Fungal Genetics and Biology 46 (2009) 825–836

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

dsRNA-induced gene silencing in *Moniliophthora perniciosa*, the causal agent of witches' broom disease of cacao

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ARTICLE INFO

Article history:
Received 19 January 2009
Accepted 29 June 2009
Available online 12 July 2009

Keywords:
Crinipellis perniciosa
Theobroma cacao
RNAi
Peroxidase
GFP
Hydrophobins

ABSTRACT

The genome sequence of the hemibiotrophic fungus *Moniliophthora perniciosa* revealed genes possibly participating in the RNAi machinery. Therefore, studies were performed in order to investigate the efficiency of gene silencing by dsRNA. We showed that the reporter *gfp* gene stably introduced into the fungus genome can be silenced by transfection of *in vitro* synthesized *gfp*dsRNA. In addition, successful dsRNA-induced silencing of endogenous genes coding for hydrophobins and a peroxiredoxin were also achieved. All genes showed a silencing efficiency ranging from 18% to 98% when compared to controls even 28 d after dsRNA treatment, suggesting systemic silencing. Reduction of GFP fluorescence, peroxidase activity levels and survival responses to H_2O_2 were consistent with the reduction of GFP and peroxidase mRNA levels, respectively. dsRNA transformation of *M. perniciosa* is shown here to efficiently promote genetic knockdown and can thus be used to assess gene function in this pathogen.

1. Introduction

The basidiomycete *Moniliophthora perniciosa* (Aime and Phillips-Mora, 2005) [Syn. *Crinipellis perniciosa* (Sthael) Singer; Tricholomataceae], causal agent of witches' broom disease of cocoa (*Theobroma cacao* L.), occurs in all cacao producing countries of South and Central America (Arévalo et al., 2007). This pathogen was the main cause for the decrease of cocoa production and environmental, social and economic decline in producing regions of those countries. The fungus shows a hemibiotrophic life cycle with distinct parasitic and saprophytic phases. The parasitic phase is characterized by intercellular monokaryotic mycelia that cause hypertrophy and hyperplasia in the infected tissue. The saprophytic phase shows intercellular and intracellular dikaryotic mycelia responsible for necrosis (Evans, 1980, 1981; McGeary and Wheeler, 1988) with subsequent programmed cell death of the tissues (Ceita et al., 2007). Although several studies have already uncovered important aspect of *M. perniciosa* pathogenicity, for example, the identification of three putative genes encoding necrosis and ethylene-inducing proteins, Mp*NEP*s (Garcia et al., 2007) and genes involved in defense responses to pathogen infection or in programmed cell death (Gesteira et al., 2007), as well as, studies relative to chitinase regulation (Lopes et al., 2008), further efforts need to be undertaken to better understand the plant–pathogen interaction and therefore be able to design new strategies to control this disease.

Direct genetic analysis of dikaryotic filamentous fungi, such as *M. perniciosa* would not be easily implemented mainly due to the restricted access to the haploid uninucleate spores. This characteristic impairs gene replacement and insertional mutagenesis at least at this stage of life cycle and/or methods relying on isolation of homokaryotic transformants derived from single transformation events to study loss of functional mutants (Vijn and Govers, 2003). In this context, knockdown of candidate genes using reverse genetic strategies such as dsRNA-induced RNAi-mediated gene silencing represents an important alternative to get new insight into key features of the *M. perniciosa* pathogenic process. Since this mechanism acts at the mRNA level, its efficiency is not compromised by the presence of non-transformed nuclei or multicopy genes (Nakayashiki et al., 2005).

RNAi-mediated gene silencing is a mechanism that has been conserved along evolution and found in a wide range of eukaryotic organisms, including fungi (Kadotani et al., 2003; Goldoni et al., 2004; Tanguay et al., 2006; Heneghan et al. 2007; Meyer, 2008; Eastwood et al., 2008), plants (Vance and Vaucheret, 2001; Tenllado et al., 2004; Herr et al., 2005) and animals (Lee and Rossi, 2004;

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doi:10.1016/j.fgb.2009.06.012

Ashrafi et al., 2003; Haley et al., 2003; Pereira et al., 2008) and is related to protecting the genome from exogenous (pathogens) and endogenous (transposons). Furthermore, RNAi participates in regulatory mechanisms of genic expression mediated by some classes of small endogenous RNAs like miRNAs (Bernstein et al., 2001; Bartel, 2004; Rana, 2007). Based on biochemical and genetic studies, a model for the RNAi mechanism was proposed (reviewed by Ceruti, 2003, Aigner, 2006; Weld et al., 2006; Rana, 2007). Briefly, the process of gene silencing is initiated by a long double-stranded RNA (dsRNA) expressed by the organism or introduced in the cell of interest or even from viral origin. The dsRNA is next cleaved into small interference RNAs (siRNAs) with around 21–23 bp in animals (Zamore et al., 2000) or 25 bp in plants and fungi (Hamilton and Baulcombe, 1999; Catalanotto et al., 2002) by a specific dsRNA endonuclease named Dicer (Bernstein et al., 2001; Forrest et al., 2004; Meister and Tuschl, 2004; Kim et al., 2005). The double-stranded siRNAs are rapidly incorporated to the multiprotein complex RISC (RNA-induced silencing complex) where the siRNAs are unwinded into single strands (Hammond et al., 2000; Maiti et al., 2007). In this manner, the antisense single strand siRNAs are used as guides by RISC to identify complementary bases of the target mRNA, which finally is degraded by endo- and exonucleolitic cleavages (Rana, 2007). Even though RNAi is predominantly characterized as a post-transcriptional process, it can participate of the transcriptional silencing pathway involving DNA methylation, histone modification, as well as meiotic gene silencing (Mette et al., 2000; Sijen et al., 2001; Volpe et al., 2002; Shiu et al., 2006).

Several studies have investigated the occurrence of the silencing mechanism by RNAi in fungi and oomycetes. In *Neurospora crassa*, Catalanotto et al. (2002) reported that siRNAs with 25 nucleotides were involved in the RNAi silencing route. Nicolás et al. (2003) demonstrated the mode of action of the RNAi-mediated gene silencing mechanism in the filamentous fungus *Mucor circinelloides* using *carB* as marker. Whisson et al. (2005) described the first application of transient gene silencing by delivering *in vitro* synthesized dsRNA into protoplasts of the oomycete *Phytophthora infestans* to trigger silencing. In *Schizophyllum commune* the *SC15* gene, which encodes a secreted structural protein, was silenced at a frequency of 80% using a hairpin construct (de Jong et al., 2006). Simultaneous silencing in at least four groups of genes was detected in *Magnaphorthe oryzae* using the RNA-silencing vector pSilent-Dual 1 (Nguyen et al., 2008). RT-qPCR analyses indicated that transcripts of two *Agaricus bisporus* genes (*URA3* and *CBX*) silenced with hairpin constructs were significantly reduced (Costa et al., 2009). In *Coprinopsis cinerea*, Costa et al. (2008) reported that the use of short hairpin constructs promoted the formation of transformants with reduced transcripts of the Green Fluorescent Protein (GFP) transgene.

In *N. crassa* RNAi-mediated gene silencing requires the following set of four genes: *qde*-1 (Cogoni and Macino, 1999) that encodes a probable RNA-dependent RNA polymerase (RdRP), the Dicer gene *dcl*-2, which encodes an endonuclease that processes dsRNA into siRNAs strands (Catalanotto et al., 2004); *qde*-2, which encodes a component of RISC (Catalanotto et al., 2000) and *qde*-3 that codes for a probable Rec-Q DNA helicase (Cogoni and Macino, 1999). The identification of sequences similar to these four *N. crassa* key genes in the genome of *M. perniciosa* (Mondego et al., 2008) suggests that silencing should also be functional in this fungus.

