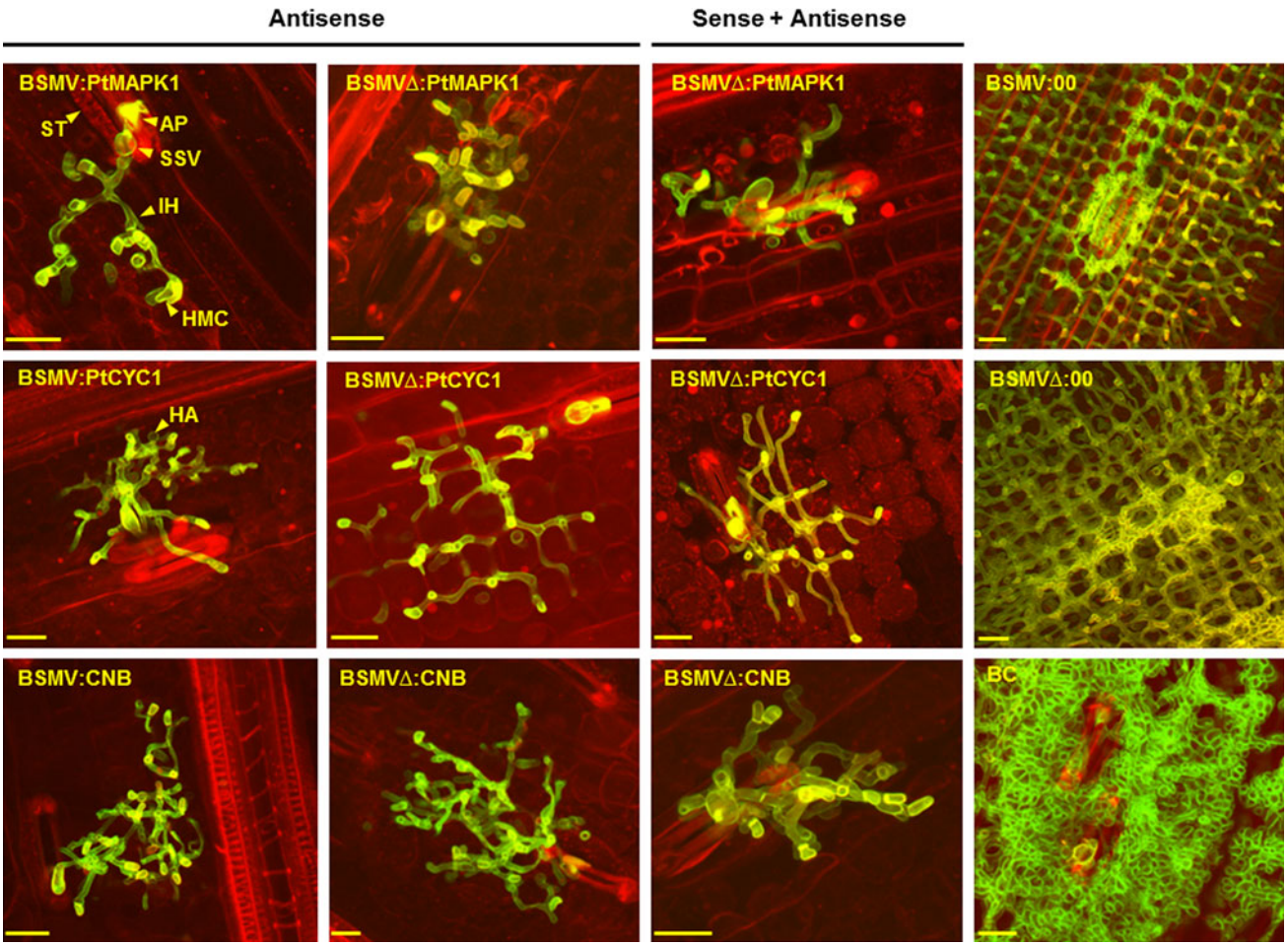
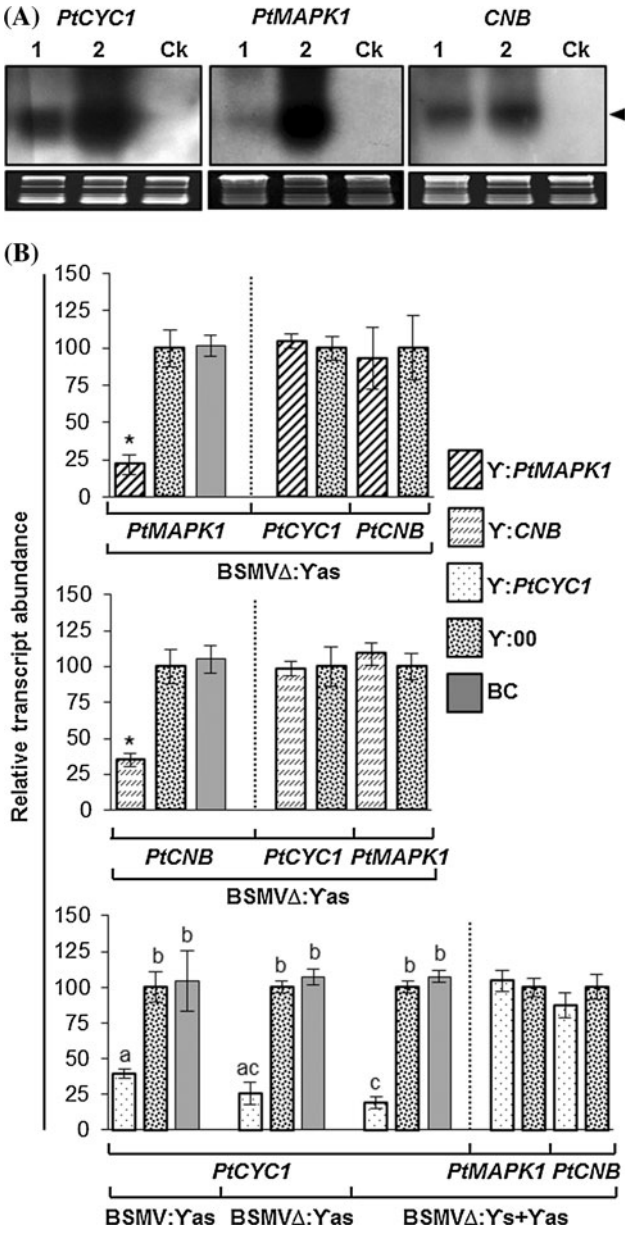


