Identification of a second family of genes in Moniliophthora perniciosa, the causal agent of witches' broom disease in cacao, encoding necrosis-inducing proteins similar to cerato-...

ARTICLE *in* MYCOLOGICAL RESEARCH · SEPTEMBER 2008

Impact Factor: 2.81 · DOI: 10.1016/j.mycres.2008.08.004 · Source: PubMed

6 AUTHORS, INCLUDING:

Odalys García
University of Campinas
15 PUBLICATIONS **298** CITATIONS
SEE PROFILE

Francisco Javier Medrano
Centro de Investigaciones Biológicas
80 PUBLICATIONS **1,521** CITATIONS
SEE PROFILE

Gustavo Gilson Lacerda Costa
University of Campinas
44 PUBLICATIONS **599** CITATIONS
SEE PROFILE

Goncalo Pereira
University of Campinas
138 PUBLICATIONS **2,515** CITATIONS
SEE PROFILE

ELSEVIER

British Mycological Society promoting fungal science

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

Identification of a second family of genes in *Moniliophthora perniciosa*, the causal agent of witches' broom disease in cacao, encoding necrosis-inducing proteins similar to cerato-platanins

Gustavo ZAPAROLI[1], *Odalys García CABRERA*[1], *Francisco Javier MEDRANO*, *Ricardo TIBURCIO*, *Gustavo LACERDA*, *Gonçalo Guimarães PEREIRA*[*]

Laboratório de Genômica e Expressão, Departamento de Genética e Evolução, IB - UNICAMP, Caixa Postal 6109, CEP 13083-970, Campinas, Brazil

ARTICLE INFO

Article history:
Received 13 November 2007
Received in revised form
27 June 2008
Accepted 12 August 2008
Corresponding Editor:
Gareth W. Griffith

Keywords:
Cerato-platanin
Moniliophthora perniciosa
NEP1 like proteins
Witches' broom disease

ABSTRACT

The hemibiotrophic basidiomycete *Moniliophthora perniciosa* is the causal agent of witches' broom disease in cacao. This is a dimorphic species, with monokaryotic hyphae during the biotrophic phase, which is converted to dikaryotic mycelia during the saprophytic phase. The infection in pod is characterized by the formation of hypertrophic and hyperplasic tissues in the biotrophic phase, which is followed by necrosis and complete degradation of the organ. We found at least five sequences in the fungal genome encoding putative proteins similar to cerato-platanin (CP)-like proteins, a novel class of proteins initially found in the phytopathogen *Ceratocystis fimbriata*. One *M. perniciosa* CP gene (MpCP1) was expressed *in vitro* and proved to have necrosis-inducing ability in tobacco and cacao leaves. The protein is present in solution as dimers and is able to recover necrosis activity after heat treatment. Transcription analysis *ex planta* showed that MpCP1 is more expressed in biotrophic-like mycelia than saprotrophic mycelia. The necrosis profile presented is different from that caused by *M. perniciosa* necrosis and ethylene-inducing proteins (MpNEPs), another family of elicitors expressed by *M. perniciosa*. Remarkably, a mixture of MpCP1 with MpNEP2 led to a synergistic necrosis effect very similar to that found in naturally infected plants. This is the first report of a basidiomycete presenting both NEP1-like proteins (NLPs) and CPs in its genome.

Introduction

Cacao (*Theobroma cacao*) was one of the most prosperous crops in Brazil with a production of 320.5 kt cocoa beans year^{-1} in the early 1990s. In 1989 an epidemic outbreak of witches' broom disease (WBD) occurred in the most important cacao-producing region, which led to a decrease in the production of cacao to around 100 kt in 2000.

In 2001 we started a genome project to understand the causal agent of WBD, the basidiomycete *Moniliophthora perniciosa* (www.lge.ibi.unicamp.br/vassoura). The aim of this research programme was to identify key proteins that could

* Corresponding author.
E-mail address: goncalo@unicamp.br
[1] Authors have made equal contributions to this work.
0953-7562/$ – see front matter © 2008 The British Mycological Society. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.mycres.2008.08.004

elucidate the biology of *M. perniciosa*, and thereby, enable the development of control strategies.

In the disease cycle, the fungus infects meristematic regions of the tree, shoots, and young flowers, and has a hemibiotrophic lifestyle (Purdy & Schmidt 1996). In the biotrophic stage, the fungus penetrates the plant intercellular space, acting as a parasite, with few and thick monokaryotic hyphae, causing the formation of hypertrophic and hyperplasic tissues in the plant, known as green broom. Eight to 12 weeks after the infection, this green broom begins to die and the pathogen changes to the saprotrophic phase, characterized by numerous, smaller, and thinner dikaryotic hyphae, with the presence of clamp connections (Silva & Matsuoka 1999). The necrotrophic mycelia cause necrosis, giving rise to dry brooms. After a dry period of three to nine months, the brooms produce basidiocarps that release spores in the air and re-initiate the disease cycle (Andebrhan 1987; Orchard *et al.* 1994).

One of the impediments to elucidate the plant–fungi interaction was the lack of media to cultivate the biotrophic mycelia *in vitro*. The inoculation of spores in ordinary fungal culture media led to the immediate transition to saprotrophic mycelia, which is non-infective. Recently, we discovered that the cultivation of spores in poor media having glycerol as the only carbon source supported the growth of biotrophic-like mycelia, which are thicker than the saprotrophic hyphae and mononucleated (Meinhardt *et al.* 2006). This finding enabled us to identify proteins expressed during this phase.

Secreted proteins have important roles in plant–pathogen interactions, especially in biotrophic fungi (Allen *et al.* 2004). Inducible defence mechanisms involve both plant and pathogen-secreted proteins, including enzymatic inhibitors, proteases, pathogen-related proteins, and elicitors (ÓConnell & Panstruga 2006). *In silico* analyses of several *Phytophthora* species and *Magnaporthe grisea* genomes showed the existence of more than 100 and 700 putative secreted proteins, respectively, suggesting that they play an important role during plant–pathogen interactions (Kamoun 2006).

In the genome draft of *M. perniciosa* we have found several genes that encode for secreted proteins. García *et al.* (2007) showed the existence of necrosis and ethylene-inducing proteins NEP1-like proteins (NLPs) in *M. perniciosa* (called MpNEPs). NLPs activate defence-related responses in plants, such as the synthesis of phytoalexin and ethylene, pathogen-related protein expression, induction of necrosis at the infection site, and finally, cell death (Veit *et al.* 2001; Fellbrich *et al.* 2002; Qutob *et al.* 2006). MpNEPs have the ability to induce ethylene emission and necrosis in cacao and tobacco tissues, indicating that they can play an active role in Witches' Broom disease.

Cerato-platanin (CP) is a recently described secreted phytotoxic protein from the ascomycete *Ceratocystis fimbriata* f. sp. *platani*, the causal agent of canker stain disease in plane (*Platanus acerifolia*). CP is a 12.4 kDa protein, with 40 % hydrophobic residues and four cysteines, which form two disulphide bridges (Pazzagli *et al.* 2006). CP induces the production of phytoalexin and plant cell death in host and non-host plants, acting as a probable elicitor of the plant defense response, and is located at the fungal cell wall (Pazzagli *et al.* 1999; Boddi *et al.* 2004). Several proteins similar to CP have been found in different fungal species, and originated the CP family, which is characterized by small secreted proteins, with four conserved cysteines. There are already 40 entries in the InterPro (IPRO010829) for CP-like proteins. For this article, we assumed that SnodProt family (Skinner *et al.* 2001) members are also part of the CP-like family, because of their high sequence similarity.

Among the fungal species producing this type of proteins are animal pathogens, plant pathogens, and non-pathogenic saprophytic fungi. Examples of these proteins are the allergen Asp f13 from *Aspergillus fumigatus*, which causes allergic reactions in humans (Rementeria *et al.* 2005); the plant defense elicitor Epl1 from *Hypocrea atroviridis* (Seidl *et al.* 2006); Snodprot1 from *Phaeosphaeria nodorum* produced during the infection of wheat leaves (Hall *et al.* 1999); Sp1 from *Leptosphaeria maculans*, which causes blackleg disease in canola (Wilson & Desjardin 2005), and CS-Ag from *Coccidioides immitis*, which causes coccidioidomycosis in humans (Pan & Cole 1995).