To determine if the RNAi route is indeed functional in *M. perniciosa* and could be efficiently used to silence specific genes, the transformation of dikaryotic hyphae with long dsRNA corresponding to three target genes was performed and the reduction of mRNA of the target genes was evaluated. We showed that this approach was efficient to silence a heterologous *gfp* reporter gene and two endogenous genes, Mp*HYD3* and Mp*PRX1*.

2. Materials and methods

2.1. Fungus strains and culture conditions

The strain CP02, a wild type of *M. perniciosa* (biotype C), used in this study was isolated from cacao branches (CEPLAC/CEPEC, Bahia, Brazil). This strain was used for sequencing the genome of this species (Mondego et al., 2008). The fungus was maintained in the saprophytic phase at 25 °C in darkness on potato-dextrose-agar media – PDA (Acumedia®) modified by addition of 0.15% casein hydrosylate, 0.2% yeast extract and 0.2% peptone.

2.2. Transformation vector

The vector pHSP70-SG contains the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) and *gfp* reporter gene, both fused to the Heat Shock Protein (*hsp70*) promoter of *Ustilago maydis* that exhibits a strong basal activity and can be further stimulated under stress condition. For the construction of this vector, Spellig et al. (1996) used an improved version of GFP, SGFP-TYG (Sheen et al., 1995). In SGFP-TYG a Ser65 → Thr point mutation was introduced in the chromophore domain, which resulted in GFP with a single excitation and emission peak, brighter fluorescence and more rapid chromophore generation (Heim et al., 1995).

2.3. Protoplasts production and transformation procedure

Protoplasts of strain CP02 were obtained from mycelia grown on modified Potato Dextrose Grow – PDG (Acumedia®) with agitation in darkness at 25 °C for 8 d. The procedure of protoplasts production was carried out according to Lima et al. (2003). Transformation of *M. perniciosa* with DNA was performed by two methods. The first followed Yelton et al. (1984) and Balance and Turner (1985) procedures with some modifications. To a suspension of protoplasts (1×10^8 protoplasts/mL) 10 μg of plasmid pHSP70 in circular shape, 10 U of *Ssp*1 restriction enzyme and 50 μL of PEG/$CaCl_2$ solution (25% PEG 6000, 50 mM $CaCl_2$) was added, incubated on ice for 20 min, followed by addition of 500 μL of the same PEG/$CaCl_2$ solution and incubated for another 20 min at room temperature. The protoplasts were then plated on selective regeneration medium (PDA medium containing 0.5 mM sucrose, with 100 μg hygromycin B/mL) and incubated at 25 °C, in darkness. Positive and negative controls, that is, protoplast suspensions treated with PEG/$CaCl_2$ solution with and without pHSP70 were plated on regeneration medium with and without addition of 100 μg hygromycin B/mL, respectively, and incubated in similar conditions.

The second method of transformation consisted in using electroporation to promote DNA transfer into the cell. Aliquots of 400 μL protoplast suspensions (10^8 protoplasts/mL) treated with 10 μg of plasmid pHSP70 in circular form and 10 U of *Ssp*1 restriction enzyme were electroporated in cuvettes (0.2 cm) with the application of a 1.5 kv/4.0 ms pulse using a Micropulser Electroporator (BioRad, Hercules, CA). The parameters used in electroporation were selected from a previous set of experiments aimed at establishing a protocol for regeneration of electroporated protoplasts of *M. perniciosa* (unpublished data). The protoplasts treated with pHSP70-SG and negative control (without plasmid) were incubated in selective regeneration medium and the positive control in non-selective regeneration medium.

2.4. Mitotic stability

The mitotic stability of the transformants was investigated by monitoring growth on non-selective medium (PDA modified medium without hygromycin B) for 40 d. That is, cultures were

sequentially transferred to non-selective medium five times and cultured for 8 d each time. Mycelial fragments were then transferred back to selective medium to determine the expression stability of hygromycin resistance.

2.5. Molecular analysis of transformants

Genomic DNA of the parent strain and two randomly chosen transformants were extracted according to Melo et al. (2006). In order to confirm the presence of plasmid pHSP70 in *M. perniciosa* hyphae, a PCR analysis was performed to detect an 1025 bp amplification product of the *hph* gene using the primers *hph*F (5′-CTATTTCTTTG CCCTCGGACGAG-3′) and *hph*R (5′-ATGAAAAGCCCT GA ACTCACCGC-3′). PCR amplification included 30 cycles of 1 min at 94 °C, for denaturing, 1 min at 50 °C for annealing and 1.5 min at 72 °C for extension. The reaction products were analyzed by electrophoresis on 1% agarose gel. In addition, a PCR analysis was performed to detect a 119 bp amplification product of the *gfp* gene using the primers *gfp*F (5′ATCATGGCCGACAAGCAAA3′) and *gfp*R (5′GCCATCGCCAATTGGA GTAT3′). The fragment was resolved on a 12% polyacrylamide gel, and visualized with ethidium bromide under UV light.

2.6. Detection of GFP by fluorescence microscopy

GFP was detected in regenerating protoplasts and hyphae by fluorescence microscopy using an Epifluorescence Photomicroscope DMR2 with filter cube GFP (excitation range blue, excitation filter BP 470/440 nm, dichromatic mirror 500 nm, and suppression filter BP 525/550, Leica, Germany). Images from microscopy were recorded on the Leica image system analysis – software IM50.

2.7. Construction of gfp, MpPRX1 and MpHYD3 templates for dsRNA preparation

The plasmid template strategy was used to obtain *gfp* and Mp*HYD3* in sense and antisense orientations both cloned to the T7 promoter seeking the synthesis of *gfp*dsRNAs and Mp*HYD3*dsRNA, respectively. The *gfp* target, sequences with 717 bp were amplified by PCR from pCAMBIA 1302 vector using *gfp-Nco*I*F* and *gfp*R primers (Table 1) and cloned in pTZ57R/T. The target sequence in sense and antisense orientations with a single T7 promoter assembled at opposite ends of the region to be transcribed was amplified using T7 F/*gfp*R and T7/*gfp-Nco*I*F* primers, respectively (Fig. 1A). On the other hand, Mp*HYD3* target sequences were amplified from pDNR-LIB vector, which was utilized for construction of a library of the interaction *Moniliophthora*–Cacao, using PDNR-LIB F and PDNR-LIB R primers (Table 1). The Mp*HYD3* fragment was cloned in pGEM T-easy. The amplification of the target sequence in sense and antisense orientations with a single T7 promoter placed at opposite ends of the region were amplified using T7 F and SP6 R primers (Fig. 1B).

The PCR template strategy was used to obtain Mp*PRX1 sequences* in sense and antisense orientations to synthesize Mp*PRX1*dsRNAs. In order to do that a pair of primers Mp*PRX1* forward and reverse with a 5′-T7 promoter sequence (Table 1) were designed to amplify 390pb Mp*PRX1 sequences* from the *Moniliophthora*–Cacao interaction library. These primers were used in a single PCR to generate transcription templates for both orientations (Fig. 1C).

2.8. dsRNA synthesis and transformation procedures

The dsRNAs for *gfp,* Mp*PRX1* and Mp*HYD3* (Fig. 1D) were produced by *in vitro* transcription at 37 °C for 4 h using a RNAi kit according to the instruction of the manufacturer (Megascript® RNAi Kit, Ambion), from sense and antisense templates generated through the two strategies mentioned above.

For *gfp*, Mp*HYD3* and Mp*PRX1* silencing assays up to 10 μg of specific dsRNA were used in protoplasts transformation experiments via PEG/$CaCl_2$ (Yelton et al., 1984; Balance and Turner, 1985) and electroporation using the same condition described for DNA transfer above.

Regenerated mycelia from different plate regions submitted to transformation treatment with dsRNA were considered as individual lines in gene expression and phenotype analyses.

2.9. RNA extraction and cDNA synthesis

Mycelium of lines exposed to *gfp*dsRNA, Mp*PRX1*dsRNA or Mp*HYD3*dsRNA and their respective control lines were collected, immediately frozen in liquid nitrogen and stored at –80 °C. Samples were macerated and total RNA was extracted using a

Table 1
Oligonucleotide primer pairs and respective amplicon size used in template preparation for dsRNA synthesis, and RT-qPCR.

