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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

New proteins orthologous to cerato-platanin in various *Ceratocystis* species and the purification and characterization of cerato-populin from *Ceratocystis populicola*

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Abstract Natural variants of cerato-platanin (CP), a pathogen associated molecular pattern (PAMP) protein produced by *Ceratocystis platani* (the causal agent of the plane canker stain), have been found to be produced by other four species of the genus *Ceratocystis*, including five clones of *Ceratocystis fimbriata* isolated from different hosts. All these fungal strains were known to be pathogenic to plants with considerable importance in agriculture, forestry, and as ornamental plants. The putative premature proteins were deduced on the basis of the nucleotide sequence of genes orthologous to the *cp* gene of *C. platani*; the deduced premature proteins of *Ceratocystis populicola* and *Ceratocystis variospora* reduced the total identity of all the others from 87.3% to 60.3%. Cerato-populin (Pop1), the CP-orthologous protein produced by *C. populicola*, was purified and characterized. Pop1 was a well-structured α/β protein with a different percentage of the α-helix than CP, and it self-assembled in vitro in ordered aggregates. Moreover, Pop1 behaved as PAMP, since it stimulated poplar leaf tissues to activate defence responses able to reduce consistently the *C. populicola* growth.

Keywords Orthologous genes · *Populus* sp. · MALDI-TOF · Circular dichroism · PAMP activity · Induction of plant resistance

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Introduction

In the course of evolution, terrestrial plants have acquired the ability to detect the presence of potentially pathogenic microorganisms with which they come in contact; they do this by means of substances produced by those microbes, including several proteins. When plants recognize these microbe-/pathogen-associated molecular patterns (MAMPs/PAMPs), they activate a defence system that is highly effective against the most potential pathogens (Bent and Mackey 2007; Chisholm et al. 2006). In addition there is a second, more advanced line of defence, where proteins of the pathogen (AVR proteins) interact directly or indirectly with the proteins of plants (R proteins) according to the resistance mechanism described genetically by Flor (1971). In this case, plant defence reactions include several steps that can reduce the colonization of the pathogen, such as the production of reactive oxygen species, the signaling of MAP kinases, the overexpression of genes related to the

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defence, the hypersensitive cell necrosis, and the synthesis of phytoalexins (Glazebrook 2005). Plant pathogenic fungi can produce a lot of proteins without catalytic activity, which are involved in various aspects of parasitism and in the development of disease, such as the dissemination of fungi by vectors, the attachment of the fungus to the surface of plant organs, the expression of symptoms, and the elicitation of defence responses. These proteins were classified by Templeton et al. (1994) on the basis of various parameters, including the number of cysteines, their molecular weight, and role in the pathogenic process. Currently, on the basis of their primary sequence, seven noncatalytic fungal protein families have been identified, including the cerato-platanin family (PF07249), the class I hydrophobins (PF01185), the class II hydrophobins (PF06766), the elicitins (PF00964), the PcF family (PF09461), and the NIP-1 protein family (PF08995) (Finn et al. 2008). Some other fungal proteins for which a role in pathogenesis has been proposed contain a cysteine-rich domain that gives rise to the CFEM family (PF05730) (Kulkarni et al. 2003).

Cerato-platanin (CP), the founder member of the "cerato-platanin family," is a protein produced by the ascomycete fungus *Ceratocystis platani* (Walter) Engelbrecht et Harrington comb. et stat. nov. [formerly known as *Ceratocystis fimbriata* (Ell. and Halst.) Davidson f. sp. *platani* Walter] (Pazzagli et al. 1999). *C. platani* causes canker stain in *Platanus occidentalis*, *Platanus orientalis*, and their hybrid, *Platanus acerifolia* (Panconesi 1999; Engelbrecht et al. 2004; Ocasio-Morales et al. 2007). In the Mediterranean countries of Europe, where naturally resistant host clones are not known to exist, *C. platani* has infected and killed many centuries-old *P. acerifolia* and *P. orientalis* trees. CP is located in the fungal cell wall, is released rapidly in vitro, and elicits structural and physiological defence responses in plants, such as plasmolysis, cell death, and increases in the levels of phenolic substances and phytoalexins in both host and nonhost plants (Boddi et al. 2004; Scala et al. 2004; Bennici et al. 2005). CP-treated leaves also overexpress many genes that are involved in plant defence responses, and they inhibit *C. platani* growth (Fontana et al. 2008). This suggests that CP acts as a PAMP and that it could be one of the first eliciting patterns of *C. platani* that is recognized by plants. Given the CP ability to induce plant cell necrosis and death, Gressel et al. (2007) studied whether the transformation of some mycoherbicides with the *cp* gene might contribute to enhance their virulence. CP has been purified from the culture filtrate of *C. platani*, and has a molecular weight of 12.4 kDa. It consists of 120 amino acids, has a high percentage of hydrophobic amino acid residues (40%), contains four cysteins that form two disulfide bridges, Cys 20–57 and Cys 60–115, and it self-aggregates in vitro in accordance with a specific hierarchic aggregation model (Pazzagli et al. 1999; Sbrana et al. 2007). The coding sequence of the *cp* gene has been cloned in *Pichia pastoris*, and structural and functional characterization of the recombinant protein revealed no significant differences between the recombinant and the native protein (Carresi et al. 2006; Pazzagli et al. 2006). The relation between the structure of CP and its biological functions is in development since the three-dimensional (3D) solution structure of recombinant CP was defined (Oliveira et al. 2006, 2009). Other members of the CP family are also involved in microbe-host interactions. CP-family proteins produced by fungi that cause or alternatively control plant disease include: snodprot1, produced by *Phaerospheria nodorum* when this fungus infects wheat leaves (Sharen and Krupinski 1970); SP1, from *Leptosphaeria maculans,* the causal agent of blackleg in *Brassica napus* (Wilson et al. 2002); Sm1, a protein secreted by *Trichoderma virens* that elicits a defence reaction in plants, confirming the capacity of this biocontrol fungus to induce resistance (Djonovic et al. 2006, 2007); and MpCP1 from *Moniliophthora perniciosa* (Zaparoli et al. 2009). A major protein found in the secretome of the biocontrol fungal strain *Hypocrea atroviridis* P1 grown on glucose is also a member of the CP family (Seidl et al. 2006). Another member is MSP1, a protein that causes the virulence of *Magnaporthe grisea*, which mainly impairs growth in plants (Jeong et al. 2007), and another is a CP-homologue (accession no. TC26886) that is variously expressed by *Fusarium verticillioides* grown on maize-based media. Yet other CP family proteins affect human beings, such as the allergene Asp f13 from *Aspergillus fumigatus*, which causes an allergic reaction (Kurup et al. 2000), and the antigene CS, a serine proteinase produced while *Coccidioides immitis* is in its parasitic phase. This last fungus causes a respiratory disease in man (Pan and Cole 1995).

The aim of the present work was to study the natural variants of CP in various species and clones of the genus *Ceratocystis* that are virulent on hosts other than plane. For these fungal strains, we isolated the coding sequence of *cp*-orthologous genes, and we reported the protein sequences deduced therefrom. The CP-orthologous protein by *C. populicola*, showing high amino acid sequence differences, was purified from culture filtrates and biochemically and functionally characterized.