Fig. 4 Confocal microscopic observation of fungal development in wheat cv. Thatcher leaves. Plates represent a projection of Z-series of scans taken from inside third systemic leaves of wheat plants 5 days after *Pt* infection but previously inoculated with viral vectors with (BSMV) or without (BSMVΔ) coat protein and carrying different combinations of recombinant γ vector with *PtMAPK1*, *PtCYC1* or *CNB* gene segments, either in antisense or as a mixture of sense and antisense forms. BSMV:00 and BSMVΔ:00 indicate empty vector controls; BC is buffer control. Uvitex 2B-stained fungal structures (*green*) and acridine orange-stained plant cell walls (*red*) are visible. Silencing of target *Pt* genes induced by host-generated RNAi restricts fungal development whereas control plants show extensive mycelial growth and colonization of mesophyll cells. *ST* stoma, *AP* appressorium, *SSV* substomatal vesicle, *HMC* haustorium mother cell, *HA* haustorium, *IH* infection hypha. *Scale bars* are 30 μm

observation suggests that the nature of the silencing molecules involved in HIGS of biotrophic pathogens seems to be the siRNAs generated in the host. Higher siRNA levels would have facilitated the fungus to acquire these molecules more readily. These findings can be used to design better BSMV vectors for the application of HIGS.

Virus-induced gene silencing has emerged as a powerful genomic research tool because it enables creation of knock-down phenotypes of genes of interest without the need for generating stable transformants. With the modification of the BSMV genome for gene silencing studies in grass species (Holzberg et al. 2002; Yuan et al. 2011), many limitations for gene function analysis in cereal crops could be overcome (Cakir and Tör 2010). Several aspects of VIGS make it particularly useful for functional genomic studies: (1) it is a rapid process; (2) silencing can be achieved using small gene segments that are easy to clone; (3) there is no need for the production of knock-out mutants, making possible the study of lethal phenotypes; (4) it is extremely useful for studies in genetically recalcitrant or polyploid species and (5), it can target whole gene families. These aspects also apply when targeting pathogen genes using the BSMV-mediated trans-specific silencing. Depending on the assays, the effects of this transient silencing can be studied phenotypically. The host *PDS* gene silencing results in clear visual symptoms, but we show here that targeting predicted fungal pathogenicity genes can result in a reduced disease phenotype.