Gene	Primer name	Primer sequence	Application	Amplicon size (pb)
gfp	*T7/gfp-NcoIF*	5′TAATACGACTCACTATAGGGG3′/5′CCATGGTAGATCTGACTAG3′	dsRNA synthesis	717
	T7/gfpR	5′TAATACGACTCACTATAGGGG3′/5′CATCCATGCCATGTGTAATCC3′		
	gfpF	5′ATCATGGCCGACAAGCAAA3′		
	gfpR	5′GCCATCGCCAATTGGAGTAT3′	RT-PCR	119
hf-3	PDNR-LIBF	5′ATCAGTCGACGGTACCGGAC3′	dsRNA synthesis	808
	PDNR-LIBR	ACAGCTATGACCATGTTCAC3′		
	T7	5′TAATACGACTCACTATAGGGG3′	dsRNA synthesis	
	Sp6	5′GTAAAACGACGGCCAGT3′		
	Hidrof1F	5′TCATCGGCCTTTTGGGT3′	RT-PCR	333
	Hidrof1R	5′GCACGAGTTACCACCAAG3′		
	Hidrof2F	5′GCTGCAATAGCGTTCAGT3′	RT-PCR	321
	Hidrof2R	5′GTGCAAGAGTTGCCTCCAAT3′		
	Hidrof3F	CGATCCTGCTGCCAGGTA3′	RT-PCR	336
	Hidrof3R	ACGGGAACGCATCCAATCGA3′		
	Hidrof4F	CGATAACGCCCACAACAAG3′	RT-PCR	330
	Hidrof4R	ACAGCAGACGGGTTGTGG3′		
	Hidrof5F	GCAGGTGTCCTTGCCTTGATA3′	RT-PCR	288
	Hidrof5R	5′CTCGCAGCAAACTGGGCT3′		
prxMp1	*T7prxMp1F*	5′TAATACGACTCACTATAGGGATATCCAAATACCTGGGCGACTCAT3′	dsRNA synthesis	414
	T7prxMp1R	5′TAATACGAGTCACTATAGGGATATCTCATCAAAGTTTCGTCCAGC3′	real-time PCR	
Act	ActinF	5′CCATCTACACCACAATGGAGGGA3′	RT-PCR	165
	ActinR	5′CCCGACATAGGAGTCCTTCT3′		

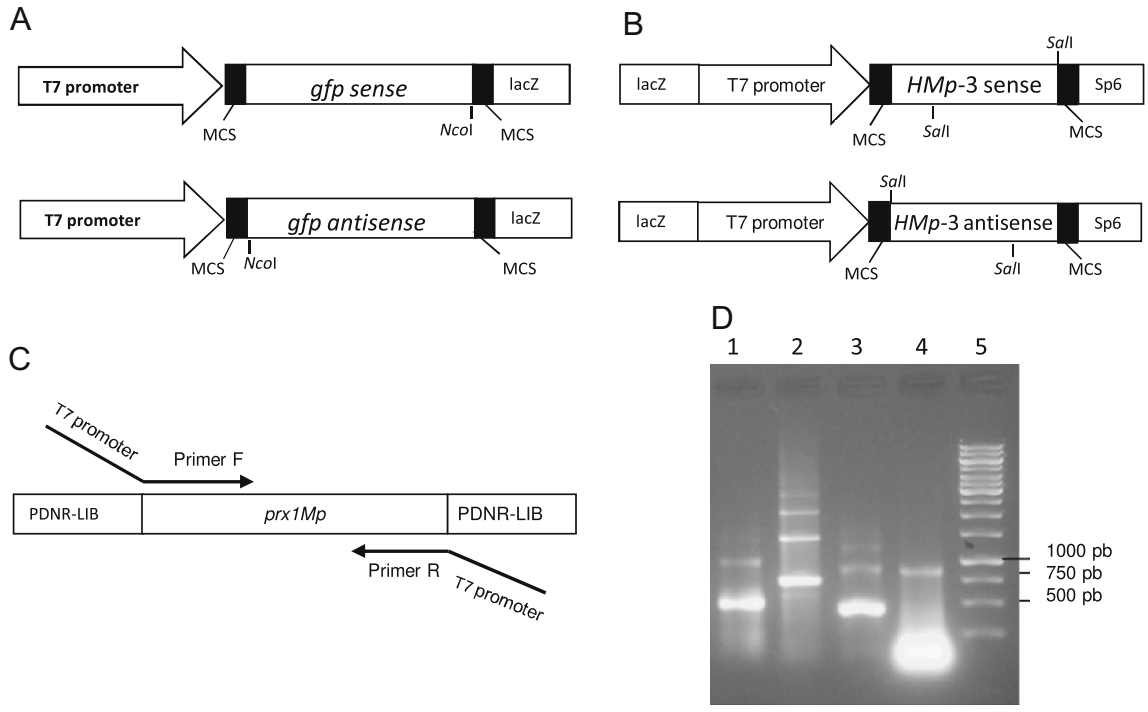

Fig. 1. Strategies for dsRNA synthesis. (A) Plasmid template strategy for obtaining *gfp* in sense and antisense orientations both cloned next to the T7 promoter (pTZ57R/T) aiming the synthesis of *gfp*dsRNAs. (B) Plasmid template strategy for the obtention of *HMp*3 gene in sense and antisense orientations both cloned next to the T7 promoter (pGEM T-easy) aiming the synthesis of Mp*HYD3*dsRNAs. (C) PCR template – amplification strategies to add T7 promoter sequences to fragment *prx*1*Mp* in both orientations for the synthesis of Mp*PRX1*dsRNA. T7 promoter sequences were added to DNA (Mp*PRX1*) using PCR. The strategy begins by synthesizing PCR primers with the T7 promoter sequence appended to the 5′ end of the primer. (D) Synthesis of dsRNAs with MEGAscript RNAi Kit transcription reaction. 1. dsRNA control from kit (500 pb), 2. *gfp*dsRNA (717 pb), 3. Mp*PRX1*dsRNA (414 pb), 4. Mp*HYD3dsRNA* (808 pb), 5. Marker 1 kb.

RiboPure™ Kit (Ambion®), which allows obtaining total RNA free of genomic DNA. Quantitative real-time PCR test was performed with the RNA samples to verify the absence of DNA contamination. The cDNA was synthesized from a 1.0 μg of total RNA per reaction (20 μL) using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) and oligo(dT)$_{18}$ primers according to the manufacturer's instructions. The resulting cDNA was stored at −20 °C before use.

2.10. Quantitative real-time PCR analysis

Real-time PCR (RT-qPCR) was used to quantify gene expression levels. All real-time assays were done in triplicate for all control and dsRNA-treated lines using 0.2 μM forward and reverse primers specific for each gene (Table 1) and 10 μL of each cDNA 1:50 (v/v) dilution in a final reaction volume of 20 μL. Thermo cycling was performed utilizing an ABI 7500 RT-qPCR System (Applied Biosystems). Fluorescence data were acquired during the elongation step in every cycle. After cycling, melting curves for each amplicon were determined between 80 and 85 °C.

The *actinA* gene was used to normalize the expression levels of the studied genes. For control samples, the mean relative expression level of the assayed gene was assigned a value of 1.0 and the relative expression levels for all lines were calculated relative to it. Analysis of variance and ranking by Tukey's test were performed. Mean standard errors of all lines were computed dividing the standard deviation by the square root of three (since three replications were taken).