Materials and methods

Fungal material

C. platani strain CfAF100 was previously described by Pazzagli et al. (1999). *C. platani* strain Cf15 was isolated

by Dr. Panaghiotis Tsopelas from a canker-stain infected plane (*P. orientalis*) tree growing in Greece. *C. populicola* (strain no. 119.78), *C. cacaofunesta* (153.62), *C. variospora* (773.73), and *C. fimbriata* strains no. 740.70, 600.70, 146.53, 141.37, and 123.39 were provided by the Centraalbureau voor Schimmelcultures, The Netherlands. *Ophiostoma novo-ulmi* subsp. *americana* strain 182 was used in our previous works (Scala et al. 1997); this strain, together with the *C. platani* strains CfAF100 and Cf15, are deposited at the Mycotheca of the Department of Agricultural Biotechnology, Section of Plant Pathology, University of Florence. The host plants, and the source and geographical origin of the isolates used in the study, are shown in Electronic Supplementary Material-1 (ESM-1).

Single-spore-derived fungal colonies were routinely cultivated on potato dextrose agar (PDA, Sigma-Aldrich, St Louis, MO, USA) at 23°C in the dark; liquid cultures were grown in potato dextrose broth (PDB). For long-term storage, conidia collected from 3-day-old liquid shaken minicultures (3 ml) were resuspended in 20% (*v*/*v*) glycerol and stored at −70°C.

Fungal growth was determined after 10 days of growth in 100 ml conical flasks containing 20 ml PDB or, for *O. novo-ulmi*, in modified Takai medium as described by Scala et al. (1994). The biomass produced by the fungi was estimated by passing the mycelium through a Whatman filter and keeping at 90°C until a constant weight was reached.

Pathogenicity test of *Ceratocystis populicola*

The pathogenicity of *C. populicola* 119.78 was tested on 3-year-old white poplar (*Populus alba*, clone "Marte") trees. Trees were grown in 30-dm^3 pots and had a height of 2.0–2.5 m and a trunk diameter of about 2 cm. The pathogenicity tests were carried out on June 1, 2007 at the Laboratorio di Patologia Vegetale Molecolare of the University of Florence, Sesto Fiorentino, Italy. At 1.5 m from the collar, on four trees, an 8-mm circular incision was performed in the bark, so as to reach the cambium; the incised portion was then removed and replaced with a same-size plug of mycelium of an active colony of *C. populicola*, grown in Petri dishes on potato dextrose agar (DIFCO Laboratories, Detroit MI, USA). The hole was sealed with cotton wool and parafilm. As controls a sterile PDA plug and a PDA plug colonized by the CFAF100 strain of *C. platani* were inoculated on four trees each treatment. The length of stained wood and the extent of necrosis around the inoculation point were assessed after 60 days. In order to determine whether these alterations were caused by *C. populicola*, 2-mm-thick stem fragments were taken from the wood below the bark around the inoculation point as well as 10 cm above and 10 cm below the site. Each fragment was placed on PDA adjusted to pH 3.5 with a sterile tartaric acid solution according to manufacturer's instructions. To identify the reisolated fungi, DNA was extracted where necessary and subjected to ITS analysis, as described below.

Cerato-platanin production and cell content in *Ceratocystis* strains

The amounts of CP and CP-orthologous proteins in the culture filtrates and in the mycelium of the *Ceratocystis* strains were determined by enzyme-linked immunosorbent assay (ELISA) following the procedures described in Scala et al. (2004) and Boddi et al. (2004), using an anti-CP antiserum raised in rabbit against purified CP from culture filtrates of *C. platani* strain CfAF100. The standard linear calibration curves at the optical density of 492 nm (OD_{492}) vs. the log of the purified CP concentration had a correlation coefficient >0.95 using purified CP over concentrations ranging from 3 ng to 1 μg per well. A concentration of 1 μg per well gave an OD_{492} of about 0.700. Negative samples always yielded an $OD_{492}<0.030$. Each determination was performed in triplicate wells, and the OD_{492} was measured with a Model 550 Microplate reader (Bio-Rad, Hercules, CA, USA).

The presence of CP and CP-orthologous proteins on the surface of the fungal conidia and hyphae was determined by an immunofluorescence (IF) assay as described by Boddi et al. (2004) and inspected with a Leitz Orthoplan microscope (Leitz, Germany) with an incidence light excitation system, equipped with UV filters and a 75-W Leitz 100Z Xenon lamp (Leitz, Germany) at the Istituto per la Protezione delle Piante C.N.R., Firenze.

DNA extraction and fungal phylogenetic analysis

DNA extraction was carried out by using all strains listed into ESM-1. Agar plugs, from cultures grown on PDA for 4–5 days at 23°C, were taken and placed on sterilized cellophane discs overlaid on PDA plates. After 3–5 days of growth at 23°C in the dark, about 0.2 g (fresh weight) of mycelium was harvested from the cellophane sheet by scraping the surface with a scalpel and stored at −20°C until use. Genomic DNA was extracted from the mycelium using the DNeasy Plant Mini Kit, (Qiagen, Valencia, CA, USA). The quality and concentration of the DNA was evaluated with a Nanodrop ND 1000 spectrophotometer (CELBIO, Milan, Italy).

The internal transcribed spacer (ITS) region of the fungal strains was amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) as described by White et al. (1990). The polymerase chain reaction (PCR) products were purified

with the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) and sequenced by MWG (AG Biotech, Ebersberg, Germany). The DNA sequences, comprising the ITS1, the ITS2, and the intervening 5.8S rRNA gene, were used for the multiple sequence alignments, which were done with Clustal W (http://clustalw.genome.jp). Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura et al. 2007), and the phylogenetic trees were constructed from the evolutionary distance data calculated using the neighbor-joining nucleotide model (number of differences). The robustness of the branches was assessed by bootstrap analysis with 1,000 replicates.

Amplification of *cp*-orthologous genes

The *cp* gene and its orthologues were isolated using two primer pairs designed on the basis of the nucleotide sequence reported by Pazzagli et al. (2006) (primer pair no. 1, downstream: 5′-GAAGTTCTCTATCCTACCCATG-3′; upstream: 5′-CGCCGTTAATGCAGTTGG-3′; primer pair no. 2, downstream: 5′-CTCTTTCATACTTCCAC CAACC-3′; upstream: 5′-TCAATGCCACACTACTC TAGC-3′). The predicted product size was 454 and 547 bp, respectively. The PCR conditions were: initial denaturation at 94°C per 3 min, followed by 45 cycles of denaturation (94°C) for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min. Final extension was at 72° C for 10 min. The PCR products were separated on a 1.5% agarose gel with 2 μl ethidium bromide (10 mg ml^{-1}) and visualized under UV light. With most strains only one large band of the expected size was amplified. The PCR products were purified directly with the High Pure PCR Product Purification Kit (Roche Diagnostics) and sequenced by MWG (AG Biotech, Ebersberg, Germany). With *C. populicola* e *C. variospora* best amplification results were obtained by using primer pair no. 2 under less stringent conditions, lowering the annealing temperature to 48°C. Both the PCR products thus obtained showed weak intensity, so that it was cloned in *E. coli* DH5α cells (Invitrogen San Diego, CA, USA). For this they were purified with the High Pure PCR Product Purification kit (Roche Diagnostics). The 50 μl of product obtained were concentrated to about one third of the original volume in a Savant Rotavapor, and 2 μl was used in a ligation reaction with the pGEM®-T Easy Vector System (Promega, Madison, WI, USA). With this system, used according to manufacturer's instructions, the plasmid pGEM-CP was obtained. The p-GEM-CP was electropored into *E. coli* DH5α using a Gene Pulser II (Bio-Rad). *E. coli* cells were grown for one hour at 37°C on SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2 and 20 mM glucose, all from Sigma and then selected on LB medium enriched with 100 μg/ml ampicillin, 0.5 mM IPTG and 80 μg/ml X-gal (Sigma). Positive colonies were subjected to thermal lysis (95°C for 10 min) and to a PCR colony using the universal primer pair M13 (downstream: 5′-GTTTTCCCAGTCACGAC-3′, upstream: 5′-CAGGAAACAGCTATGAC-3′) under the following conditions: 94°C for 3 min; 94°C for 30 s; 55°C for 30 s; 72°C for 40 s, for 35 cycles, 72°C for 8 min. Gene insertion was verified by agarose gel electrophoresis and by sequence analysis which was performed by MWG (AG Biotech, Ebersberg, Germany). When the conditions of the experiment yielded only one PCR product of the expected length, this was purified by the High Pure PCR Product Purification Kit (Roche Diagnostics) and stored at −20°C until it was used for sequencing. DNA was sequenced by MWG (AG Biotech, Ebersberg, Germany).