In this article, we show that *M. perniciosa* presents putative homologous open reading frames (ORFs) to the CP genes (called MpCPs). MpCP1 was cloned and the protein was expressed and purified in *Escherichia coli*. This protein is able to induce necrosis in tobacco and cacao leaves. MpCP1 activity is resistant to high temperatures and the protein is differently expressed in biotrophic-like and saprotrophic fungal phases. A comparison between the symptoms of MpCP1 and MpNEP2 in cacao leaves shows differences in the progression of necrosis. We discuss a possible synergic effect between MpCP1 and MpNEP2, suggesting a central role in WBD and *M. perniciosa* survival strategy.

Materials and methods

Biological material and growth condition

The isolate of *Moniliophthora perniciosa* used in this work was the original CP02 selected for the genome project (Rincones *et al.* 2003). To obtain saprotrophic mycelia, spores were inoculated directly in semi-defined media containing glucose (1 %), $NH_4H_2PO_4$ (1 %), KCl (0.2 %), $MgSO_4$ (0.2 %), yeast extract (0.5 %), $CuSO_4$ (0.05 %), and $ZnSO_4$ (0.001 %). Biotrophic-like mycelia were obtained by germinating spores in a special poor medium containing glycerol as sole carbon source, as described previously (Meinhardt *et al.* 2006). The *Moniliophthora perniciosa* isolate used in this study, BP02, can be obtained from the Laboratório de Genômica e Expressão at UNICAMP upon request by e-mailing suporte@lge.ibi.unicamp.br.

Phylogenetic analysis

Sequences with significant similarity (value $1e^{-4}$) to MpCP1 were retrieved from the NCBI database using BlastP and tBlastX (Altschul *et al.* 1990; McGinnis & Madden 2004). Additional sequences were retrieved from the draft genome sequences of the basidiomycetes *Laccaria bicolor* (http://genome.jgi-psf.org/Lacbi1/Lacbi1.info.html), *Phanerochaete chrysosporium* (http://genome.jgi-psf.org/whiterot1/whiterot1.info.html), and *Coprinus cinereus* (http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Info.html), not present at the NCBI database.

The search of these genomes was performed locally using Blast tools (tBlastN and tBlastX). The signal peptide region was predicted using the program Signal P3 (Bendtsen *et al.* 2004). Additional sequences of possible CP-like proteins were found in *M. perniciosa* with BlastX and tBlastX. Gene prediction was performed with Genezilla_v6x program (Majoros *et al.* 2004). Amino acid sequences were aligned using the ClustalW program with the default options (Higgins *et al.* 1996), except for the use of the substitution matrix PAM. The signal peptide (position 1 to 33) and poorly aligned regions (positions 88, 100–109, and 157–163) were removed from the final alignment, resulting in the alignment of 110 amino acids. The nucleotide sequence corresponding to the 110 amino acid alignment was used to build the phylogenic tree. The General Time Reversible (GTR) model of nucleotide substitution was selected for the construction of the phylogenetic tree using the program MrAIC.pl (Nylander 2004). The phylogenetic tree was built with the ML method using the selected evolutionary model and gamma distribution with the alpha parameter set to 2 with the program PhyML (Guindon & Gascuel 2003). The robustness of the inferred tree was tested by BS analysis with 1 K resamplings (Felsenstein 1985).

Cloning, expression and purification of MpCP1 protein

Genomic DNA from *Moniliophthora perniciosa* was isolated as previously described (Specht *et al.* 1982). The gene coding for MpCP1 without the signal peptide (381 bp) was amplified by PCR from purified genomic DNA using the oligonucleotide primers MpCP-F 5′-CGGGATCCGCTGTGAAACT C-3′ and MpCP-R 5′-GGAATTCTTACGACTGGCGTTCG-3′ based on the sequence of the gene. The primers were designed to generate products with cohesive overhangs (underlined) for efficient cloning into expression vectors. The PCR product was digested with the restriction endonucleases *Bam*HI and *Eco*RI, and cloned into the pET28a(+) vector (Novagen, San Diego, CA), previously digested with the same enzymes, resulting in the plasmid pMpCP1. PCR products and plasmids were purified using the Qiagen kits according to the manufacturer instructions. *Escherichia coli* DH10b was used for the propagation of recombinant plasmids. This vector is designed for the heterologous protein expression in *E. coli* producing a protein with a tag of six histidines at the amino terminus to facilitate the purification of the protein. *E. coli* BL21 (DE3) pT-Trx (Yasukawa *et al.* 1995) cells were transformed with the plasmid pMpCP1 and used for protein expression. The correct sequence of the gene cloned into the vectors was verified by DNA sequencing. All DNA manipulation including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, and ligation with T4 DNA ligase were performed as described by Sambrook (Sambrook & Russel 2001).

E. coli BL21 (DE3) pT-Trx cells harbouring the plasmid pMpCP1 were grown in Luria Bertani medium containing kanamycin (15 mg ml^{-1}) and chloramphenicol (34 mg ml^{-1}) at 30 °C and 200 rev min^{-1}. An aliquot of 5 ml of an overnight culture of *E. coli* BL21(DE3) pT-Trx strain containing the recombinant pMpCP1 plasmid was used to inoculate 500 ml of the same medium. Expression of the recombinant protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM at an $OD_{600\ nm}$ of 0.4 and cultivated for an additional 4 h. Cells were harvested by centrifugation at 6000 *g* and 4 °C for 10 min. They were resuspended in 5 ml lysis buffer (0.1 M Tris–HCl buffer at pH 8 containing 50 mM NaCl). Protein expression was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (Laemmli 1970) and stained with Coomassie brilliant blue R-250 (Fairbanks *et al.* 1971). The recombinant protein carrying a 6 × His tag was purified by immobilized metal affinity chromatography (IMAC) using a Hi-Trap (Amersham Biosciences, NJ) chelating column loaded with Ni^{2+} as the affinity metal. The cells were broken using a sonifier (Sonics Vibra-cell) at 40 % maximum intensity (3 × 30 s). Streptomycin sulphate, 2 % (w/v), was added to the cell extract and incubated for 30 min at 4 °C with continuous stirring to precipitate the nucleic acids. Insoluble material was removed by centrifugation at 38 000 *g* for 1 h at 4 °C. The soluble extract was loaded into the column pre-equilibrated with buffer A (0.1 M Tris–HCl at pH 8 containing 0.5 M NaCl and 5 mM imidazole), the column was washed until no protein came out, additional washes with 25, 50, and 100 mM imidazole in buffer A were performed, and the MpCP1 protein was eluted with 200 mM imidazole. Purity of the protein was analysed by SDS-PAGE.

Analysis of MpCP expression ex planta

Total RNA from the mycelia of saprophytic and biotrophic-like phases was isolated using Trizol (Invitrogen, CA), as previously described (Ausubel *et al.* 1998). As a control, saprophytic mycelia (SAP) were grown in the same media as the biotrophic-like mycelia (Meinhardt *et al.* 2006). The cDNA was synthesized from total RNA using a PCR-select cDNA subtraction kit according to manufacturer's instructions (Clontech, Palo Alto, CA). The cDNA was amplified by semi-quantitative PCR using *primers* MpCP-F and MpCP-R, followed by dot-blot analyses, as described previously (García *et al.* 2007). The concentrations of cDNA used in each experiment were normalized using the intensity of the amplification of the actin gene, which was assumed to have a similar level of expression in both types of mycelia.

Assay of necrosis activity

Necrosis experiments in both tobacco and cacao leaves were performed as described previously for MpNEPs (García *et al.* 2007). In tobacco, the protein and buffer solution were inoculated separately into leaves of five-week-old plants with a hypodermic syringe. In cacao, leaves were cut from four-month-old seedlings and the petiole was dipped into the protein and buffer solution separately. Solutions of single protein (MpCP1 or MpNEP2) was prepared with 30–50 µl of 0.5 mg ml^{-1} in 10 mM Tris–HCl buffer at pH 8 containing 50 mM NaCl. Mixed protein solutions were prepared using 0.3 mg ml^{-1} of each protein in the same buffer.

Dynamic light scattering (DLS)

DLS measurements were conducted on a temperature-controlled DynaPro DLS instrument (Protein Solutions, CA). Data were analysed using the DYNAMICS, version 6, software

from Protein Solutions. Each measurement consisted of at least 500 independent readings, with each reading being 10 s in duration. A 100 μl aliquot of MpCP1 protein in 10 mM Tris–HCl buffer at pH 8 was centrifuged at 15 000 ***g*** for 1 h at 4 °C, and a 60 ml aliquot of the supernatant was loaded into a quartz cuvette. All measurements were taken at 25 °C.