2.11. Determination of 1-cys peroxidase activity

For the determination of the peroxidase activity, six spots of individual mycelia from control and treated with Mp*PRX1*dsRNA lines were grown in 50 mL of PDG enriched medium at 25 °C and stirred in the dark for 12 d. After that, an aliquot of the culture supernatant was used in a reaction medium containing 10 mM phosphate buffer pH 6.0, 20 mM guaiacol and 0.3% (w/v) H_2O_2. The volume of the aliquot was adjusted to obtain absorbance readings at 470 nm between 0.2 and 1.0 in a reaction time of 3 min. The enzyme activity was followed through the change in absorbance (λ 470 nm, at 25 °C) of the reaction immediately after mixing, and afterward every 30 s for 3 min. The absorbance was measured with a microplate spectrophotometer Versamax (Molecular Devices Corporation, CA, USA).

The measured absorbance values (Abs) were correlated to enzyme activity through a standard curve prepared using enzymatic extract in excess and varying guaiacol concentrations in the reaction medium. This enabled the change in absorbance correlate with the amount of guaiacol converted into tetrahydroguaiacol. Therefore, peroxidase activity could be expressed in mMol guaiacol/mL of supernatant/h using the equation: activity = $-0.0135 + 1.6444 \times$ Abs ($r^2 = 0.999$).

2.12. M. perniciosa survival curve in H_2O_2

Broken hyphae were obtained according to Filho et al. (2006) from control mycelia and from mycelia of the four lines silenced with Mp*Prx*1dsRNAs; all grown in enriched PDA media at 25 °C for 8 d. Three milliliters of the resulting broken hyphae suspension were transferred to 50 mL of liquid PDG media and incubated at 25 °C for 7 d without agitation. After this period, the sensitivity of broken hyphae to different concentrations of H_2O_2 (0, 1, 2 and 4 mM) was determined by applying 1.0 mL of broken hyphae suspension in three plates of PDA containing H_2O_2 and incubated at 25 °C. After 7 d the absolute number of *pseudo-colonies* formed from the surviving broken hyphae was counted. Each of the three

plates gave rise to a number of pseudo-colonies/mL of suspension. Control plates without H_2O_2 yielded *M. perniciosa* pseudo-colonies for determination of 100% survival rate. Results are expressed as the percentage of survival related to the untreated control and represent the mean of at least three independent experiments. Bars represent standard deviations as calculated by the GraphPad Prism® program (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. dsRNA-induced silencing of the exogenous gfp gene marker

3.1.1. Analysis of transformants

In a first step toward a straightforward evaluation of dsRNA-mediated gene silencing in *M. perniciosa*, we used the reporter gene *gfp* as a target for silencing. Therefore, transgenic hyphae expressing *gfp* were produced. To this end, the pHSP70-SG vector containing the hygromycin B phosphotransferase gene (*hph*), which confers resistance to hygromycin B, and *gfp* under the control of *Ustilago maydis* Heat Shock Protein 70 (*hsp*70) promoter was employed to transform *M. perniciosa* protoplast using a modified PEG/$CaCl_2$ transformation protocol (Yelton et al., 1984; Balance and Turner, 1985). Hygromycin resistant mycelia from distinct protoplasts regenerating on separate regions of the plate were isolated after 10 days (d) of selection. Each selected mycelia was defined as an independent transformant and the presence of *hph* and *gfp* was then analyzed in these independent transformants after a further growth period of 10 d on selective medium.

The transformation efficiency ranged from 0.9 to 0.6 independent transformants/μg DNA, in two distinct experiments. Five hygromicyn resistant regenerants, were tested for the presence of pHSP70-SG by amplification of the *hph* and *gfp* genes (Fig. 2A and B) and by analysis of *gfp* expression by fluorescence microscopy (Fig. 3. A1–A3 and B).

Furthermore, the mitotic stability of *hph* gene was evaluated by testing the ability of transformed hyphae to express hygromycin resistance even after a growth period in non-selective medium. We found that all five selected independent transgenic hyphae when grown up to 40 d in non-selective medium and subsequently transferred to selective medium containing hygromycin B still kept their capacity to grow, indicating that *hph* was stably integrated in the genome.

Transformation with *gfp* was monitored by observation and recording green fluorescence. In transformants GFP fluorescence was detected in the cytoplasm of protoplasts 24 h after transformation and at the hyphal extension zone during the early stages of hyphae development (3–5 d post-transformation). In up to 20-d old hyphae different degrees of green fluorescence was observed in some hyphae while others did not fluoresce at all (Fig. 3. A1–A3) indicating a heterogeneous pattern of fluorescence.

It was also recorded that GFP fluorescence in 6-month old transformants was preferentially localized in more specific regions of the hyphae such as cell wall, hyphae tips, between hyphae compartments, points of hyphae ramifications, as well as clamp connections (Fig. 3. B1–B3).

A weak fluorescence background was also detected in 3–5 and 20-d old control hyphae that were regenerated from non-transformed protoplasts. This fact can be attributed to fluorescent phenolic compounds in the mycelium cell wall and this hypothesis is compatible with the absence of any fluorescence in non-transformed protoplasts which lack cell wall structures (Fig. 3. A4–A6).

We next attempted to induce *gfp* silencing by direct transformation of protoplasts obtained from hyphae expressing the heterologous *gfp* reporter gene with an *in vitro* synthesized *gfp*dsRNA sequence. Hyphae regenerated from six independent protoplast transformation events with *gfp*dsRNA will be referred to as individual lines T1–T6 and therefore can be considered as biological replicas. The expression level of *gfp* was measured by RT-qPCR 28 d after protoplast transformation with *gfp*dsRNAs. The expression level of *gfp* RNA in lines T1, T3 and T4 treated with *gfp*dsRNA was found to be reduced to approximately half (51.3–63.6%) the level measured in the control *gfp* expressing line. On the other hand, lines T2, T5 and T6 showed striking decreases in the expression levels, ranging from 90.8% to 97.3%, compared to the control line (Fig. 4A).

According to Tukey's test in all six lines treated with *gfp*dsRNA the level of *gfp* expression was significantly lower than the expression values measured in the control (Fig. 4A). Furthermore, the reduction in *gfp* expression in the six silenced lines was compatible with their reduction in GFP fluorescence (Fig. 4B). These results strongly suggest that dsRNA can induce silencing of a specific target gene in *M. perniciosa*. We next verified whether endogenous genes could also be efficiently silenced by related dsRNAs.

3.2. dsRNA silencing of M. perniciosa 1-cys peroxiredoxin (MpPRX1) and hydrophobin (MpHYD) gene family

To assess the possibility to silence endogenous genes by dsRNAs in *M. perniciosa* we choose the 1-cys peroxiredoxin (Mp*PRX1*) that encodes for a mitochondrial protein and the hydrophobin-3 (Mp*HYD3*) genes as targets. These gene sequences were identified in the *M. perniciosa* genome database (http://www.lge.ibi.unicamp.br/vassoura).

The mitochondrial enzyme 1-*cys* peroxiredoxin function in the detoxification of ROS generated by the respiration process or as a response to several stress agents (Monteiro and Netto, 2004),

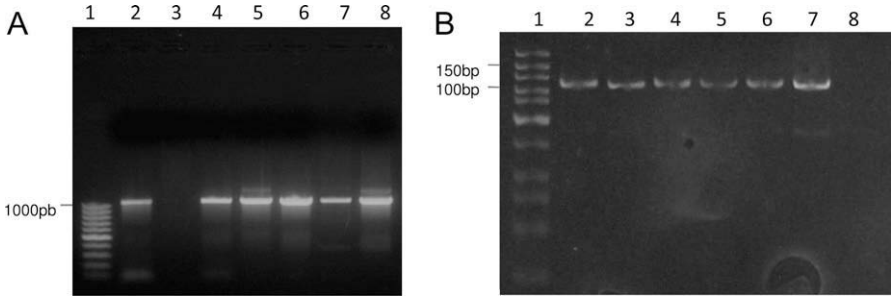

Fig. 2. Detection of the *hph* and *gfp* genes in lines of *M. perniciosa* transformed with pHSP70(*gfp*). (A) DNA from several independent hygromycin resistant *M. perniciosa* mycelia was extracted and the presence of the *hph* gene evaluated by PCR amplification. 1. Marker; 2. Positive Control (pCAMBIA 1381); 3. Non-transformed *M. perniciosa*; 4, 5, 6, 7, 8 *M. perniciosa* transformants (T1–T5). (B) DNA from several independent hygromycin resistant *M. perniciosa* mycelia was extracted and the presence of the *gfp* gene (119 pb) evaluated by PCR amplification. 1. Marker; 2. Positive Control (pHSP70-SG), 3, 4, 5, 6, 7 *M. perniciosa* transformants (T1–T5); 8. Non-transformed *M. perniciosa*.