BSMV symptoms in infected plants have not been reported to affect the analysis of target genes by masking the phenotype under study. The temporal and spatial pattern of gene silencing induced by BSMV has proved



◀ **Fig. 5** Molecular analysis of host-derived endogenous RNAi of *Pt* genes. **a** RNA blot showing the generation of siRNA molecules specific to *PtCYC1*, *PtMAPK1* or *CNB*-like sequences in wheat cv. Thatcher plants inoculated with the respective VIGS constructs as indicated. RNA blots were hybridized with the corresponding target gene-specific probes. siRNA levels in plants silenced using the BSMV ΔCP vector expressing target fungal fragments in antisense orientation (*lanes 1*), or with a mixture of sense plus antisence forms (*lanes 2*). No signal was detected in empty BSMV controls (Ck). Total RNA was extracted from third systemic leaves pooled from three different plants 10 days after virus inoculation. Lower panels, ethidium bromide-stained rRNA as gel loading controls. The *arrowhead* indicates an oligonucleotide size marker of 23 nucleotides **b** RT-qPCR analysis of endogenous transcript abundance of *PtMAPK1*, *PtCYC1* and *PtCNB* genes. Silencing was induced by viral vectors with (BSMV) or without coat protein gene (BSMVΔ) having recombinant γ vector expressing target *PtMAPK1*, *PtCYC1* or *CNB* gene segments, as indicated, either in antisense (γ_{as}) or as a mixture of sense and antisense forms ($\gamma_{s+}\gamma_{as}$). BSMV having no insert (γ00) or mock inoculation with buffer (BC) served as controls. Values of tanscript levels are measured relative to the endogenous *Pt* succinate dehydrogenase reference gene with the empty vector (BSMV:γ00) set at 100 %. Silencing induced by the viral vectors is specific to the target gene insert and does not affect the transcript abundance of the other two unrelated genes used in this study. The measurements were determined using cDNA prepared from total RNA isolated from systemic (3rd) leaves pooled from three different plants, 5 days after *Pt* infection. Mean values ± SD (*error bars*) from three independent experiments. *Asterisks* indicate values that are statistically significant at $P < 0.05$ (*t* test), and different *small letters* above the columns indicate values were statistically significant at $P < 0.05$ (Tukey HSD test)

sufficient to allow the initiation of VIGS and the subsequent challenge of silenced tissue with a pathogen. For example, the function of several cereal resistance genes was verified using this system, such as *Lr1*, *Lr10* and *Lr21* against wheat leaf rust (Scofield et al. 2005; Cloutier et al. 2007; Loutre et al. 2009) and the barley *Mla13* resistance gene against *B. graminis* (Hein et al. 2005). BSMV-VIGS has also been applied to determine the role of wheat WKRY53 transcription factor in aphid (*Diuraphis noxia*) defence (Van Eck et al. 2010). In *Haynaldia villosa*, a wild grass, this assay was used for functional analysis of powdery mildew resistance-related genes (Wang et al. 2010). When using VIGS for gene silencing studies, there is always a concern about inducing host defence-related genes in response to virus infection which might influence the infection phenotype of the secondary pathogen (Tufan et al. 2011). However, as long as control plants are diligently monitored for the confounding effect of the viral vectors, VIGS can be a valuable functional genomic tool to study plant pathogens, including rust fungi. Several studies show that prior infection with BSMV does not compromise susceptibility in wheat to leaf or stripe rust fungi (Scofield et al. 2005; Cloutier et al. 2007; Zhou et al. 2007; Yin et al. 2011). Consistent with these findings, we also did not observe any effect on *Pt* parasitism of BSMV-inoculated wheat cv. Thatcher plants. The incidence of disease, in terms of uredinia morphology and density, was similar in both virus-infected and buffer-treated control plants (Fig. 3). Also, the confocal microscopic study revealed that germinated spores produced extensive hyphal networks in BSMV:00-treated control plants (Fig. 4).

We did not observe an effect of *in planta*-generated silencing on pre-haustorial structure formation (Fig. 4) indicating that silencing molecules were received by the fungus only after primary haustorium biogenesis. The haustorium is a highly specialized defining feature of obligate fungal parasites which functions as a physical and physiological bridge to the host (Szabo and Bushnell 2001). It remains distinct from host mesophyll cells by a zone, the extra-haustorial matrix (EHM), which separates the host