Results

Sequences of CP-like proteins in Moniliophthora perniciosa

The gene that codes for the plant necrosis-inducer, CP, from the fungus *Ceratocystis fimbriata* (GI:121624696) was used to investigate the presence of homologous genes in the *Moniliophthora perniciosa* genome using Blast tools. The search showed the presence of at least five different sequences with significant similarity to CP-like proteins (Fig 1A). We have named them '*Moniliophthora perniciosa* cerato-platanins' (*MpCPs*). The whole sequence for the gene *MpCP1* was present in the *M. perniciosa* genomic data bank with the 5′ UTR and 3′ UTR to define the organization of the gene (Fig 1B). Also, the ORF sequences of the genes *MpCP2* and *MpCP3* were present (Fig 1C). A possible TATA box was identified in the 5′ UTR of the gene *MpCP2* 95 bp upstream of the start codon (Fig 1C), but not enough of the 3′ UTR could be located with good quality to define the whole gene. The corresponding ESTs (expressed sequence tags) for *MpCP1*, *MpCP2* and *MpCP3* were found in cDNA libraries, indicating that they are expressed genes. An incomplete sequence could be recovered from the genomic data bank for the other two genes, *MpCP4* and *MpCP5*, but no information relative to these genes was retrieved from the EST data bank. The sequence of the coded proteins is shown in Fig 1A. Four of the genes, for which we have the beginning of the coding region, showed an identifiable signal peptide sequence, a clear indication of the extracellular location of the mature protein.

The comparative analyses of the genomic sequence with the EST sequence for *MpCP1* and *MpCP2* showed the presence

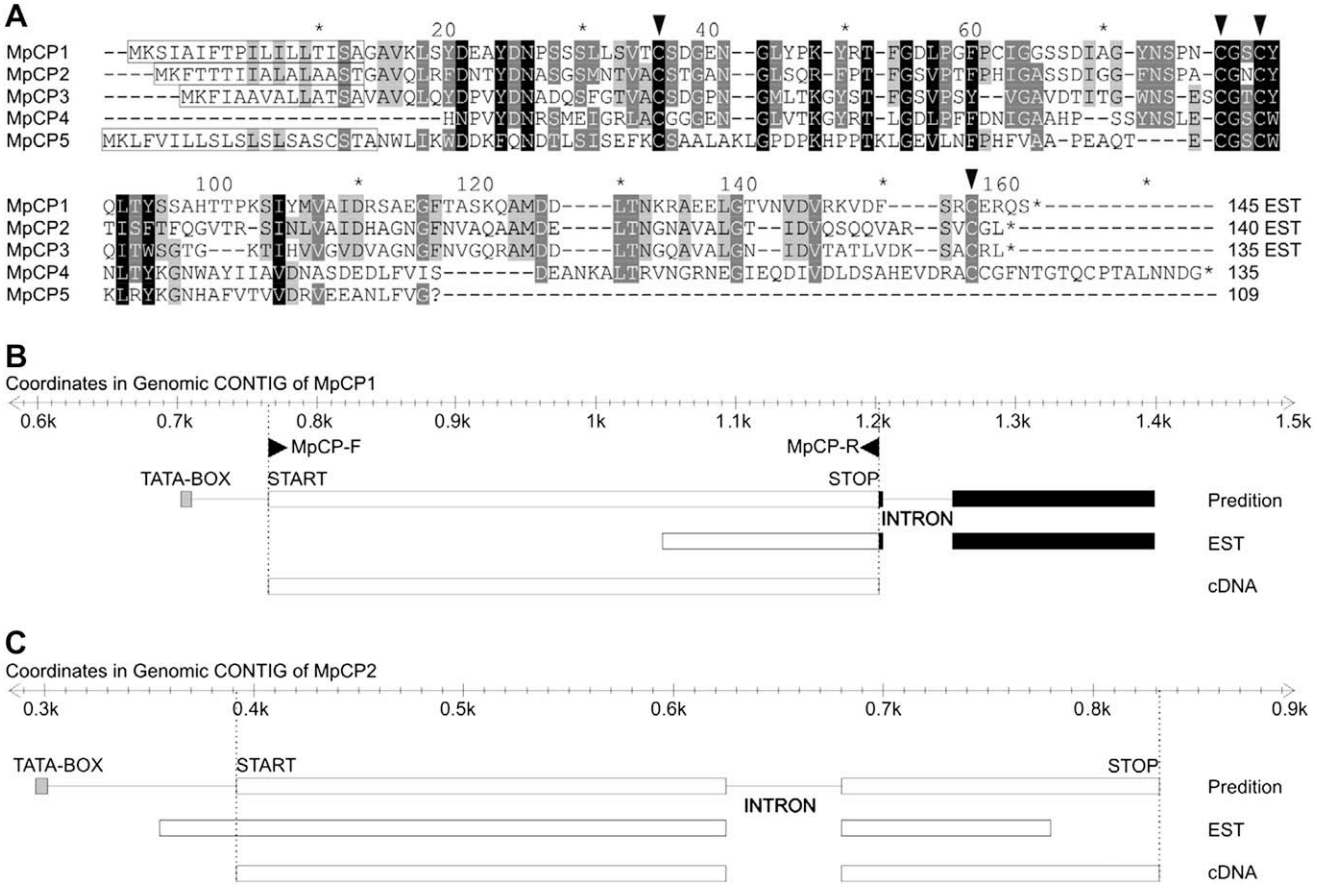

Fig 1 – MpCPs present in the *Moniliophthora perniciosa* genome. (A) Alignment of *M. perniciosa* protein sequences with significant similarity to CP family members. Background colour indicates the conservation level of each amino acid position (black, 100 % conserved; dark grey, more than 80 % conserved; pale grey, more than 60 % conserved). Solid rectangles indicate the signal peptides. Black arrows show the conserved cysteine residues. EST after the sequence indicates that this sequence has a correspondent EST in our data bank. (B) Organization of the gene *MpCP1* in the genome. Prediction (Genezilla) of the gene with the possible TATA box sequence (grey), start and stop codons, 3′-UTR (black) and an intron. MpCP1 EST and expressed sequence (white). Primers used for cloning are represented by the dark triangles. (C) Organization of the gene *MpCP2* in the genome. Prediction (Genezilla) of the gene with the possible TATA box sequence (grey), start and stop codons, 3′-UTR (black) and an intron. MpCP2 EST and expressed sequence (white).