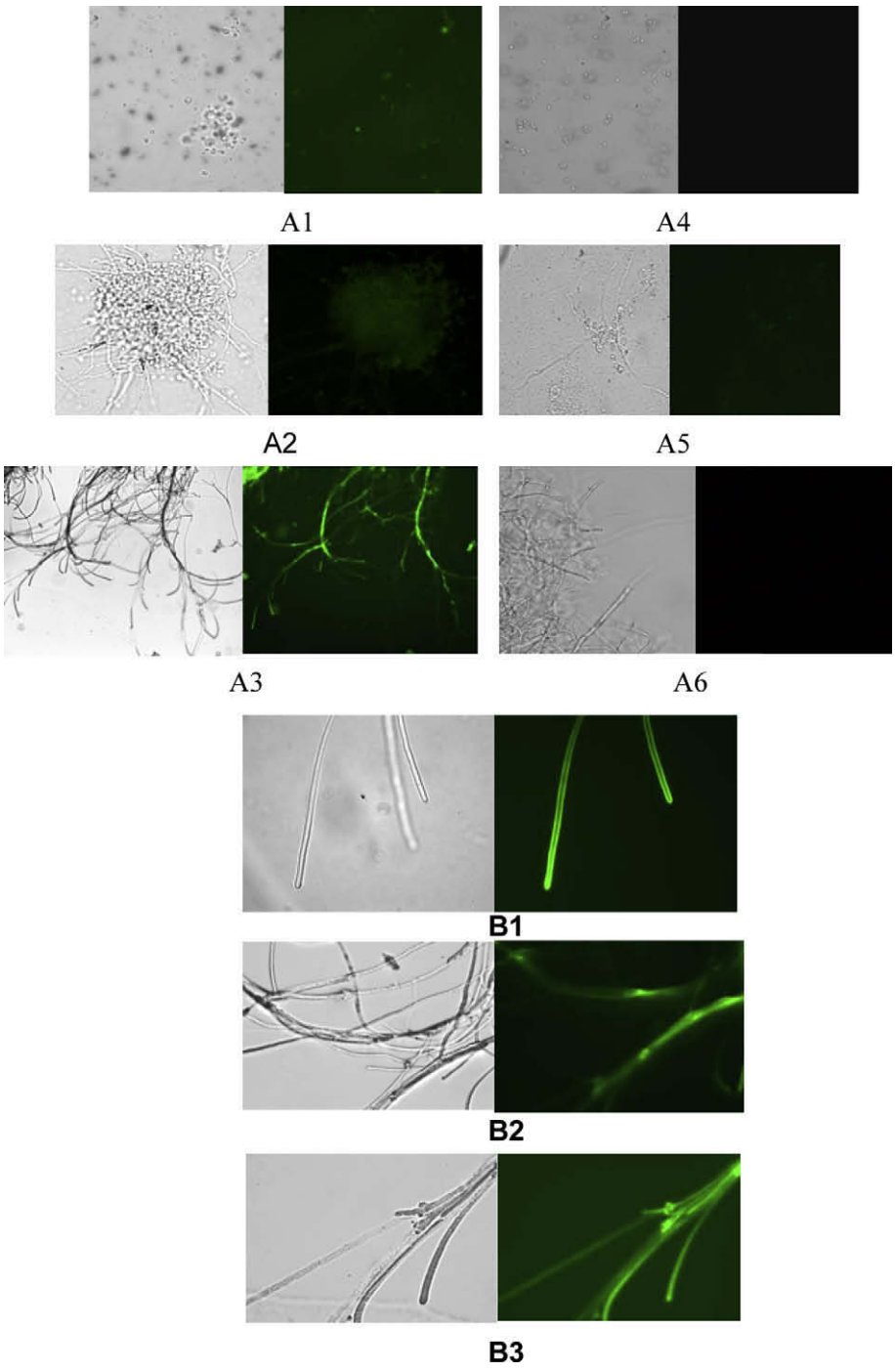

Fig. 3. Expression of GFP in *M. perniciosa* mycelia transformed with pHSP70, via PEG/$CaCl_2$. Protoplasts and mycelia were examined microscopically using 40× objective under white light conditions (left) or UV light to detect green fluorescence (right). (A) Expression of GFP in *M. perniciosa* mycelia transformed with pHSP70 was monitored at different developmental stages of transformed (A1, A2, A3) and compared to control non transformed mycelia (A4, A5, A6) at equivalent developmental stages. (A1 and A2), Protoplasts, 1 d after transformation. (A2 and A5), Hyphae in early development, 5 d after transformation. (A3 and A6), Hyphae 20-d old after transformation. (B) Expression of GFP in 6-month old *M. perniciosa* transformed mycelia. (B1) Details of hyphae tip shows greater fluorescence intensity at the cell wall and hyphae tip. (B2) Detail of hyphae shows fluorescence among hyphae compartments. (B3) Detail of hyphae shows greater fluorescence intensity at the ramification points and connection clamps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protecting the cells against oxidative stresses. In addition to its functional importance, the *MpPRX1* gene was also chosen for its phenotypic expression, that is, lines impaired in the expression of the mitochondrial peroxidase activity at the initial phase of mycelial development are sensitive to H_2O_2 treatment and, therefore, can be readily identified.

The procedure to induce Mp*PRX1* silencing by a corresponding dsRNA sequence was essentially identical to the one described for *gfp* silencing. Silencing was evaluated by using RT-qPCR to quantify the Mp*PRX1* RNA level in 28-d old mycelia that were regenerated from Mp*PRX1*dsRNA-treated protoplasts (Fig. 5A). A clear reduction of Mp*PRX1* mRNA levels was detected in all five independent lines regenerated from Mp*PRX1*dsRNA-treated protoplasts (Fig. 5A). According to Tukey's test, in five lines the expression was significantly reduced (22.8–87.3%) compared to the control line (Fig. 5A).