Analysis of the deduced proteins

The hydrophobicity profile of the putative mature proteins was constructed with ProtScale Tool software (http://expasy.org/tools/protscale.html) following Kyte and Doolittle (1982). The theoretical isoelectric point (I_p) and the grand average of hydropathicity (GRAVY) were obtained with ProtParam software (http://www.expasy.org/tools/protparam.html).

Purification and biochemical characterization of cerato-populin from *C. populicola*

Purification procedure The CP-orthologous protein from *C. populicola*, named Pop1, was obtained from the fungal culture filtrate using a modification of the purification procedure previously reported for CP (Pazzagli et al. 1999). The fungus was inoculated in 1-l flasks containing 250 ml of PDB and incubated on a Gerhardt RO30 rotary shaker (Gerhardt, Bonn, Germany) at 100 revolutions/min, at 25°C for 21 days. Culture filtrates were obtained by removing mycelium and spores from the medium after filtration through a 0.45-mm Millipore membrane (Millipore, MA, USA). The culture filtrate was lyophilized, re-dissolved in 10 ml of 1% acetic acid, and then loaded on a Bio-Gel P-10 (Bio-Rad) column (2.5×70 cm), equilibrated, and eluted with 1% acetic acid. The fractions containing Pop1—identified by relative absorbance at 280 nm and by SDS-PAGE—were pooled and concentrated to 10 ml by ultrafiltration on a YM3 membrane (Millipore, MA, USA). The collected fractions were then applied to a Sephadex G-75 column (2.4×90 cm) (Pharmacia, Uppsala, Sweden), equilibrated, and eluted with 0.05 M Na-acetate buffer, pH 5.0, containing 1.0 M NaCl. The fractions containing Pop1 were pooled again, lyophilized, and resuspended in water containing 0.1% trifluoroacetic acid

(TFA). Finally, they were applied to a reverse-phase high-performance liquid chromatography C4 column, 5 μm, 250×10 mm (Vydac, Grace, Columbia, MD, USA). Elution was carried out using a water/acetonitrile gradient in 0.1% (*v*/*v*) trifluoroacetic acid. During purification, the yield of Pop1 was determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

The purity of Pop1 was determined by 15% SDS-PAGE according to Laemnli (1970). Western blot analysis was performed with anti-CP rabbit polyclonal antiserum and detected with horseradish-conjugated species-specific secondary antibodies (HPR-conjugated anti-rabbit IgG; Chemicon, Temecula, CA, USA) followed by an enhanced chemiluminescence reaction (Pierce). Composition and protein concentration of purified Pop1 were determined by amino acid analysis: 1–2 nmol of protein was hydrolyzed with 6.0 M HCl and analyzed on an amino acid analyzer equipped with a Na^+ high-performance column (4×120 mm, Beckman, Fullerton, CA, USA) and a post-column derivatization with *o*-phthaldialdehyde and 2-mercaptoethanol (Benson and Hare 1975). The molecular weight was determined using 60 pmol of the pure protein that was dissolved in CH_3CN 50% containing 0.1% TFA and added to a sinapinic acid matrix. Spectra were visualized on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer. The measurements were performed on a linear time of flight instrument (Bruker-Franzen Analytik, Bremen, Germany). Ions formed by a pulse UV laser beam were accelerated to 20 KeV. The mass spectra obtained averaged 100–200 shots.

Circular dichroism spectroscopy The secondary structure of Pop1 was characterized by circular dichroism (CD) spectroscopy. Pop1 was dissolved in 10 mM Na-phosphate buffer to a final protein concentration of 12.2 μM. CD was measured in the far-UV region from 190 to 300 nm using a Jasco J-810 spectropolarimeter (Jasco, Easton, MD, USA) equipped with a Peltier temperature control system. The spectra were obtained using a 1-mm path length quartz cell with a 0.5-nm step resolution, a speed of 50 nm/min, an 8-s response time, and a 1-nm band width, at 20°C. Ellipticity was expressed as the mean residue molar ellipticity (θ) (deg cm^2 $dmol^{-1}$) according to the formula: $[\theta] = CD/[(c/m)\,10d\,n]$ (*CD*, mdeg, observed signal; *c*, g/l, protein concentration; *m*, g/mol, molecular mass; *d*, cm, path length; *n*, number of amino acid residues).

Formation of assemblages Pop1 aggregates were obtained by incubating 1.3 mM Pop1 in 10% acetic acid. Samples were vigorously vortexed for 5 min and placed in a thermostat at 37°C. Self-aggregation of Pop1 was followed by fluorescence spectroscopy using thioflavine T, as described in the literature (LeVine 1999). Five microliters of each sample was withdrawn and centrifuged at 10,000×*g* for 5 min; the pellets were resuspended in 5 μl of the incubation solution and immediately mixed with 495 μl of 6.4 μM thioflavine T (ThT) in 50 mM glycine–NaOH buffer, pH 8.5 (final concentration: 0.54 μM CP). The ThT fluorescence spectra were recorded on a Perkin-Elmer LS-55 spectrofluorimeter with λ_{ex}=435 nm and λ_{em}=400–600 nm. Slits were set at 5 nm in both the excitation and the emission monochromators. The fluorescence of the soluble protein was also measured.

Morphology of assemblages The morphology of the aggregates was detected by atomic force microscopy; experiments were performed in air, in the intermittent contact mode, using a PicoSPM microscope equipped with an AACMode controller (Agilent, CA, USA). A rectangular silicon cantilever model NSG-01 (NT-MDT, Moscow, Russia) with a typical resonance frequency of 150 kHz was used. Scanner calibration was periodically checked by means of a reference grid (TGZ02, MikroMash, Tallin, Estonia) with known pitch, 3 μm, and step height of 102 nm. Five microliters of the aggregated Pop1 was centrifuged, the pellet was washed twice with the incubation solution, and finally, resuspended in 5 or 20 μl (sample diluted 1:4) of the same solution and placed on a freshly cleaved mica disc. AFM imaging of Pop1, aggregates was performed on several different areas of many samples, and the images were analyzed with the WSxM program (Nanotec Electronica SL, Spain).

