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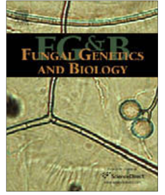

# dsRNA-induced gene silencing in *Moniliophthora perniciosa*, the causal agent of witches' broom disease of cacao

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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.

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## 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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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 ($1 \times 10^8$ 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

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