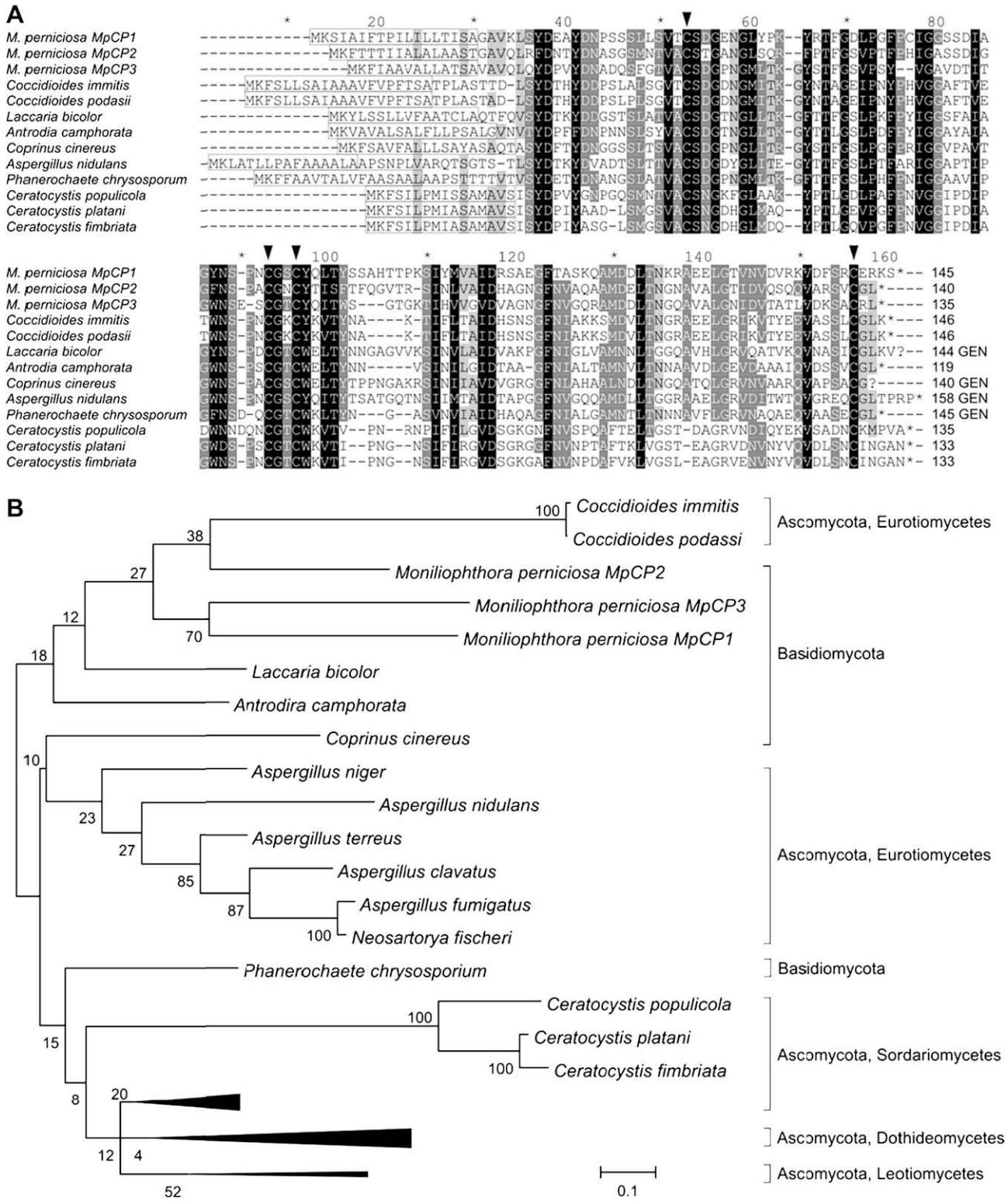

Fig 2 – Sequence alignment and phylogenetic analysis of CP members. (A) Alignment of CP protein sequences from fungal species used in the phylogenetic analysis. Background colour indicates the conservation level of each amino acid position (black, 100 % conserved; dark grey, more than 80 % conserved; pale grey, more than 60 % conserved). Solid rectangles indicate the signal peptides. Black arrows show the conserved cysteine residues. GEN after each sequence shows that it was obtained only from genome sequences. (B) Phylogenetic tree of CP member proteins. The root was placed between ascomycetes and basidiomycetes (without the *M. perniciosa* sequences). BS numbers are shown over the branches. At the bottom of the tree, sequences of some ascomycetes presenting low BSs were grouped together according to the taxonomic order and represented by dark triangles.

of introns in both genes. *MpCP1* presented an intron outside the coding region. This intron is located at the 3′ UTR, just after the stop codon (Fig 1B). *MpCP2* presented an intron inside the coding region (Fig 1C).

A sequence alignment of the mature protein of the three complete genes from *M. perniciosa* with selected members of the CP family is shown in Fig 2A. These CP genes from *M. perniciosa* code for small proteins of around 13 kDa. This alignment showed a high degree of similarity among all the members of the family. The similarity is higher on the first half of the mature protein, around 80 amino acids. Another characteristic of this family is the presence of four conserved cysteine residues (Fig 2A, black arrows). These residues are present in all the MpCPs.

Phylogenetic analysis of MpCP1

A phylogenetic analysis was performed using the members of the CP family from the organisms listed in Table 1; the phylogenetic tree is shown in Fig 2B. The result did not reflect the phylogenetic relationship among the fungal species, being unable to discriminate the different classes (Fig 2B).

Analysis of MpCP1 ex planta

We selected MpCP1 for further studies due to the fact that, among all the sequences of CPs from *M. perniciosa*, it presents the highest similarity with CP from *Ceratocystis fimbriata*, for which necrosis-inducing ability has been reported (Carresi *et al.* 2006; Pazzagli *et al.* 1999, 2006).

The results of the semi-quantitative PCR are shown in Fig 3. After 25 cycles there is already a detectable signal of MpCP1 in the biotrophic-like mycelia, as can be seen by the small black dot. Only after 30 cycles MpCP1 begins to be detectable using RNA from the saprotrophic mycelia, when the signal is very evident in the biotrophic-like mycelia. At cycle 35 the signal became saturated. These results showed that MpCP1 is more expressed in biotrophic-like than in saprotrophic mycelia.

Cloning, expression and purification of MpCP1

The gene coding for MpCP1 without the signal peptide was cloned into the pET28a(+) expression vector. The protein expressed in the soluble form in *Escherichia coli* BL21 (DE3) pT-Trx cells. The purified protein was observed as a single

Table 1 – Fungi that present CP sequences in their genome used in this study

Organism	Taxonomy	Accession no.	e-Value	Pathogenicity
Antrodia camphorata	*Basidiomycota: Agaricomycotina: Agaricomycetes*	GI:47156029	3e-16	Non
Coprinus cinereus			Local BLAST	Non
Laccaria bicolor			Local BLAST	Non
Moniliophthora perniciosa MpCP1		EU250339		Plant
M. perniciosa MpCP2		EU250341		Plant
M. perniciosa MpCP3		EU250343		Plant
Phanerochaete chrysosporium			Local BLAST	Non
Aspergillus clavatus	*Ascomycota: Pezizomycotina: Eurotiomycetes*	GI:121715869	7e-16	Animal
A. fumigatus		GI:71001827	1e-26	Animal
A. nidulans		GI:67540205	3e-28	Animal
A. niger		GI:145231638	1e-25	Plant
A. terreus		GI:115384119	3e-28	Animal
Coccidioides immitis		GI:119193607	9e-27	Animal
C. posadasii		GI:25528648	6e-26	Animal
Neosartorya fischeri		GI:119481376	6e-26	Non[a]
Ceratocystis fimbriata	*Ascomycota: Pezizomycotina: Sordariomycetes*	GI:121624696	2e-13	Plant
C. platani		GI:121624715	2e-12	Plant
C. populicola		GI:121624693	3e-11	Plant
Chaetomium globosum		GI:116198414	1e-19	Non[a]
Gibberella pulicaris		GI:56181486	2e-10	Plant
G. zeae		GI:46137392	7e-12	Plant
G. zeae		GI:46139380	3e-23	Plant
Magnaporthe grisea		GI:39940863	1e-15	Plant
Neurospora crassa		GI:85091040	1e-14	Non
Trichoderma atroviride		GI:118402803	1e-04	Non
Hypocrea atroviridis (T. atroviridis)		GI:92112037	4e-12	Non
T. viride		GI:118402815	4e-12	Non
Hypocrea virens (T. virens)		GI:73672052	6e-13	Non
H. virens (T. virens)		GI:93009003	1e-14	Non
Cochliobolus lunatus	*Ascomycota: Pezizomycotina: Dothideomycetes*	GI:37003430	6e-27	Plant
Phaeosphaeria nodorum		GI:3329508	2e-20	Plant
Leptosphaeria maculans		GI:27085248	7e-19	Plant
Botrytis cinerea	*Ascomycota: Pezizomycotina: Leotiomycetes*	GI:5828302	8e-16	Plant

a Organism rarely pathogenic to animals.

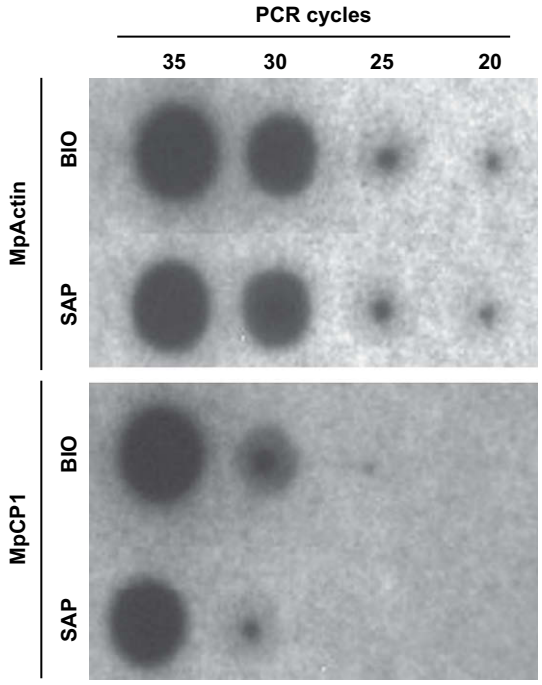

Fig 3 – Analysis of the expression of the gene *MpCP1* in the biotrophic-like and the necrotrophic phases of the life cycle of *Moniliophthora perniciosa*. Dot-blot analysis of the quantitative PCR of the expression of the *MpActin* gene (above) used to normalize the data, and of the *MpCP1* gene, below.