Eliciting activity of Pop1

The capacity of Pop1 to elicit the synthesis of autofluorescent phenolic compounds and localized resistance in plants was determined according to Pazzagli et al. (1999) and Fontana et al. (2008), respectively. Synthesis of the autofluorescent phenolic compounds was tested by placing a number of 10-μl droplets of Pop1 1.5×10^{-4} M (about five droplets per cm^2) on the lower surface of white poplar (*P. alba* clone "Marte") or plane (*Platanus acerifolia*) leaves. After 24 h, the droplets were collected from the leaves, diluted with distilled water to a final volume of 500 μl, and their fluorescence was evaluated by a Perkin Elmer 650-10S fluorimeter, with λ_{ex} at 365 nm and λ_{em} at 460 nm. Eliciting activity of proteins was expressed as fluorescence intensity arbitrary units of droplets, where defence phenolic compounds accumulated. To determine the capacity of Pop1 to induce resistance in poplar leaves, about 8×10^2 conidia of *C. populicola* were added to the droplets 24 h after treatment. After a further 48 h, the conidia and hyphae were stained by adding a droplet of 100 mM phosphate buffer, pH 7.0, containing 0.1 μg ml^{-1} 4′-6-diamidino-2-

phenylindole (DAPI). After incubating for 10 min, the leaves were inspected under a Leitz Laborlux S fluorescent microscope (Leitz). The resistance induced was evaluated as the degree of inhibition of conidial germination and mycelium growth according to a four-point scale from 0 to 3, where 0=no conidia germination; 1≤33 % conidia germination and <33% droplet area weakly colonized by the mycelium; 2=33–66% conidia germination and 33–66% droplet area medially colonized by mycelium; 3≥66 % conidia germination and >66% droplet area massively colonized by mycelium.

Nucleotide sequence accession number

The nucleotide sequences of the internal transcribed spacer (ITS) region (ITS1, ITS2, and the intervening 5.8S rRNA gene) of the ribosomal DNA of the fungal strains *C. platani* CfAF100, *C. platani* Cf15, *C. populicola* 119.78, *C. cacaofunesta* 153.62, *C. variospora* 773.73, *C. fimbriata* (clone numbers, 740.70 from *Crotalaria*, 600.70 from *Mangifera*, 146.53 from *Coffea*, 141.37 from *Ipomea*, and 123.39 from *Fagus*) were deposited in the GenBank with the following accession numbers: EF042602, EF042613, EF042603, EF042611, EF042609, EF042604, EF042605, EF042606, EF042607, and EF042612 respectively. For the same strains, the nucleotide sequences of the *cp*-orthologous genes were deposited in the GenBank with the following accession numbers: EF017218, EF017226, EF017219, EF017224, EU330176, EF017220, EF017221, EF017222, EF017223, and EF017225, respectively.

Statistical analysis

Data of the CP production and cell content in *Ceratocystis* strains were analyzed by ANOVA, fixed model. The phenolic compounds production by leaves and the fungal growth were similarly evaluated. Homogeneous groups were identified by means of the Tukey HSD test.

Results

Molecular identification and phylogenetic analysis of *Ceratocystis* strains

The nucleotide sequences of the ITS region of the ribosomal DNA of the *Ceratocystis* strains were analyzed by BLASTN software, confirming that the strains belonged to the *Ceratocystis* species and clones listed into ESM-1. The derived dendrogram confirmed the known close relationship of the species and clones used; only *C. variospora* and *C. populicola* were grouped in a clearly separate clade (ESM-2).

Pathogenicity test of *C. populicola*

As expected, *C. populicola* 119.78 was pathogenic to white poplar. Sixty days after inoculation, areas of darkened wood and necrosis could be seen around the inoculation site, as well as a pronounced flattening of the trunk. The necrotic areas were located about 49.2±2.7 cm above the inoculation sites and about 21.4±1.3 cm below. Plants mock inoculated with distilled water or with *C. platani* CfAF100 only showed a small brown area located less than 2 cm above and below the inoculation point.

Mycelial growth and protein production of species and clones of *Ceratocystis*

All isolates of *Ceratocystis* grew abundantly when cultured in PDB (Table 1). There were, however, statistically significant differences. The *C. fimbriata* clone from *Ipomea* grew more than the other strains, producing 123.0±11.7 mg of dry fungal biomass, an amount that was however not statistically different from that produced by the *C. fimbriata* clone from *Fagus* (91.4±12.5 mg) and the *C. fimbriata* clone from *Mangifera* (86.5±5.0 mg). The smallest biomass (44.7±13.3 mg), produced by *C. cacaofunesta*, did not differ significantly from the biomass of the *C. fimbriata* clones of *Coffea* and *Crotalaria*, from the *C. platani* strains CfAF100 and Cf15, from *C. populicola*, or from *C. variospora*.

The occurrence of CP or the CP-orthologous proteins on the surface of the various *Ceratocystis* strains was studied by examining both the conidia and the young hyphae. Table 1 summarizes the results of the IF assays. All the strains reacted positively, with an intense fluorescence value, except *C. variospora*, where this value was only moderate. The mycelium of *O. novo-ulmi* (putative negative control) did not show any fluorescence, nor did the *Ceratocystis* control samples treated with preimmune serum (data not shown).

Table 1 also shows the cell content of CP or the CP-orthologous proteins as determined by quantitative ELISA using a rabbit CP-specific antiserum. The amounts of the proteins varied from 0.39±0.12 to 3.22±1.29 ng mg^{-1} dried mycelium. The greatest amount occurred in *C. variospora*, but it was not significantly different from that of *C. cacaofunesta*, of the *C. fimbriata* clones from *Fagus* and *Ipomea*, or of the *C. platani* strain Cf15. No CP was detected in extracts of the mycelium of the putative negative control *O. novo-ulmi* (data not shown).

When grown in PDB shake culture at 23°C, the *Ceratocystis* strains produced an average of about 30 μg of CP ml^{-1} culture filtrate, but there were significant differences between strains. The greatest amount of protein was produced by the *C. fimbriata* clone from *Fagus* (70.4±

Table 1 Mycelial growth, surface presence and cellular content, and production in culture medium of cerato-platanin (CP) and CP-homologous proteins in various species and clones of *Ceratocystis*

Fungal strain[a]	Mycelial biomass grown in liquid culture[b]	Cerato-platanin (CP) and CP-homologous proteins on cell surface and in mycelial cells		Cerato-platanin (CP) and CP-homologous proteins secreted in culture liquids[e]
		IF[c]	ELISA[d]	
C. cacaofunesta	44.7±13.3bc	3	1.11±0.36ab	30.4±11.2abc
C. fimbriata clone from *Coffea*	83.0±6.9bc	3	0.70 ±0.24b	9.6±2.6bc
C. fimbriata clone from *Crotolaria*	70.5±6.1bc	3	0.39±0.12b	53.5±10.4ab
C. fimbriata clone from *Fagus*	91.4±12.5ab	3	2.01±0.63ab	70.4±8.1a
C. fimbriata clone from *Ipomea*	123.0±11.7a	3	1.15±0.34ab	41.0±9.6abc
C. fimbriata clone from *Mangifera*	86.5±5.0ab	3	0.34±0.10b	32.5±9.8abc
C. platani strain CfAF100	68.1±9.9bc	3	0.95±0.21b	13.1±4.9bc
C. platani strain Cf 15	74.5±4.8bc	3	1.90±0.63ab	31.7±3.9abc
C. populicola	57.7±1.7bc	3	0.54±0.10b	16.0±11.0abc
C. variospora	68.3±4.1bc	2	3.22±1.29a	3.0±0.8c

[a] Results come from two independent experiments, in each of which each fungal strain was grown in three 100 cc conical flasks containing 20 ml potato dextrose broth; the immunological assays were performed with a rabbit CP-specific antiserum, and ELISA was performed in triplicate for each flask. Means in the columns followed by the same letter do not significantly differ at $P<0.05$ according to the Tukey's test

[b] Expressed as mg of dried mycelium±SE collected after 10 days growth in shake liquid culture at 23°C in the dark.