plasma membrane from the fungal cell wall. Across this intimate interphase, the rust fungus takes up nutrients from the host (Szabo and Bushnell 2001; Bolton et al. 2008; Voegele and Mendgen 2011) and is postulated to deliver virulence factors to suppress host defenses (Panstruga and Dodds 2009; Rafiqi et al. 2012). A plausible hypothesis is that host-generated siRNA molecules are translocated inside the fungus through the EHM using either nutrient transport or vesicle trafficking mechanisms, or other, as yet undiscovered pathways (Panwar et al. 2013). This would explain why the higher accumulation of siRNA silencing molecules in the host cells led to elevated endogenous silencing of *Pt* genes. In a previous study by Yin et al. (2011), BSMV-HIGS in the wheat stripe rust fungus *P. striiformis* was reported for genes specifically expressed in haustoria and the authors suggested that silencing only occurs in fungal cells near or in direct contact with host cytoplasm. However, our results indicate that once acquired from the host, it is likely that the silencing signal is amplified by the fungus. Indeed, the basic components of the silencing machinery such as Argonaute, DICER and RdRps have been predicted to be present in the draft *Pt* genome (Broad Institute, http://www.broadinstitute.org/). Moreover, the extent of growth inhibition (Fig. 4) and the depletion of *PtMAPK1*, *PtCYC1* and *PtCNB* transcripts measured in whole leaf extracts (Fig. 5b) suggest spread of silencing throughout *Pt* hyphae. More direct evidence is needed to verify how the endogenous gene silencing machinery of rust fungi functions.

A potential complication with gene silencing is that genes sharing sufficient sequence identity may be unintentionally silenced; so-called 'off-target' effects (Jackson et al. 2003). RT-qPCR analysis indicated that transcript levels of the endogenous *PtCNB* gene were effectively reduced by the expression of the *CNB*-like sequence which shares 65 % overall nucleotide similarity (Fig. S2). It was reported that the presence of a continuous stretch of 8–14 nucleotide similarities between siRNA and its target may be sufficient to induce silencing (Jackson et al. 2003; 2006; Nguyen et al. 2008). Therefore, we cannot rule out the possibility that *PtMAPK1*, *PtCYC1* and *PtCNB* homologs sharing sequence similarity might also be silenced. However, there is still a lack of clear understanding on the mechanism that determines the gene-silencing ability of a given siRNA. A number of factors have been found to play critical roles in this regard including: (a) positional effects due to local protein factor(s) on mRNA (Henderson and Jacobsen 2007), (b) local structure of the targeted mRNA (Bohula et al. 2003), and (c) differential efficiency of 5′ siRNA phosphorylation (Nykänen et al. 2001). BLASTn searches of available *Pt* genomic resources revealed that *PtMAPK1* and *PtCYC1* transcripts share sequence homology with other genes with unknown function, but often in interrupted stretches. However, it is not known whether these homologs are expressed and truly functional during infection. The three sequences that served as silencing inserts were also extensively searched against all available wheat genomic resources but no extensive homology was found (Fig. S2). Indeed, no aberrant phenotypes were observed in systemic wheat plants with target siRNA molecules. For practical applications, silencing multiple fungal genes, including possible homologs, could be advantageous and provide more selection pressure for these fungi to overcome. We conclude tentatively that the targeted *Pt* genes have roles in virulence.

For transient gene silencing assays to be useful in allowing knock-down phenotypes to be effectively monitored and analyzed, a sufficiently large part of the plant or leaf area needs to generate the silencing effect. Our previous study on the use of *A. tumefaciens*-mediated introduction of rust fungal sequences in the wheat host showed significant RNAi of cognate fungal genes but only within the agroinfiltrated leaf areas (Panwar et al. 2013). Here, BSMV-VIGS of *Pt* genes resulted in systemic spread of silencing in wheat plants and was more homogenous and consistent, likely due to virus replication and movement. A more-persistent silencing effect could prove particularly useful for determining the role of fungal genes that are expressed late in infection. The observation that the BSMV coat protein deletion mutant in conjunction with the simultaneous introduction of sense and antisense target fragments enhances trans-specific gene silencing of *Pt* genes, could be used to improve the efficiency of BSMV-mediated HIGS for exploring gene functions in pathogens which maintain a biotrophic relationship with their host. One possibly draw-back of the BSMV-based system is the need to produce RNA transcripts and this is more costly than *Agrobacterium* delivery. It should be possible to combine these techniques and deliver similar BSMV constructs via agroinfiltration (Yuan et al. 2011).

The possibility of dissecting gene function through host-generated gene silencing using a VIGS expression system represents a robust approach for mining the various genomic resources being generated for cereal rust fungi. It will increase our understanding of rust fungus biology and the success of these pathogens. With previous studies targeting plant genes, we show now that with the expression of fungal gene sequences in wheat using a suitable viral vector, it should be feasible to study the function of genes in both host and pathogen during plant–fungus interaction. The improved understanding will guide the development of effective control measures against rust fungi.

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