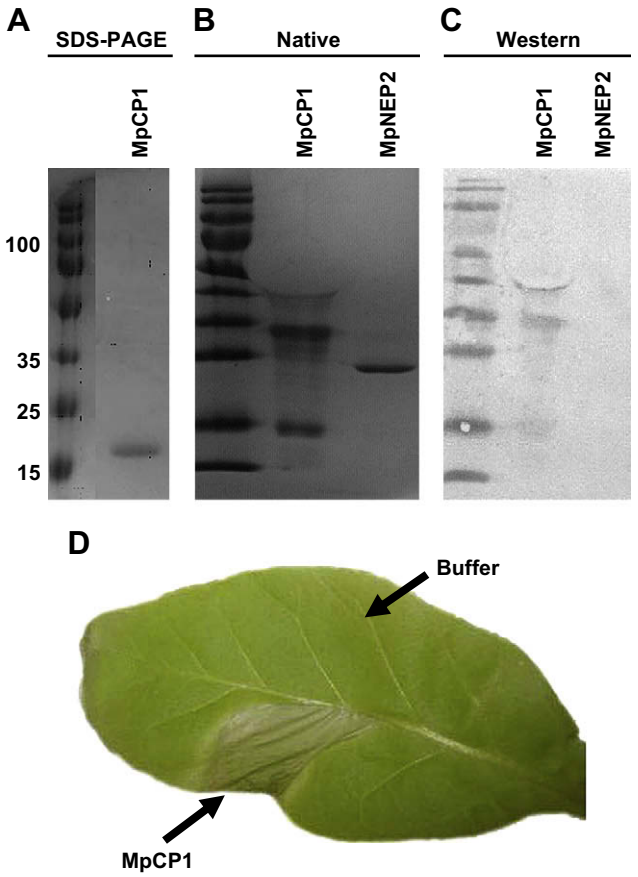

Fig 4 – Physical properties of MpCP1. (A) SDS-PAGE, showing the purified MpCP1 with expected size (17.2 kDa). (B) Native PAGE of MpCP1 and MpNEP2 (without His-tag). (C) Western-blot analysis of the native PAGE using an anti-His-tag antibody. (D) Necrosis induced by MpCP1 in tobacco leaves.

band of the expected molecular weight for the monomer of MpCP1 with the His-tag provided by the expression vector (17.2 kDa) by SDS-PAGE (Fig 4A). An electrophoresis of the purified protein under non-denaturing conditions showed the presence of several bands (Fig 4B). This indicated the presence of different oligomeric states of the recombinant protein. In order to confirm that all the bands corresponded to MpCP1 we carried out a Western blot using an anti-His-tag antibody and all the bands were positive (Fig 4C).

Further analysis of the oligomeric state using DLS showed that the particles present in solution had a radius of gyration of 3.3 nm. This radius of gyration corresponded to a particle with a molecular weight of 57 kDa. These data suggested the presence of a trimer in solution. Preliminary data obtained using small angle X-ray scattering (SAXS) indicates the presence of an elongated dimer in solution that will account for the molecular weight obtained in the DLS experiments (data not shown).

Necrosis-inducing effect of MpCP1

Tobacco and cacao leaves were treated with MpCP1 to see whether the protein was able to induce any necrotic effect. When the protein was injected into tobacco leaves there was an evident effect after 24 h. The affected part of the leaves became thinner, more transparent, and wrinkled easily (Fig 4D). This effect was reproduced every time the experiment was repeated. The effect of MpCP1 on the cacao leaves can be seen in Fig 5C when they were dipped into the protein solution. The leaves became brownish, indicative of necrosis. The necrosis effect was extensive, began to appear after 5 d, and affected most of the leaf in around 12 d.

There were differences in the extension of the effect of MpCP1 on the two plants. In order to see whether the different methods used for the treatment of the plants had any effect, tobacco leaves were also dipped in the protein solution. The leaves treated this way were completely affected by the necrosis. The leaves became thin and almost transparent. It was very difficult to handle them because they broke up easily upon handling. This was an evidence of the ability of the protein to move inside the leaves, most likely through the vascular system.

Synergistic effect of MpCP1 and MpNEP2

During the infection of the cacao tree MpCP1 is not the only necrosis factor expressed by the fungus, there are at least two MpNEPs being expressed at the same time. We were curious to see the combined effect of MpCP1 with one of

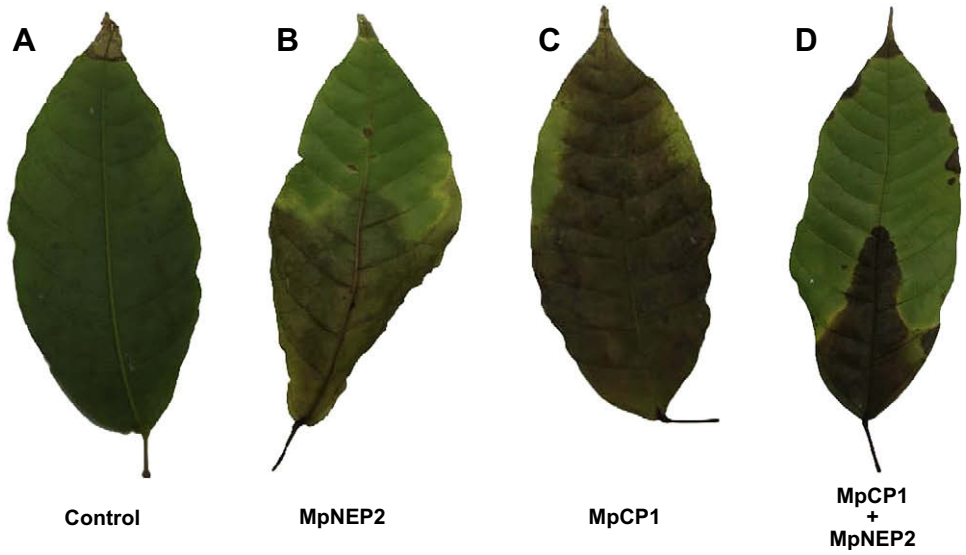

Fig 5 – Effect of proteins MpCP1 and MpNEP2 on cacao leaves. (A) Control leaf treated with buffer. (B) Leaf treated with MpNEP2. (C) Leaf treated with MpCP1. (D) Leaf treated with a mixture of MpCP1 and MpNEP2. Pictures were taken 15 d after the beginning of the treatment.

the MpNEPs. For this purpose we dipped cacao leaves in a protein solution containing both proteins. The effect of the treatment is shown in Fig 5D. The only effect of the treatment on the control leaves dipped in buffer was that the tips dried out. Both proteins produced different effects; MpCP1 affected most of the leaf, whereas the effect of MpNEP2 was limited to the lower part of the leaf. Also, the leaf treated with MpCP1 presented a more intense brown colour. The combined effect of both proteins was quite distinct from those observed for the individual proteins. The necrosis was more limited, present only at the lower part of the leaf, and was more intense. Also, the edge of the necrosis was very well defined, in contrast to the diffuse edge observed for the individual proteins. Another distinctive effect is the presence of localized dry areas at the edge on the upper part of the leaf.

Effect of the heat treatment on the activity of MpCP1

To investigate the recovery of necrotic activity after thermal unfolding, we placed MpCP1 in a boiling water-bath for 30 min and then inoculated the tobacco leaves with the heat-treated protein. The protein was able to produce necrosis in the same way as the non-treated protein (Figs 4D and 6A). As a control for the heating treatment we used the necrosis and ethylene-inducing protein 2 (MpNEP2) from *Moniliophthora perniciosa*, which showed the same behaviour (García *et al.* 2007). Close inspection of the necrotic areas produced by MpCP1 and MpNEP2 showed clear differences (Fig 6A–C). The necrotic area produced by MpNEP2 was circumscribed to the inoculation region without crossing the lateral ribbings (Fig 6B), whereas the necrotic area caused by MpCP1 progressed beyond the ribbings (Fig 6C). These experiments were performed several times, and the results obtained were similar. These results suggest differences in the mechanism by which both proteins cause cell death.

Discussion

Normally, mature cacao leaves do not suffer necrosis with aging. They become lightly yellowish and fall off. On the soil, these leaves become brown, but retain their firmness till complete degradation. However, it can be observed that when a cacao branch is partially broken, so that the xylem is interrupted, the leaves of that branch are unable to become mature and do not fall. They keep the integrity and turn brown still attached to the branch. We interpret this fact with the existence of mobile factors, such as hormones, that are transported to the leaves in order to carry out the abscission process. The interruption of xylem hampers the passage of these 'abscission factors', so that the leaves turn brown, but do not fall.