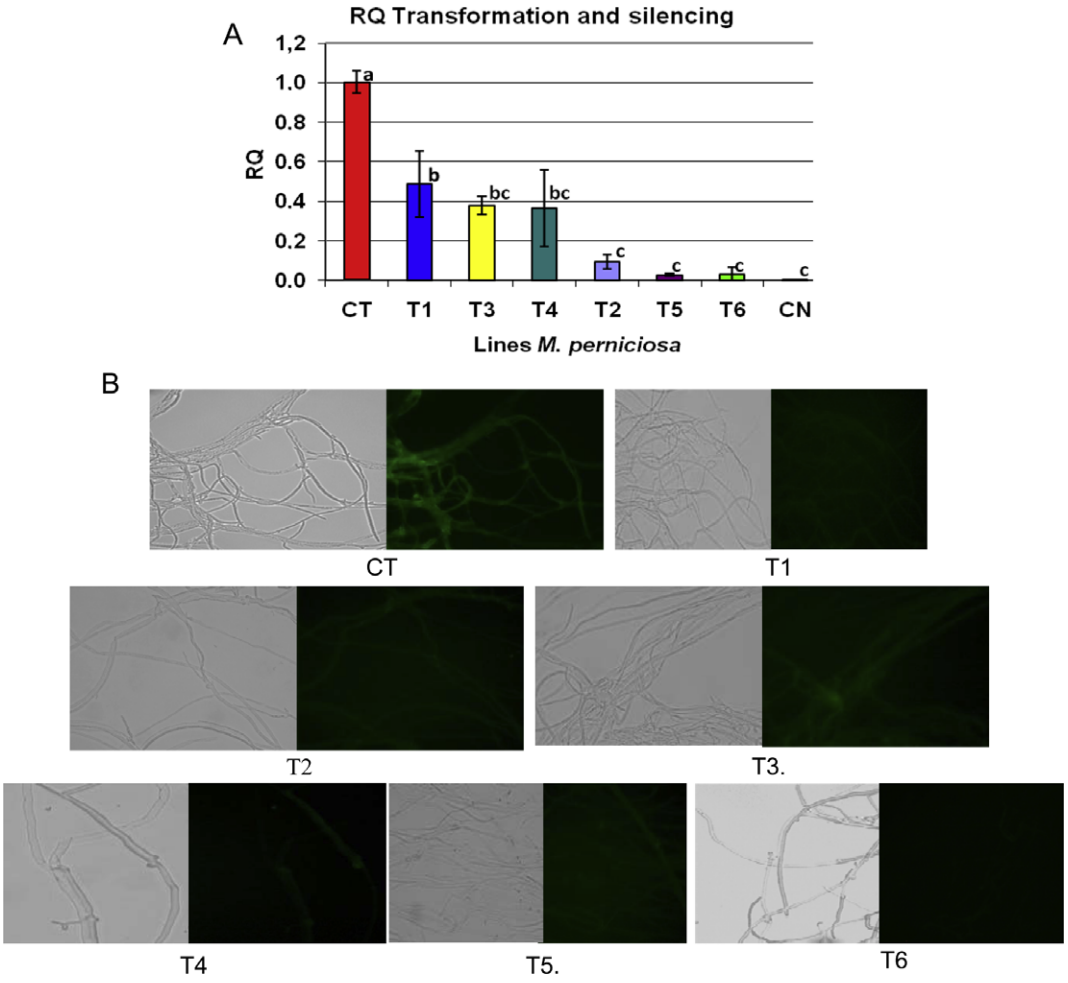

Fig. 4. Evaluation of *gfp* silencing by *gfp*dsRNA in *M. perniciosa*. (A) Quantification of *gfp* silencing by RT-qPCR. Relative *gfp* expression in the transgenic independent lines of *M. perniciosa* treated with *gfp*dsRNA (T1–T6), Control line transgenic expressing *gfp* (CT) and negative control (NT, non-transformed control line). *gfp* values were normalized to actin values. The levels of *gfp* expression in lines treated with *gfp*dsRNA significantly decreased (51.3–97.3%) in relation to the control line. Variations in the levels of *gfp* expression are shown as standard errors of the means ($n = 3$). Means with the same letter are not significantly different from each other according to Tukey's test ($p = 0.05$). (B) Expression of GFP in 4-month old hyphae of *M. perniciosa* as revealed by green fluorescence. *gfp* expression levels in silenced lines are compatible with the intensity of green fluorescence observed under UV light. Hyphae examined microscopically using 40× objectives in white light (left) and GFP filter (right). CT, control non-silenced line and T1–T6 correspond to lines treated with *gfp*dsRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As expected, the five lines silenced for Mp*PRX1* presented a reduction in the corresponding peroxidase activity which appeared to be proportional to the Mp*PRX1* mRNA levels (Fig. 5B). Reduction of peroxidase activity should confer high sensitivity to H_2O_2. Hence, 5-month old hyphae silenced for Mp*PRX1* were challenged for their sensitivity to H_2O_2. In all five lines a clear correlation between H_2O_2 exposure doses and survival response was observed (Fig. 5C); with significant reduction in survival values in lines silenced with Mp*PRX1*dsRNAs treated with H_2O_2 as compared to the control line (Fig. 5C). The differences among the silenced lines were more accentuated at 4 mM H_2O_2, indicating that this dose was the most effective in discriminating the different lines. At this concentration all lines differed from each other except T5 and T7, which were not statistically different and showed the lower survival values (Fig. 5C). Since this assay was performed 150 d after dsRNA transformation and the lines were replicated almost 19 times, this result suggests that stable silencing of Mp*PRX1* occurred.

In order to consolidate our findings we reasoned that it would be important to be able to silence several genes simultaneously. For that, we chose to simultaneously silence the hydrophobin (Mp*HYD*) multigene family, which is formed by five members that share a high degree of sequence similarity (Fig. 6A and F and Santos, 2005). In addition, as Mp*HYD* are required for the formation of aerial hyphae, basidiomes and spores, which are essential structures in reproduction and preservation of the fungus, silencing of these genes would result in phenotypes that could be monitored. Therefore, a dsRNA sequence corresponding to the whole Mp*HYD3* gene (alignments of Mp*HYD3* with other hydrophobin sequences are shown in Fig. 6A), was used to evaluate the efficiency of silencing the Mp*HYD* gene family.

The efficiency of Mp*HYD* silencing was measured by RT-qPCR analysis in two independent experiments that differed from each other by the method of transferring the Mp*HYD3dsRNA*, PEG/$CaCl_2$ versus electroporation transformations. All analyses were performed using the four pairs of primers that specifically amplify hydrophobins 1, 2, 4 and 5, respectively (Table 1). Although Mp*HYD3*dsRNA was used to trigger hydrophobin genes silencing, HMP3 RNA could not be quantified adequately by RT-qPCR and was therefore not included in our analysis.

Fig. 6B shows that silencing occurred in a set of three independent lines that were regenerated from protoplasts electroporated with Mp*HYD*3dsRNA. The degree of reduction in hydrophobin gene expression ranged from 89.0% to 97.6% (Fig. 6B) and the