[c] Expressed as fluorescence index, determined on an arbitrary scale, where 0=no visible fluorescence, 1=faint fluorescence, 2=moderate fluorescence, 3=intense fluorescence. Data are based on observations of 30 microscopy fields in two independent experiments

[d] Expressed as ng of CP mg^{-1} dried mycelium±SE

[e] Expressed as μg of CP ml^{-1} culture filtrate±SE

8.1 μg ml^{-1}), which was not however statistically different from the amounts produced by *C. cacaofunesta*, the *C. fimbriata* clones from *Crotalaria*, *Ipomea*, or *Mangifera*, *C. platani* strain Cf15, or *C. populicola*. The smallest amount of protein was produced by *C. variospora*, which was also, however, not statistically different from many of the other strains.

Amplification of orthologues of the *cp* gene and sequence analysis

Genomic DNA from most of the *Ceratocystis* strains was amplified with primer pair no. 1, specific to the *cp* gene of *C. platani* CfAF100, and generated a single product. With *C. populicola* and *C. variospora*, however, the best results were obtained with primer pair no. 2. The *cp*-orthologous genes were aligned starting at the ATG start codon and ending at the TAG stop codon and compared. All sequences had an intron of 59 bp that was identical in all strains, except *C. fimbriata* from *Crotalaria* and from *Fagus*, whose introns had a T base instead of a G base in position 55, and *C. variospora* and *C. populicola,* whose introns had no fewer than six and eight substitutions, respectively.

The deduced premature proteins consisted of 134 amino acid residues, with the exception of *C. populicola* and *C. variospora*, whose deduced premature proteins consisted of 136 residues. The alignment of the deduced premature proteins is shown in Fig. 1. Both the Italian and the Greek strains of *C. platani* had the same amino acid sequence. The sequences of the peptide signal were identical except for those of *C. populicola* and *C. variospora*, which substituted alanine for serine in position 10. The four cysteine residues were in conserved positions, as were the two amino acids between the second and third cysteine residue, the eight prolines, the 12 glycines, and the amino acids at the cutting site of the CP of *C. platani* between the 14th and the 15th residue of the premature protein. However, the sequences of the *C. fimbriata* clones from *Coffea*, *Mangifera*, and *Fagus*, and the sequence of *C. cacaofunesta*, presented four to five substitutions, and the clones of *C. fimbriata* from *Crotalaria* and *Ipomea* presented as many as 12 substitutions. The amino acid sequences of the CP-orthologous proteins of *C. populicola* and *C. variospora* had the greatest number of amino acid substitutions, compared with the CP sequence of *C. platani*, so that the total identity of the deduced proteins decreased from 87.3% to 60.3% when these last two proteins were taken into account.

On the basis of the differences in the amino acid sequence, the hydrophobicity profiles of the putative mature proteins of *C. populicola* and *C. variospora* showed major differences from all the other proteins (ESM-2). Moreover, the protein of *C. populicola* showed a Grand

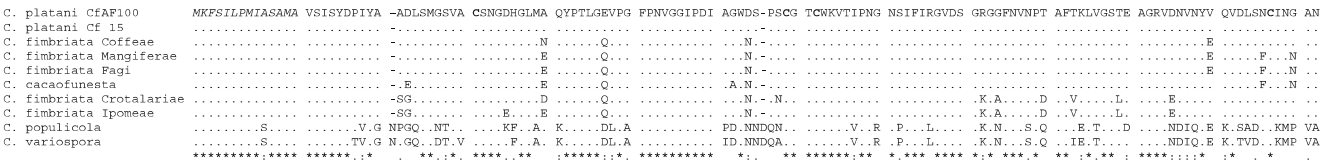

Fig. 1 Amino acid alignment of the premature cerato-platanin (CP) of *C. platani* and deduced CP-orthologous proteins. Amino acid sequences were aligned using Clustal W. The signal peptide was typed in *italic*, and the cysteine residues are shown in *boldface*. A gap introduced during the alignment is shown by a hyphen (-); stars (*) indicate amino acid identity; the marks ":" and "." indicated highly or moderately conserved residue substitutions

average of hydropathicity (GRAVY) index that, at −0.304, differed considerably from that of the other fungi; the GRAVY of the *C. variospora* protein (−0.089) fell within the range of values for the other proteins (−0.107 to 0.040). It is to notice a highly hydrophilic region of the *C. populicola* protein located between amino acids 50 and 60.

The theoretical isoelectric point values of the putative mature proteins of *C. populicola* and *C. variospora* were 4.58 and 4.23, which were not significantly different from those of the other proteins, which ranged between 4.33 and 4.56.

Purification and biochemical characterization of cerato-populin from *C. populicola*

The CP-orthologous proteins from *C. populicola* and *C. variospora* showed many differences in their amino acid sequence (Fig. 1) and then seemed the most interesting to investigate. In particular, we decided to purify the protein produced by *C. populicola* because it is the more hydrophilic than that produced by *C. variospora* and because it has as its host the poplar, one of the few plants whose genome has been completely sequenced (Tuskan et al. 2006). This will make it possible to analyze the expression levels of many host plant genes with known functions in resistance/susceptibility. The pure Pop1 protein was obtained after three purification steps and did not have any of the pigments contained in the culture filtrate (ESM-3). The final purification yield was 4.5 mg of pure protein from 1 l of filtrate, comparable with the purification yield of CP (Pazzagli et al. 1999). The SDS-PAGE showed a single band with an apparent molecular weight of about 15.0 kDa, and Western blot showed that Pop1 was recognized by the anti-CP antibodies, suggesting that it had antigenic sites similar to those of CP, in spite of the quite low (62%) homology and the different GRAVY index (Fig. 2a, b). MALDI TOF mass spectrometry revealed a single peak at 12,942.25 *m/z,* which was in agreement with the sequence results (calculated molecular weight = 12,941.45 Da) and which indicated that there were no posttranslational modifications (Fig. 2c). Lastly, purified Pop1 was analyzed by circular dichroism to find out more about the secondary structure of this protein, and the CD results were compared with the CD spectrum of CP (Pazzagli et al. 2006; Oliveira et al. 2006). Like CP, Pop1 is a well-structured α/β protein with a positive peak at 200 nm; the negative peaks at 212 and 232 nm typical of native CP were replaced in Pop1 by a single negative peak at 225 nm, which indicated differences in the percentage of the α-helix (Fig. 2d). Also, the spectrum suggests the presence of a coiled coil structure that can be indicative of the formation of a dimeric form of the protein in solution. However, the SDS-PAGE shows only the monomeric form of Pop1, and no covalent dimer can been detected.