WBD is characterized by two distinct phases that can be well observed in the branches of the brooms produced by the infection (Scarpari *et al.* 2005). In the biotrophic phase of the fungus, when the fungal cells are present at low density and occupy the apoplast, the infected tissue becomes hyperplasic and hypertrophic. Clearly, this branch is converted into a nutritional sink and the fungus seems to work to avoid the premature death of the tissue, mobilizing as much of the nutrients as possible to that area. It is interesting to note that small brooms are sometimes produced in resistant plants (not shown), reinforcing the idea that the consequences of the disease can be diminished if less of the plant's nutrients are drained to the infected area. After some weeks, the infected tissue seems to enter into apoptosis and the fungus goes into the saprophytic phase. The density of the fungus increases enormously inside the infected tissue, and the branch becomes completely necrotic (Scarpari *et al.* 2005; Ceita *et al.* 2007). At first sight, cacao branches infected by *Moniliophthora perniciosa* look like a branch that has had the xylem interrupted, i.e., the leaves turn brown attached to the branch. However, the nature of the two physiological process causing the browning of the leaves are very diverse. In the infected branch, the internal content appears to

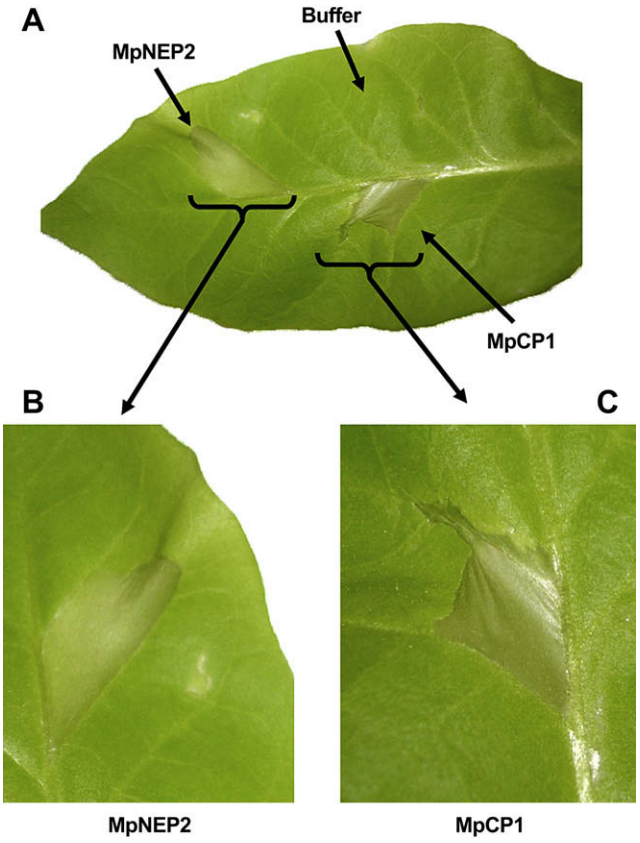

Fig 6 – Effect of temperature on the structure and necrotic activity of MpCP1. (A) Effect of thermally treated MpCP1 and MpNEP2 (30 min in boiling water-bath before inoculation) on a tobacco leaf. (B) Detail of the part of the leaf treated with MpNEP2. (C) Detail of the part of the leaf treated with MpCP1.

be drained and the leaves become very thin and transparent, looking like a papyrus (Silva *et al.* 2002). Nevertheless, the saprophytic mycelia, which is easily detected in the necrotic stem, do not seem to be present in the necrotic leaves. It appears as if the leaf content has been transferred to the infected stem; therefore, what causes the necrosis in the cacao tissue, in particular the leaves, during the saprophytic phase of WBD?

Initial experiments showed that supernatants of *M. perniciosa* liquid cultures have the ability to cause quick necrosis in tobacco and cacao leaves and the apical meristem. Therefore, we inspected the genome of the fungus searching for possible genes encoding necrosis-inducing factors. Recently, we described and characterized such genes belonging to the NLP family (García *et al.* 2007). However, we observed that the necrosis caused by these factors, though intensive, was markedly different from that caused by the inoculation of the supernatants, mainly in the appearance and time course of necrosis. This observation was an indication of the existence of additional factors, besides NLPs, that might be involved in the necrosis process.

A new search of the genome identified the presence of at least five genes showing high similarity with the CP genes. In *Ceratocystis fimbriata* these genes encode a phytotoxin able to cause necrosis in plants (Carresi *et al.* 2006; Pazzagli *et al.* 1999; 2006). Therefore, we selected CP genes as potential candidates that contribute to the necrosis process in cacao. Two of the *M. perniciosa* CPs (MpCPs) were also found in the EST data bank, indicating that they are expressed genes. Interestingly, *MpCP1* presented an intron in the 3′ UTR (Fig 1B), an uncommon feature found in only 1.23 % of the fungal genes analysed (Pesole *et al.* 2001).

The presence of several copies of MpCPs suggests the importance of the protein coded by these genes, but this does not represent a special feature of *M. perniciosa*. This class of genes seems to be organized in families of several fungal species, such as *C. fimbriata, C. platani, Coccidioides posadasii, Coprinopsis cinerea*, *Phaeosphaeria nodorum, Neosartorya fischeri*. *Gibberella zeae, Trichoderma atroviride*, and *T. viride* also present several copies that belong to the CP family (Seidl *et al.* 2006). This fact would indicate that the expansion of this gene was an ancient event, but this possibility could not be confirmed by the phylogenetic analysis (Fig 2B). On the contrary, this analysis was not able even to discriminate among the different fungi classes. This could be an indication of an intense horizontal transfer between species, but the result is more likely a consequence of the short sequence size, which hampers the phylogenetic analysis.

NLPs are present in bacteria (Mattinen *et al.* 2004), *Ascomycota* (Bailey 1995), and oomycetes (Fellbrich *et al.* 2002), whereas CPs have been found only in *Ascomycota* (Pazzagli *et al.* 1999) and *Basidiomycota* (Local Blast). Several *Ascomycota* (pathogenic and non-pathogenic) present both families of proteins in their genome, such as *Magnaporthe grisea* (Dean *et al.* 2005; Jeong *et al.* 2007), *G. zeae* (Gijzen & Nurnberger 2006; Seidl *et al.* 2006), *Neurospora crassa* (Zhu *et al.* 2001; Galagan *et al.* 2003), *Aspergillus nidulans* (Gijzen & Nurnberger 2006; Seidl *et al.* 2006), and *A. fumigatus* (Wilson *et al.* 2002; Gijzen & Nurnberger 2006). The genome of *M. perniciosa* is the first member of the *Basidiomycota* that presents genes of both NLPs and CPs families. The fact that both types of necrosis-inducing proteins are present in a non-pathogenic organism might indicate that these proteins could be involved in other types of functions besides the induction of necrosis. Further investigation with non-pathogenic organisms is needed to better understand the roles of these proteins.

We selected MpCP1 to study the possible necrosis-inducing activity of these proteins. The gene was successfully cloned in an *Escherichia coli* expression vector, expressed in the soluble form, and we were able to purify the recombinant protein by the IMAC technique. The recombinant protein was able to induce necrosis in plant tissues — tobacco and cacao — an effect similar to that observed with the corresponding protein from *C. fimbriata*. Moreover, the extensive effect exerted by the protein solution when only the petiole of the leaf was dipped in it indicates that this type of protein is transported through the vascular system of the plant, being, therefore, able to affect parts of the plant far from the point of entrance.

Indeed, it is highly probable that this situation may happen in WBD. The fungus in the biotrophic phase is located in the apoplastic space, which is connected with the xylem. The CPs are secreted proteins; all of the known members present a secretion signal peptide. Moreover, we observed that MpCP1 and MpCP2 were expressed in both biotrophic-like and saprophytic mycelia. Therefore, they may be secreted by

the fungal cells, reach the xylem, and be transported upstream to other parts of the infected branch, such as the leaves.

It is possible that the host organism has a set of defence mechanisms in order to deal with proteins secreted by infectious agents to avoid their propagation. Some of these mechanisms are proteases that degrade foreign proteinaceous elicitors and toxins secreted by the pathogen. A class of such proteins was found to be expressed in cacao tissues infected by *M. perniciosa* (Gesteira *et al.* 2007). Moreover, the infected cacao tissue may be subject to intense oxidative stress caused by peroxides (Scarpari *et al.* 2005; do Rio *et al.* 2008). Therefore, the pathogens have developed strategies to circumvent these defence mechanisms; one of which is to increase the stability and resistance of the secreted proteins against such defence mechanisms. We could clearly observe this feature in MpCP1, which is able to maintain its activity even after being placed in a boiling water-bath for 30 min. This characteristic was also present in MpNEP2 (García *et al.* 2007), that was also able to induce necrosis after the heat treatment. These data suggest that these necrosis factors that belong to two different classes, i.e. CP and NLP, are able to preserve their activity in unfavourable environments, such as those encountered by the fungus in the apoplast. In order to do this, the whole structure, or at least maintain the active part of protein, has to remain unchanged in hostile conditions, to trigger necrosis and thereby enable the fungus to succeed in the invasion of the host.