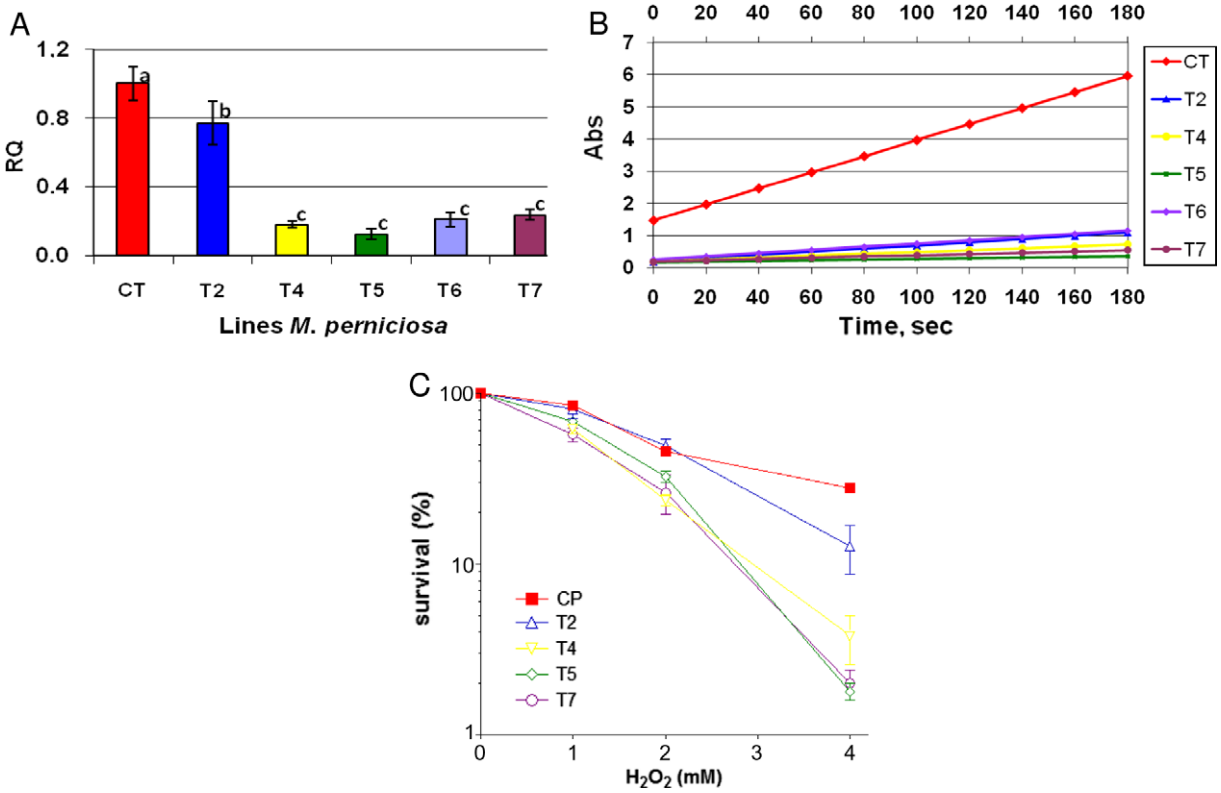

Fig. 5. Evaluation of Mp*PRX1* silencing. (A) Determination of Mp*PRX1* silencing efficiency using RT-qPCR. Relative Mp*PRX1* expression in control (CT) and five independent hyphae lines T2, T4, T5 T6 and T7 treated with Mp*PRX1*dsRNA. Variations in the levels of Mp*PRX1* expression are shown as standard errors of the mean ($n = 3$). Means followed by the same letter in the same column are not significantly different from each other according to Tukey's test ($p = 0.05$). (B) Determination of peroxidase activity in mycelia of *M. perniciosa* silenced with Mp*PRX1*dsRNA. Lines are linear regression equations, symbols actual data ($n = 3$). Abs can be converted in activity using the equation in material and methods. (C) Survival curves of *M. perniciosa* lines silenced with Mp*PRX1*dsRNA and control line, all exposed to different H_2O_2 concentrations and incubated for 7 d at 25 °C.

hydrophobin 1, 2, 4 and 5 were significantly different from the control line according to Tukey's test (Fig. 6B).

Transfer of Mp*HYD3*dsRNA into protoplasts via the PEG/$CaCl_2$ transformation protocol also resulted in a wide range of reduction of expression levels (18.4% to the 97.0%) compared to control lines (Fig. 6C). According to Tukey's test, the lines T1, T2 and T10 showed significantly reduced levels of mRNA for hydrophobins 1, 2, 4 and 5 with the exception of line T3 for hydrophobin 1 (Fig. 6C). Higher decreases in the expression of hydrophobins were detected in lines T1, for hydrophobins 1, 2 and 4, and lines T1, T2, T3 and T10 for hydrophobin 4 (Fig. 6C).

Overall, it appeared that hydrophobins 4 and 5 showed higher degrees of silencing in most of the silenced lines treated with Mp*HYD3*dsRNA possibly as a consequence of their higher similarity with Mp*HYD3* as compared to hydrophobins 1 and 2 (Fig. 6D). In any case, the results obtained for hydrophobins 1, 2, 4 and 5 strongly suggest that the simultaneous silencing of several members of a multigenic family, by the introduction of a dsRNA that covers conserved sequences among the family members, was successfully achieved. Microscopical and visual analyses of the hyphae revealed that 50% and 40% of them exhibited a hydrophilic phenotype (non-flocculent) for electroporated and PEG silenced lines, respectively, when compared with the non-silenced lines (Fig. 6E and F).

4. Discussion

We provided evidences that efficient gene silencing mediated by dsRNA can be achieved in *M. perniciosa*. In a first step we showed that the heterologous *gfp* reporter gene could be stably transferred into *M. perniciosa* through protoplast transformation with subsequent reduction of *gfp* expression in stably transformed lines promoted by transfer of *gfp*dsRNA synthesized *in vitro*. Silencing of *gfp* in all six transgenic lines submitted to *gfp*dsRNA was highly efficient. Reductions of *gfp* mRNA levels ranging from 51.3% to 97.3% in relation to the control line were found and validated by the reduction in GFP fluorescence emission. Our results are compatible with previously reported *gfp*dsRNA- or hairpin *gfp*-induced *gfp* silencing in different fungi, such as *Colletotrichum lagenarium* (de Jong et al., 2006), *M. oryzae* (Kadotani et al., 2003), *Venturia inaequalis* (Fitzgerald et al., 2004), *P. infestans* (Whisson et al., 2005) and *Coprinopsis cinerea* (Walti et al., 2006; Costa et al., 2008). Silencing of the *gfp* gene in *M. perniciosa* was initially detected after 28 d following transformation with *gfp*dsRNA. Additionally, after 4 months of subsequent mycelium growth and replication, the silenced phenotype was maintained since the treated lines presented reduction in fluorescence when compared to the control non-silenced line grown under the same conditions. This result contrasts with that of Whisson et al. (2005), who considered silencing of *P. infestans* treated with *gfp*dsRNA transient due to the fact that fluorescence was partially recovered after 4 d. In general, RNAi is activated within one or 2 d and may persist for many days until the expression of the gene is recovered (Chen et al., 2003). In our study, the persistence of *gfp* silencing for a longer period (120 d) can be explained by the presence of a sequence similar to *qde*-1 of *N. crassa* that encodes for a RNA-dependent RNA-polymerase (RdRP) present in the *M. perniciosa* genome (Mondego et al., 2008). This enzyme feeds back the production of new siRNAs (secondary siRNAs), amplifying silencing through two different pathways (Axtell et al., 2006; Sijen et al., 2001,