Sbrana et al. (2007) and Pazzagli (2008, unpublished data) demonstrated that CP formed ordered aggregates in mild denaturing conditions, known to induce a partial unfolding of the proteins as in the case of fibril formation (Chiti and Dobson 2006). Also, Pop1 self-assembles in a manner completely comparable as CP suggesting that the pathway by which Pop1 assembled into ordered aggregates might be the same than CP. Formation of ordered aggregates was followed by Thioflavine T (ThT). ThT greatly enhances its fluorescence on binding to amyloid fibrils (LeVine 1999), to protofibrils (Walsh et al. 1999), and to class I hydrophobins (Kwan et al. 2006). Six hours after incubating in 10% acetic acid, 1.3 mM Pop1 began to form ThT-positive aggregates, which reached very high fluorescence values after 10 days of incubation (Fig. 3). The same samples that were assayed for ThT fluorescence were also analyzed by circular dichroism to detect any conformational change accompanying the aggregation process. Aggregation often initiates from a partly unfolded monomeric intermediate-stage protein, or from a naturally unstructured protein, and it is generally accompanied by an increase in the beta structure (Chiti and Dobson 2006). Figure 3b shows that Pop1 aggregation began with the unfolding of the polypeptide chain, but that once aggregates were formed, no signs of an all-β structure were detected as indicated by the absence of negative peak at 220 nm. To complete the analysis of Pop1 aggregates, a 6-day-old sample and a 10-day-old sample was subjected to AFM to determine its morphology. The aggregated Pop1 was diluted 1:100 before imaging since the undiluted sample showed a multitude of structures stacked upon each other that did not permit the dimensional analysis of these elements. Elongated (oval shaped) elements of almost

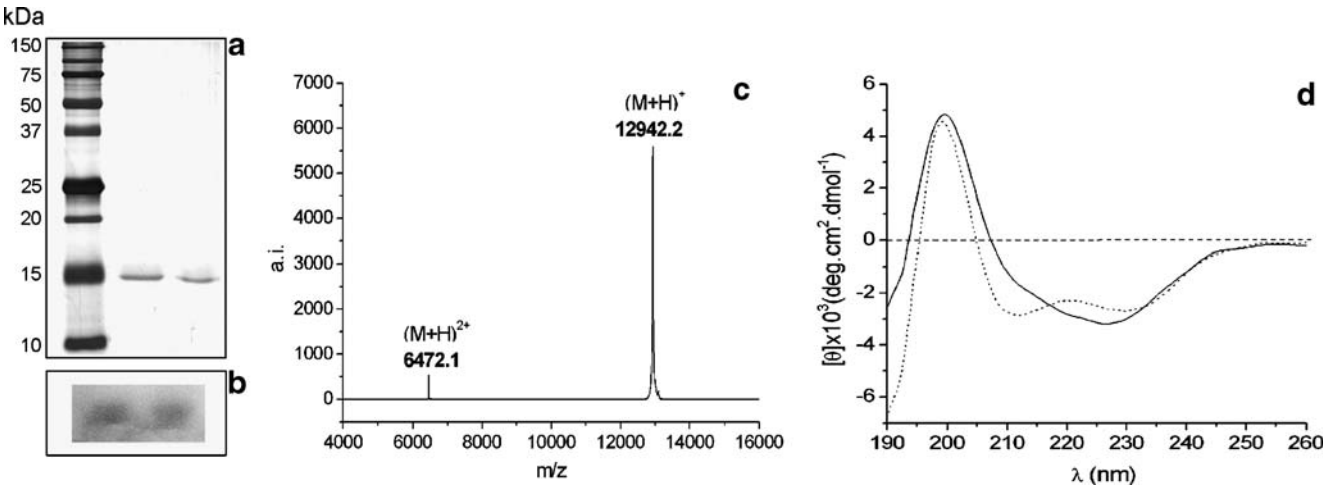

Fig. 2 Characterization of Pop1. **a** Silver stained, SDS-PAGE of purified Pop1. *lane 1* protein standard, *lane 2*, Pop1 1 μg; *lane 3* Pop1 2 μg. **b** Western blot analysis of purified Pop1. performed with anti-CP polyclonal antiserum and 0.5 μg of Pop1. **c** Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrum of purified Pop1. Analysis was performed using a LINEAR method. The mass spectrometer was calibrated with a 6- to 18-kDa protein standard. **d** CD spectra of purified Pop1. Far-UV CD spectra of Pop1 (*bold line*) and CP (*dotted line*). Sample concentration was 12.2 μM in water at 25°C

identical size (about 45×35 nm), were observed in all samples, with high reproducibility. Moreover, the axis ratio of these Pop1 aggregates was much the same as the minor/major axis ratio of 2:3 that was previously observed for CP assemblages, and that was proposed as the basis for a hierarchical aggregation mechanism starting from annular-shaped oligomers that self assembled in large oval-shaped aggregates (Sbrana et al. 2007).

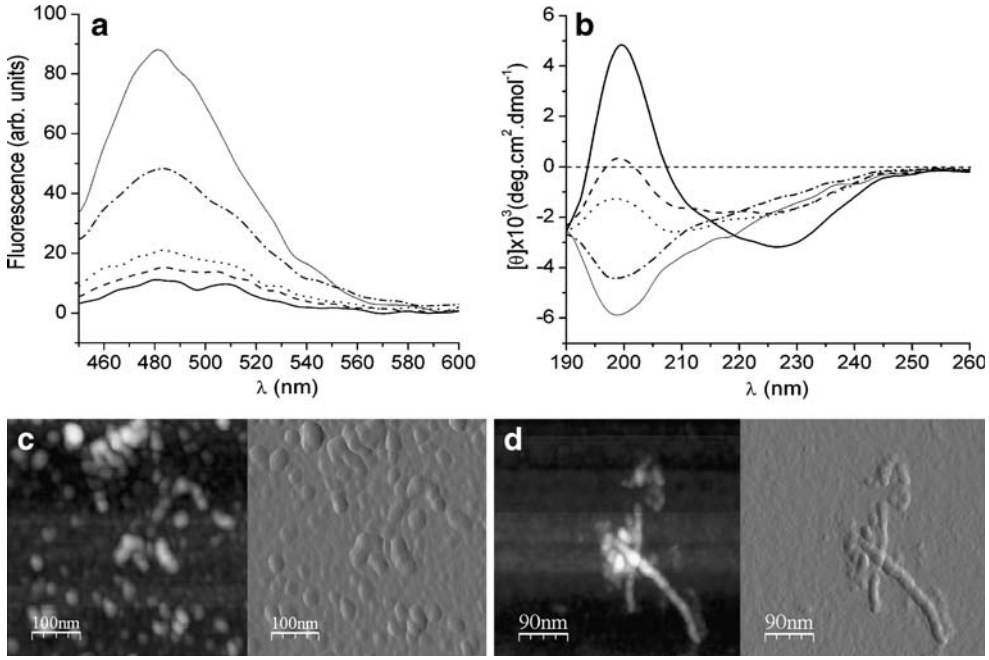

Fig. 3 Characterization of Pop1 aggregates. (**a-b**) *Spectroscopic characterization of Pop1 aggregates*. Soluble protein: *solid black line*. Aggregated protein after 6 h (dash line), 1 day (*dot line*), 6 days (*dash-dot line*), and 10 days (*thin solid line*) of incubation. **a** Fluorescence spectra of thioflavine T (ThT) with aliquots of Pop1 incubated in a 10% acetic acid/H_2O (*v*:*v*) mixture at 37°C. The samples contain 6.4 μM ThT in a 50 mM glycine–NaOH buffer, pH 8.5, and 13 μM Pop1. **b** CD spectra of 1.3 mM Pop1 incubated in 10% acetic acid/H_2O (*v*:*v*) mixture at 37°C. Sample concentration was 12.2 μM in water at 25°C. **c–d** *AFM images of Pop1 aggregates*. For each sample, images are height and amplitude (left and right, respectively). **c** Pop1 aggregates after six incubation days. Image size is 1.5 μm×1.5 μm. *Gray lines* are the profiles along the length (42 nm) and width (35 nm) of two representative aggregates satisfying ratio $D/L=2/3$. **d** Pop1 aggregates after 10 incubation days. The ovoidal units are assembled in large structures