As mentioned before, MpCP1 is expressed in greater amounts in the biotrophic-like phase of the life cycle (Fig 3). Real-time PCR experiments showed that MpCP2 is also expressed in greater amounts in the biotrophic-like hyphae (data not shown). The density of hyphae at this stage *in planta* is very low in the infected tissue (Ceita *et al.* 2007). The decreased expression observed in the necrotrophic phase might be compensated for by the increased amount of mycelia present in the infected tissue of the plant, maintaining or increasing the concentration of this protein in the infected tissues. This situation is similar to that of the MpNEP2, which is primarily expressed during the biotrophic-like phase (García *et al.* 2007). The differential expression of the protein being present at the biotrophic phase where no necrosis is evident might indicate an additional role for this protein.

We have observed a difference in the kinetics of necrosis caused by MpCP1 in comparison with MpNEPs. Whereas in the latter the necrosis is proportional to the protein concentration inoculated, with MpCP1 it seems that the protein must reach a given concentration to cause necrosis (not shown). At lower levels, no necrosis is detected. We are currently analysing whether these sub-necrosis-inducing concentrations could be eliciting plant defenses. It has been recently reported that *M. perniciosa* produces salicylic acid (Kilaru *et al.* 2007), a compound that helps in plant defence. Thus, it might be possible that during the biotrophic-like phase, the fungus produces low amounts of substances capable of inducing plant defenses and thereby, increases the survival time of the green broom, and the amount of nutrients drained to this area.

At the late stages of the infection there is generalized necrosis, but before this, there is a transition phase, which starts with necrosis at the edge of the leaves of the infected tissue. We believe that this necrosis could be caused by low amounts of fungal necrosis-inducing proteins travelling through the vascular system of the plant. This initial necrosis rapidly increases, causing a chain reaction. We have detected the conversion of amino acids to amides (Scarpari *et al.* 2005), a signal of remobilization; i.e. it appears that nutrients are being brought back to the infected branch. This process may increase the availability of nutrients in the apoplast, and this may trigger the fungi to convert from the biotrophic to the necrotrophic phase. We have mimicked this situation *ex planta*, observing that the low nutrient availability maintains the biotrophic-like hyphae (Meinhardt *et al.* 2006). Considering this model, it seems important for the fungi to avoid nutrients escaping from the infected area. In order to do that, one possibility would be to damage the vascular system thus hampering the return of the nutrients to non-infected parts of the plant.

Visual inspection of the necrotic areas caused by MpCP1 showed differences when compared with the necrotic areas caused by MpNEP2 (Fig 5). *In vivo* both types of proteins would act together on the infected tissue. This led us to carry out experiments with both types of proteins acting together. We observed an enhancement of the necrosis produced by both proteins compared with that produced by each acting separately (Fig 5). This effect might be closer to what happens *in vivo* at the site of infection. The combined effect of both protein types exerts a more intense necrosis and, more importantly, an effect limited to the base of the leaf. This intense necrosis might destroy the tissue organization and the vascular system of the plant leaving no way for the proteins to travel to more distant parts of the plant. The drying of the edge of the leaves is a consequence of the disruption of the vascular system resulting in a loss of the transport system, thus, generating a hydric stress in the leaf. This situation might be an advantage to this pathogen. The initial infection by *M. perniciosa* generates extra plant tissue, the green broom, with the plant supplying all the nutrients. When the necrotrophic phase begins the plant may attempt to recover these nutrients that were diverted by the action of the fungus. At this time, the necrosis-inducing proteins destroy the vascular system of the green broom restraining the recovery of the nutrients back to non-infected parts of the plant.

In summary, we report here the presence of at least five copies of CP-like genes in the *Basidiomycota M. perniciosa*. At least three of these genes are expressed. MpCP1 and MpCP2 are more expressed in the biotrophic-like phase, and MpCP1 is able to induce necrosis *in vitro*. It is probable that MpCPs play a role in WBD, as the genes are expressed *in planta*. We show a synergistic effect between two necrosis factors, MpCP1 and MpNEP2, which might be relevant for the progress of the infection. We are currently studying the structure of MpCP1, the necrosis mechanisms, and developing protocols to knock out these genes in *M. perniciosa*.

Acknowledgements

This research was supported by the Brazilian agencies CNPq and Capes (research fellowship to G. Zaparoli and O.G. Cabrera). We thank Derivaldo Evangelista de Jesus for helpful

comments and field observations on WBD, and Joan Barau for the real-time PCR experiments.

REFERENCES

Allen RL, Bittner-Eddy PD, Grenville-Briggs LJ, Meitz JC, Rehmany AP, Rose LE, Beynon JL, 2004. Host–parasite coevolutionary conflict between *Arabidopsis* and downy mildew. *Science* **306**: 1957–1960.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.

Andebrhan T, 1987. Rain-water as a factor in the dissemination of basidiospores of *Crinipellis perniciosa* on cocoa seedlings. In: Rudgard SA, Maddison AC, Anderbrhan T (eds), *Disease Management in Cocoa. Comparative Epidemiology of Witches' Broom*. Chapman & Hall, London, pp. 157–164.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith AJ, Struhl K, 1998. *Current Protocols in Molecular Biology*. Wiley & Sons, New York.

Bailey BA, 1995. Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*. *Phytopathology* **85**: 1250–1255.

Bendtsen JD, Nielsen H, von Heijne G, Brunak S, 2004. Improved prediction of signal peptides: Signalp 3.0. *Journal of Molecular Biology* **340**: 783–795.

Boddi S, Comparini C, Calamassi R, Pazzagli L, Cappugi G, Scala A, 2004. Cerato-platanin protein is located in the cell walls of ascospores, conidia and hyphae of *Ceratocystis fimbriata f. sp. platani*. *FEMS Microbiology Letters* **233**: 341–346.

Carresi L, Pantera B, Zoppi C, Cappugi G, Oliveira AL, Pertinhez TA, Spisni A, Scala A, Pazzagli L, 2006. Cerato-platanin, a phytotoxic protein from *Ceratocystis fimbriata*: expression in *Pichia pastoris*, purification and characterization. *Protein Expression and Purification* **49**: 159–167.

Ceita GO, Macedo JN, Santos T, Alemanno L, Gesteira AS, Micheli F, Mariano A, Gramacho KP, Silva DC, Meinhardt L, Mazzafera P, Pereira GAG, Cascardo JCM, 2007. Involvement of calcium oxalate degradation during programmed cell death in *Theobroma cacao* tissues triggered by the hemibiotrophic fungus *Moniliophthora perniciosa*. *Plant Science* **173**: 106–117.

Dean RA, Talbot NJ, Ebbole DJ, Farman ML, Mitchell TK, Orbach MJ, Thon M, Kulkarni R, Xu JR, Pan H, Read ND, Lee YH, Carbone I, Brown D, Oh YY, Donofrio N, Jeong JS, Soanes DM, Djonovic S, Kolomiets E, Rehmeyer C, Li W, Harding M, Kim S, Lebrun MH, Bohnert H, Coughlan S, Butler J, Calvo S, Ma LJ, Nicol R, Purcell S, Nusbaum C, Galagan JE, Birren BW, 2005. The genome sequence of the rice blast fungus *Magnaporthe grisea*. *Nature* **434**: 980–986.

Fairbanks G, Steck STL, Wallach DF, 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**: 2606–2617.

Fellbrich G, Romanski A, Varet A, Blume B, Brunner F, Engelhardt S, Felix G, Kemmerling B, Krzymowska M, Nurnberger T, 2002. NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and *Arabidopsis*. *The Plant Journal* **32**: 375–390.

Felsenstein J, 1985. Confidence-limits on phylogenies — an approach using the bootstrap. *Evolution* **39**: 783–791.

Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, FitzHugh W, Ma LJ, Smirnov S, Purcell S, Rehman B, Elkins T, Engels R, Wang S, Nielsen CB, Butler J, Endrizzi M, Qui D, Lanakiev P, Bell-Pedersen D, Nelson MA, Werner-Washburne M, Selitrennikoff CP, Kinsey JA, Braun EL, Zelter A, Schulte U, Kothe GO, Jedd G, Mewes W, Staben C, Marcotte E, Greenberg D, Roy A, Foley K, Naylor J, Stange-Thomann N, Barrett R, Gnerre S, Kamal M, Kamvysselis M, Mauceli E, Bielke C, Rudd S, Frishman D, Krystofova S, Rasmussen C, Metzenberg RL, Perkins DD, Kroken S, Cogoni C, Macino G, Catcheside D, Li W, Pratt RJ, Osmani SA, DeSouza CP, Glass L, Orbach MJ, Berglund JA, Voelker R, Yarden O, Plamann M, Seiler S, Dunlap J, Radford A, Aramayo R, Natvig DO, Alex LA, Mannhaupt G, Ebbole DJ, Freitag M, Paulsen I, Sachs MS, Lander ES, Nusbaum C, Birren B, 2003. The genome sequence of the filamentous fungus *Neurospora crassa*. *Nature* **422**: 859–868.

García O, Macedo JA, Tiburcio R, Zaparoli G, Rincones J, Bittencourt LM, Ceita GO, Micheli F, Gesteira A, Mariano AC, Schiavinato MA, Medrano FJ, Meinhardt LW, Pereira GAG, Cascardo JC, 2007. Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*. *Mycological Research* **111**: 443–455.

Gesteira AS, Micheli F, Carels N, Da Silva AC, Gramacho KP, Schuster I, Macedo JN, Pereira GAG, Cascardo JC, 2007. Comparative analysis of expressed genes from cacao meristems infected by *Moniliophthora perniciosa*. *Annals of Botany* (London) **100**: 129–140.

Gijzen M, Nurnberger T, 2006. Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa. *Phytochemistry* **67**: 1800–1807.

Guindon S, Gascuel O, 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696–704.

Hall N, Keon JPR, Hargreaves JA, 1999. A homologue of a gene implicated in the virulence of human fungal diseases is present in a plant fungal pathogen and is expressed during infection. *Physiological and Molecular Plant Pathology* **55**: 69–73.

Higgins DG, Thompson JD, Gibson TJ, 1996. Using CLUSTAL for multiple sequence alignments. *Methods in Enzymology* **266**: 383–402.

Jeong JS, Mitchell TK, Dean RA, 2007. The *Magnaporthe grisea* snodprot1 homolog, MSP1, is required for virulence. *FEMS Microbiology Letters* **273**: 157–165.

Kamoun S, 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology* **44**: 41–60.

Kilaru A, Bailey BA, Hasenstein KH, 2007. *Moniliophthora perniciosa* produces hormones and alters endogenous auxin and salicylic acid in infected cocoa leaves. *FEMS Microbiology Letters* **274**: 238–244.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Majoros WH, Pertea M, Salzberg SL, 2004. TigrScan and GlimmerHMM: two open source ab initio eukaryotic gene-finders. *Bioinformatics* **20**: 2878–2879.

Mattinen L, Tshuikina M, Mae A, Pirhonen M, 2004. Identification and characterization of Nip, necrosis-inducing virulence protein of *Erwinia carotovora* subsp.*carotovora*. *Molecular Plant–Microbe Interactions* **17**: 1366–1375.

McGinnis S, Madden TL, 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. *Nucleic Acids Research* **32**: W20–W25.

Meinhardt LW, Bellato CM, Rincones J, Azevedo RA, Cascardo JC, Pereira GAG, 2006. In vitro production of biotrophic-like cultures of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. *Current Microbiology* **52**: 191–196.

Nylander JAA, 2004. *MrAIC.pl* Program distributed by the author. Evolutionary Biology Centre, Uppsala University, Sweden.

ÓConnell RJ, Panstruga R, 2006. Tête à tête inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. *New Phytologist* **171**: 699–718.

Orchard J, Collin HA, Hardwick K, Isaac S, 1994. Changes in morphology and measurement of cytokinin levels during the

development of witches-brooms on cocoa. *Plant Pathology* **43**: 65–72.

Pan SC, Cole GT, 1995. Molecular and biochemical characterization of a *Coccidioides immitis*-specific antigen. *Infection and Immunity* **63**: 3994–4002.

Pazzagli L, Cappugi G, Manao G, Camici G, Santini A, Scala A, 1999. Purification, characterization, and amino acid sequence of cerato-platanin, a new phytotoxic protein from *Ceratocystis fimbriata* f. sp. *platani*. *Journal of Biological Chemistry* **274**: 24959–24964.

Pazzagli L, Pantera B, Carresi L, Zoppi C, Pertinhez TA, Spisni A, Tegli S, Scala A, Cappugi G, 2006. Cerato-platanin, the first member of a new fungal protein family: cloning, expression, and characterization. *Cell Biochemistry and Biophysics* **44**: 512–521.

Pesole G, Mignone F, Gissi C, Grillo G, Licciulli F, Liuni S, 2001. Structural and functional features of eukaryotic mRNA untranslated regions. *Gene* **276**: 73–81.

Purdy LH, Schmidt RA, 1996. Status of cacao witches' broom: biology, epidemiology, and management. *Annual Review of Phytopathology* **34**: 573–594.

Qutob D, Kemmerling B, Brunner F, Kufner I, Engelhardt S, Gust AA, Luberacki B, Seitz HU, Stahl D, Rauhut T, Glawischnig E, Schween G, Lacombe B, Watanabe N, Lam E, Schlichting R, Scheel D, Nau K, Dodt G, Hubert D, Gijzen M, Nurnberger T, 2006. Phytotoxicity and innate immune responses induced by Nep1-like proteins. *Plant Cell* **18**: 3721–3744.

do Rio M, de Oliveira B, Tomazella DT, da Silva J, Pereira GAG, 2008. Production of calcium oxalate crystals by the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom disease of cacao. *Current Microbiology* **56**: 363–370.

Rementeria A, Lopez-Molina N, Ludwig A, Vivanco AB, Bikandi J, Ponton J, Garaizar J, 2005. Genes and molecules involved in *Aspergillus fumigatus* virulence. *Revista Iberoamericana de Micologia* **22**: 1–23.

Rincones J, Meinhardt LW, Vidal BC, Pereira GAG, 2003. Electrophoretic karyotype analysis of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. *Mycological Research* **107**: 452–458.

Sambrook J, Russel DW, 2001. *Molecular Cloning. A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, New York.

Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AW, Schiavinato MA, Cascardo JC, Pereira GAG, 2005. Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. *Journal of Experimental Botany* **56**: 865–877.

Seidl V, Marchetti M, Schandl R, Allmaier G, Kubicek CP, 2006. Epl1, the major secreted protein of *Hypocrea atroviridis* on glucose, is a member of a strongly conserved protein family comprising plant defense response elicitors. *FEMS Microbiology Letters* **273**: 4346–4359.

Silva SDVM, Luz E, de Almeida O, Gramacho KP, Bezerra J, 2002. Redescrição da sintomatologia causada por *Crinipellis perniciosa* no cacaueiro. *Agrotrópica* **14**: 1–24.

Silva SDVM, Matsuoka K, 1999. Histologia da interação *Crinipellis perniciosa* em cacaueiros suscetível e resistente a vassoura-de-bruxa. *Fitopatologia Brasileira* **24**: 54–59.

Skinner W, Keon J, Hargreaves J, 2001. Gene information for fungal plant pathogens from expressed sequences. *Current Opinion in Microbiology* **4**: 381–386.

Specht CA, DiRusso CC, Novotny CP, Ullrich RC, 1982. A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. *Analytical Biochemistry* **119**: 158–163.

Veit S, Worle JM, Nurnberger T, Koch W, Seitz HU, 2001. A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defense responses in carrot, *Arabidopsis*, and tobacco. *Plant Physiology* **127**: 832–841.

Wilson AW, Desjardin DE, 2005. Phylogenetic relationships in the gymnopoid and marasmioid fungi (basidiomycetes, euagarics clade). *Mycologia* **97**: 667–679.

Wilson LM, Idnurm A, Howlett BJ, 2002. Characterization of a gene (sp1) encoding a secreted protein from *Leptosphaeria maculans*, the blackleg pathogen of *Brassica napus*. *Molecular Plant Pathology* **3**: 487–493.

Yasukawa T, Kanei-Ishii C, Maekawa T, Fujimoto J, Yamamoto T, Ishii S, 1995. Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin. *Journal of Biological Chemistry* **270**: 25328–25331.

Zhu H, Nowrousian M, Kupfer D, Colot HV, Berrocal-Tito G, Lai H, Bell-Pedersen D, Roe BA, Loros JJ, Dunlap JC, 2001. Analysis of expressed sequence tags from two starvation, time-of-day-specific libraries of *Neurospora crassa* reveals novel clock-controlled genes. *Genetics* **157**: 1057–1065.