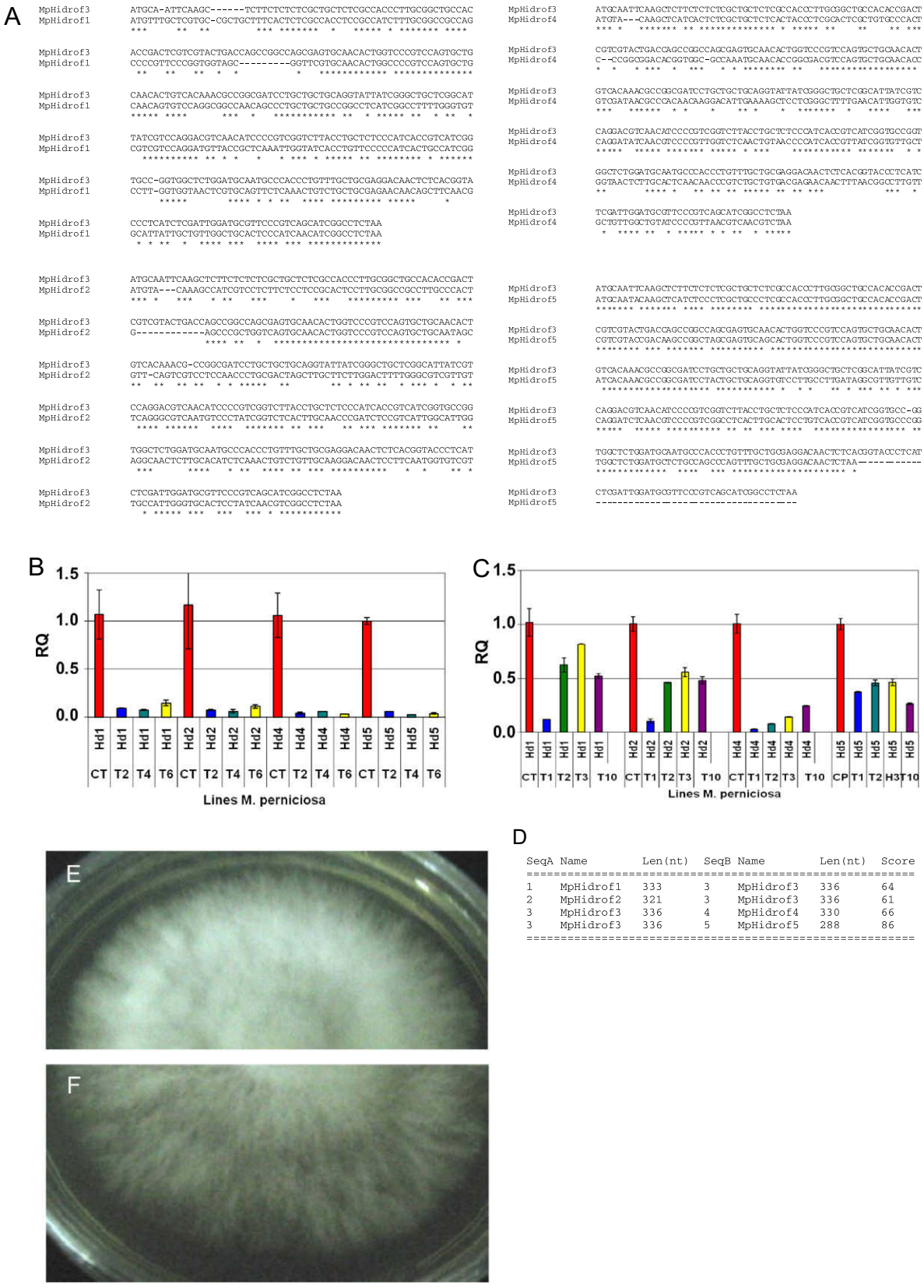

Fig. 6. Determination of Mp*HYD3*dsRNA ability to silence hydrophobin gene family. (A) Individual alignments of the Mp*HYD3* with the other four hydrophobins. Relative hydrophobins expression in control (CT) and independent hyphal lines treated with Mp*HYD3*dsRNA inserted via electroporation (B) and by PEG/$CaCl_2$ (C) in a hydrophobin multigenic family. Variations in the levels of Mp*HYD3* expression are shown as standard errors of the means ($n = 3$). (D) Summary of the identities between the hydrophobins compared with Mp*HYD3*. (E) Phenotype of the non-silenced line presenting aerial flocculent hyphae (hydrophobic phenotype). (F) Phenotype of one silenced line presenting a few aerial hyphae (hydrophilic phenotype).

2007; Alder et al., 2003; Aoki et al., 2007; Baulcombe, 2007). Alternatively, the late occurrence of gene silencing in *M. perniciosa* may involve other mechanisms, such as DNA methylation or formation of heterochromatin.

In summary, since RNAi is a locus-independent silencing mechanism, transmitted by a molecular mobile signal through the cytoplasm of each cell, its application is viable in filamentous fungi formed by multicellular and bi- or multinucleate hyphae, like *M. perniciosa.*

To be useful as a reverse genetic tool, dsRNA-induced gene silencing must be working for endogenous genes. For that, we tried two approaches, one silencing a gene that encodes for a mitochondrial-targeted protein and another silencing a small gene family that encodes for secreted proteins. We reported that the Mp*PRX1* silencing was efficiently promoted by Mp*PRX1*dsRNA reaching up to 87.3% mRNA reduction when compared to the non-silenced control. Moreover, a positive correlation between mRNA levels and the degree of the corresponding enzyme 1-*cys* peroxiredoxin activity was established indicating that dsRNA-induced silencing can indeed result in lower protein levels, which characterizes a knockdown outcome.

Reduction of mitochondrial 1-*cys* peroxiredoxin activity was further confirmed by the increased sensitivity, therefore, lower survival rates of silenced lines to H_2O_2. Generally, in this study, performed 5 months after transformation, we found a strong negative correlation between survival values in lines silenced with Mp*PRX1*dsRNAs treated with H_2O_2 as compared to the control line. We notice, however, that low concentrations of H_2O_2 of 1 and 2 mM were not discriminative since the survival values of the different silenced lines were rather similar to the control line. This result can be explained if other enzymes such as the cytoplasmic and mitochondrial catalases involved in the neutralization or removal of ROS species were activated (Boveris, 1998) and could compensate for the lack of Mp*PRX1* activity. In *S. cerevisae*, for example, the peroxiredoxin-null mutants, including *prxΔ* did not exhibit serious growth defects in aerobic culture due the induction of expression of others antioxidant proteins including glutathione peroxidase and glutathione reductase (Wong et al., 2004). Furthermore, the decrease in the survival rate values of silenced lines treated with H_2O_2, when compared to the control line, indicates that the silencing persisted throughout a 5 month period. This data is in agreement with the stability of *gfp* silencing during growth described above and further supports the conclusion that silencing is maintained during long periods. Recently, Pungartnik et al. (2009) demonstrated the effects of ROS in the *M. perniciosa* biology, thus, the silencing of Mp*PRX1* will be very important to validate several hypothesis raised in this work.

One way to get around the functional redundancy among members of gene families is to silence simultaneously all members of the family. In order to test such a possibility we evaluated the dsRNA-induced silencing of four among five members of the *M. perniciosa* hydrophobin family. We showed that indeed, by direct transfer of an Mp*HYD3*dsRNAs full sequence, some part of which is conserved among all five hydrophobin genes (Fig. 6A), the expression levels of Mp*HYD* 1, 2, 4 and 5 were significantly reduced as compared to the untreated control.

The levels of relative expression of Mp*HYDs* showed wide variation (18.4–97.6%) among the lines treated with Mp*HYD3dsRNA* which is in agreement with the variability in the extent of silencing that was described for the six hydrophobins of the fungus *Cladosporium fulvum targeted with* inverted repeat chimeras (Lacroix and Spanu, 2008). It appeared however that hydrophobin 4 and 5 showed the highest degrees of silencing in most of the lines treated with Mp*HYD*3dsRNA. This can be correlated to the overall high degree of identity of Mp*HYD*4 and Mp*HYD*5 with the silencing sequence from Mp*HYD*3, 66% and 88%, respectively (Fig. 6D).

Our results are in line with those obtained by Walti et al. (2006), who used as targets the isogenes *cgl*1 and *cgl*2 that encode two isogalectins, which are highly induced during the formation of fruiting bodies of *Coprinopsis cinerea*. They demonstrated the possibility of silencing part of this gene family with a single hairpin construction. The genes *cgl*1 and *cgl*2, which show 87% identity, were silenced in a frequency of up to 90%, while the counterpart *cgl*3, with 53.5% identity to *cgl*2 in its coding region, was not affected. In *C. fulvum*, simultaneous silencing of hydrophobins using inverted repeat chimeras was obtained, however, it was noticed that the efficiency of the process decreased with the increase of genes to be simultaneously silenced (Lacroix and Spanu, 2008). Together, these observations provide guidelines for functional gene analyses using dsRNA-induced silencing mainly in the cases of functional redundancy among closely related paralogues. In addition to the RT-qPCR results, a hydrophilic phenotype was observed in 45% of the silenced lines, which is accordingly with one of the expected roles of hydrophobins in aerial hyphae maintenance (Wösten et al., 1994). Indeed, antisense silenced lines of HCf1 in *Cladosporium fulvum* presented a hydrophilic phenotype (Hamada and Spanu, 1998).

Since the technology used in this work does not rely on laborious methods, it has great potential as a tool for the study of many functional genes in *M. perniciosa*, especially those linked to the developmental and infective stages. Moreover, the adjustment and optimization of the protocol for silencing mediated by dsRNA in *M. perniciosa*, including the technology for plasmidial construction that express *hp*RNA and produces stable silencing, is important for the functional analysis of genes that are expressed in more advanced developmental stages, such as basidiocarp formation. RNAi silencing would be a valuable tool to silence genes that are active in the heterokaryotic stage and require the inactivation of them in both nuclei. RNAi is able to silence genes in both nuclei by the expression of a hairpin construction in just one of them.

Acknowledgments

We thank Dr. Regine Kahmann and Dr. Karina Gramacho for kindly providing the pHSP70-SG vector and the *M. perniciosa* CPO2 strain, respectively. Research supported by FINEP and FAPESB *M. perniciosa* Proteomic Network. A.C.C.S held a fellowship from the Programa de Qualificação Institucional – PQI-CAPES and the UNICAMP Genetics Post-Graduation Program. C.V.D held FAPESB fellowship as Doctoral student in the Genetics Post-Graduation Program of UESC.

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