Eliciting activity of Pop1

When the lower surface of poplar or plane leaves were treated with droplets containing 1.5×10^{-4} M Pop1 or 1.5×10^{-4} M CP, fluorescent phenolic substances were produced (Table 2). They accumulated in the droplets containing Pop1 or CP in a manner statistically higher than in those containing only sterile distilled water. No statistically different activities were found between the effects of treatments with Pop1 and CP. If after 24 h under these conditions about 10^3 conidia of *C. populicola* were added to each of the droplets containing Pop1, the fungal growth was inhibited in a statistically significant manner in comparison with that occurring on other poplar leaves treated with sterile distilled water (control) (Fig. 4). According to the four-point scale (0–3 relative units) described in the "Materials and methods" section, in droplets containing distilled water, nearly all conidia germinated, and the mycelium colonized more than 90% of the area (fungal growth value = 2.8±0.2 relative units). On the contrary, in the droplets containing Pop1, a little more than the 10% of conidia germinated, and the droplet area was not much colonized (1.3±0.2 relative units). Separated experiments showed that on glass microscope slides, Pop1 was not able to inhibit the growth of *C. populicola* (data not shown).

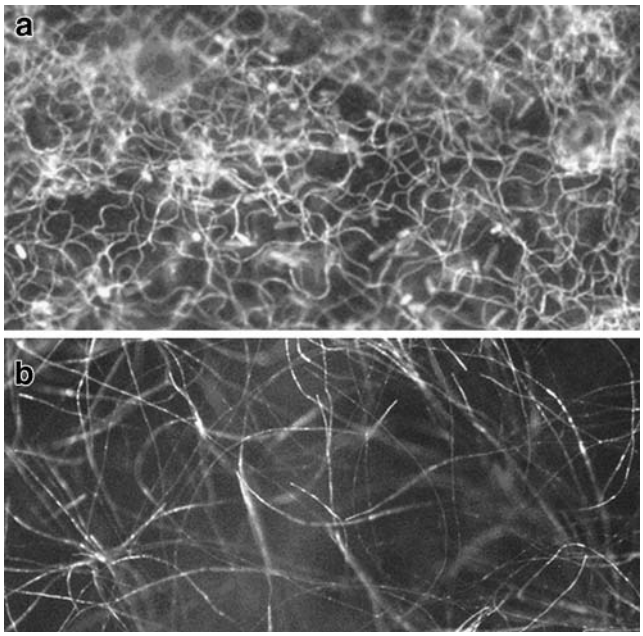

Fig. 4 *Ceratocystis populicola* grown for 2 days on poplar leaves that had been treated 24 h earlier with **a** distilled water or **b** 10^{-4} M cerato-populin; initial *C. populicola* conidial amount approximately 10^3

Discussion

C. platani is a plane-specific pathogen that has only recently been designated as a *nova species*, when it was separated from *C. fimbriata* Ellis and Halsted (Baker et al. 2003; Engelbrecht and Harrington 2005). *C. fimbriata sensu lato* is itself widespread in almost all the temperate and tropical regions of the world and has a very large host range that includes at least 31 plant species belonging to 14 families (Baker et al. 2003). The different plants that are hosts to *Ceratocystis* species have long been considered an important factor in the evolution of these species, and *C. fimbriata s.l.* has been subdivided into *formae* or clones depending on the particular host from which the fungus was isolated (Barnes et al. 2001; Witthuhn et al. 1998). Pontis (1951) already demonstrated that coffee plants became diseased when they were inoculated with *C. fimbriata* isolates from coffee, but not when they were inoculated with those same isolates from sweet potato. More recently, various host-associated monophyletic lineages of *C. fimbriata* have been identified in experiments that examined the interfertility, morphology, and the phylogenetics of the ITS regions of the rDNA. Isolates from these lineages likewise showed strong host specialization. On the basis of these findings, Engelbrecht and Harrington (2005) proposed that the lineage from *Platanus* sp. should be raised to the status of a *nova species* named *C. platani* Engelbrecht

Table 2 Eliciting activity of cerato-populin (Pop1) from *Ceratocystis populicola* and cerato-platanin (CP) from *Ceratocystis platani*

	Fluorescent phenolic compounds production[a,b,c]	
	Populus alba	*Platanus acerifolia*
Distilled water	8.75±4.53a (n=30)	6.34±1.13a (n=16)
Cerato-populin 1.5×10^{-4} M	22.18±7.53b (n=19)	27.19±9.70b (n=14)
Cerato-platanin 1.5×10^{-4} M	18.33±6.30b (n=11)	34.16±10.29b (n=17)

[a] Phenolic compounds production by white poplar (*Populus alba* clone "Marte") and plane (*Platanus acerifolia*) leaves which were treated 24 h with 10 µl droplets of distilled water, 1.5×10^{-4} M Pop1 or 1.5×10^{-4} M CP.

[b] The production of phenolic compounds was evaluated as fluorescence intensity arbitrary units.

[c] Values are the means of data from two independent experiments±SD. Values in the columns marked with different letters are significantly different at $P\leq0.01$ according to ANOVA.

and Harrington and that the lineage from cacao should be raised to the status of a *nova species* named *C. cacaofunesta* Engelbrecht and Harrington. Four other host-associated lineages were described by Johnson et al. (2005) and have been defined as *novae species*, including the oak and cherry lineage, denominated *C. variospora* (Davids.) C. Moreau, and the poplar lineage, denominated *C. populicola* J.A. Johnson and Harrington. More recently, as well, van Wyk et al. (2007) showed that isolates from mango in Pakistan and Oman represented a species distinct from other species in the *C. fimbriata s.l.* complex. The name *Ceratocystis manginecans* sp. nov. was provided for this fungus, which was closely related to but distinct from available isolates from mango growing in Brazil.

The present work found that there are variations in the coding sequences of *cp*-orthologous genes from isolates belonging to different species and clones of the genus *Ceratocystis* and in the proteins deduced from them. These isolates are pathogenic to plants with considerable importance in agriculture and forestry, and as ornamental plants, and they are all related to *C. platani*, the species producing the CP protein. Analysis of the ITS region confirmed the close relationship between all the *Ceratocystis* species and clones tested, but it also revealed that *C. variospora* and *C. populicola* were grouped together in a separate clade. The coding sequences of the *cp*-orthologous genes of most of the fungal strains tested in this study were amplified without difficulty, suggesting that there were no significant differences in the DNA sequences of these genes. Interestingly, the fact that the CP amino acid sequences from the Italian and the Greek strains of *C. platani* were identical confirmed the clonality of the European *Ceratocystis platani* population (Santini and Capretti 2000). Only with *C. variospora* and *C. populicola* was there some difficulty in amplifying the *cp*-orthologous gene; this was because the DNA sequences of these two species were somewhat different from those of the other species and clones of *Ceratocystis*. The putative deduced mature proteins of *C. variospora* and *C. populicola* had about 38% of their amino acids different from those of the CP of *C. platani*, and they substantially reduced the total identity of all the putative deduced proteins, from 87.3% to 60.3%. Interestingly, the positions of the prolines and glycines (often involved in turn structures of the polypeptide chains) were conserved, suggesting that Pop1 may have a fold similar to that of CP, in spite of the different GRAVY. The putative mature proteins of *C. populicola* and *C. variospora* had two more amino acids, so that they consisted of 122 residues. The hydrophobicity profiles of these proteins therefore differed considerably from the profiles of the putative mature proteins of the other fungal isolates; but only the *C. populicola* protein had a significantly lower GRAVY. The theoretical isoelectric point values of the *C. populicola* and *C. variospora* proteins were a little out of the range of those for the other proteins.

The *cp*-orthologous genes were expressed in all the fungal strains studied here. All the putative CP-orthologous proteins reacted positively with *C. platani* CP-specific antiserum in immunofluorescence assays and in semiquantitative ELISA experiments and were expressed and detected on the conidia and hyphae of the mycelial cells, and in the culture filtrates. The CP family thus acquires a further eight expressed proteins produced by plant-pathogenic fungi. The level of variation between these proteins confirms the high conservativeness of the members of the CP family. Zaparoli et al. (2009) showed that the sequence alignment and phylogenetic analysis of the CP family members did not reflect the phylogenetic relationship among the fungal producing species. Even the proteins produced by the Basidiomycota and the Ascomycota were not separated into different clusters; this suggests that the CP family proteins were already present in the filamentous fungi before separation into Basidiomycota and Ascomycota occurred. Even the class I hydrophobins were produced by both the Basidiomycota and the Ascomycota, but in this case, the class I hydrophobins of the Basidiomycota formed a group that was distinct from the Ascomycota Class I hydrophobins (Whiteford and Spanu 2002). Only proteins belonging to species of the same genus tended to cluster in the same clades. In another study of the phylogeny of the CP family, Seidl et al. (2006) found that only the genera *Gibberella* and *Hypocrea* displayed, respectively, two and three clusters of orthologues. The generally close relationship between the CP family members strongly suggests that they have a structural role and/or a basal function in fungal life, in a similar way as do the hydrophobins (Wösten 2001). In the present work, Pop1 and the other CP-orthologous proteins were detected in the mycelium. This suggests that they have a role in, for example, morphogenesis or that they protect the fungus against external factors, but it is important to point out that some proteins of the CP family were not associated with the fungal cell wall—as is the case with MSP1 from *M. grisea* (Jeong et al. 2007).

The CP-orthologous protein from *C. populicola* seemed the most interesting to purify in view of the many differences in their amino acid sequence (like that of *C. variospora*) and also because the poplar, host of *C. populicola*, is one of the few plants whose genome has been completely sequenced (Tuskan et al. 2006). This has made it possible to perform comparative genome analysis between the dicots *Populus* and *Arabidopsis* and the monocot *Oryza* in order to determine the functional implications of the various gene families putatively involved in the plant defence responses to pathogens (Hamberger et al. 2007; Yang et al. 2008). As a result, the

CP-orthologous protein Pop1 was purified from *C. populicola* culture filtrates, after confirming that the CBS strain 119.78 was still pathogenic to poplar trees. Like CP, Pop1 self-assembled when incubated in mild denaturing conditions, forming aggregates that resembled an ordered, amyloid-like structure, even if they did not show the increase in β-sheet that is typical of amyloid. As for the CP assemblages, we defined these ThT-positive structures as "ordered aggregates," in part because of their ovoidal shape that resembled the spherical globules and the short, curly fibers seen in some amyloidogenic proteins (Krebs et al. 2004; Anderson et al. 2006). Vargas et al. (2008) suggested that the CP protein family was divided into two subfamilies depending on whether they had a glycosylation site and on whether they self-aggregated. Self-assembly was defined as the ability to form covalent dimers via Trp oxidation. In the present paper, we showed that, like CP, Pop1 lacks the consensus motifs for both glycosylation and Trp oxidation. However, both these proteins form large noncovalent aggregates. Previously, Sbrana et al. (2007) found that the aggregation process of CP was hierarchical and involved the early formation of annular-shaped oligomers that constituted the fundamental bricks that then formed large assemblies. It would be interesting to see if these noncovalent ordered aggregates obtained in vitro elicit a defence response in plants.

As mentioned in the "Introduction" section, the CP family members have many functions. So far, the phylogenetic trees obtained were unrelated to the biological roles of the single proteins, but this does not mean that such roles, correlated with specific regions of the proteins, do not exist. It is our opinion that the orthologous proteins can be useful here. They often conserve some amino acids in the polypeptide chain at positions that are crucial for their functions, and they have the same or similar biological roles also in organisms that have only a relatively low relatedness with each other. This of course does not rule out the possibility that new functions or functional adaptations derive from the habitat in which the species producing the orthologous proteins live and compete with other species. Consequently, the study of orthologous genes and proteins helps us to understand how these genes have evolved and to predict gene-function. That is why, new methods are being developed to map orthologous genes across microbial genomes (Mao et al. 2006). Equally interesting is a database of orthologous groups of proteins, that cluster the results of an all-against-all comparison of the protein sequences encoded in prokaryotic and eukaryotic genomes (www.ncbi.nlm.nih.gov/COG), and also an e-Fungi database whose purpose is to facilitate the comparative analysis of genes and proteins from about 200 sequenced fungal genomes (www.e-fungi.org.uk).

Circular dichroism analysis showed that Pop1 was a well-structured α/β protein. The different percentage of the α-helix, if compared with CP, may well be indicative of a coiled-coil structure. This could produce differences between the two proteins in their 3D structure and, consequently, differences in how they interact with plants. In the current state of our knowledge, treating poplar leaves with 10^{-4} M Pop1 does not inhibit *C. populicola* as completely as CP at the same concentration inhibits *C. platani* on plane leaves (Fontana et al. 2008). But Pop1 and CP, though differing in their primary sequence, did not differ significantly in eliciting the synthesis of phenolic compounds in poplar and plane leaves. It will be interesting to see whether Pop1 and CP also have the same PAMP activity on nonhost plants of both *C. populicola* and *C. platani*. If both these proteins elicit the same defence response, their active region will coincide with one of the common structural portions of these proteins; whereas if each elicits a different defence response, their eliciting activity must be linked to a particular structural region in each protein. The definition of the 3D structure of CP (Oliveira et al. 2006, 2009) may also be very useful when it comes to describing the 3D structure of Pop1 and other orthologous proteins and may shed light on whether any changes in the residues will result in new or different functional properties. It is not unreasonable to think that a comparison between, on the one hand, the biophysical and structural properties of CP and its orthologues, and on the other, PAMP activity of these proteins, will suggest to add or delete amino acid residues, in order to design new, environmentally friendly peptides that will elicit effective defence responses in a number of plants. These new peptides could then be cloned in heterologous microorganisms used as cell factories and could be produced on an industrial scale. Similarly, a number of short peptides active against plant pathogens have been identified and designed ex novo, and their heterologous production with a view to plant disease control has been proposed (Marcos et al. 2008).

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