Cerato-platanin protein is located in the cell walls of ascospores, conidia and hyphae of Ceratocystis fimbriata f. sp. platani.

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Cerato-platanin protein is located in the cell walls of ascospores, conidia and hyphae of *Ceratocystis fimbriata* f. sp. *platani*

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Abstract

Cerato-platanin (CP), a protein of about 12.4 kDa from *Ceratocystis fimbriata* f. sp. *platani* (*Cfp*), accumulated in the mycelium and was located in the cell walls of *Cfp* ascospores, hyphae and conidia suggesting that this protein had a role in forming the fungal cell wall apart from the already known fact that it is secreted early in culture and elicits phytoalexin synthesis and/or plant cell death. The finding was obtained with three immunological techniques: a quantitative ELISA which determines the amount of CP in the mycelium, an immunofluorescence assay, and immunogold labelling to define the exact localization of CP in the *Cfp* cells.

Keywords: ELISA; Immunofluorescence; Immunogold labelling; Plant–pathogen interaction

1. Introduction

Cerato-platanin (CP), a small, moderately hydrophobic protein consisting of 120 amino acids, is produced by the Ascomycete *Ceratocystis fimbriata* (Ell. and Halst.) Davidson f. sp. *platani* Walter (*Cfp*) [1]. *Cfp* is the causative agent of canker stain of plane, a severe disease with a high incidence in European populations of *Platanus acerifolia* (Ait.) Willd. [2,3]. CP is reported to be released abundantly in shake culture at an early stage, and to elicit defense-related responses, such as phytoalexin synthesis and/or cell death, in both host and non-host plants [1,4,5].

According to various databases (SwissProt, EMBL and GenBankTM), CP is the reference protein of a small protein family that includes three other proteins produced by other Ascomycota: the snodprot1 protein, from *Phaeosphaeria nodorum* (SwissProt Accession no. O74238), the allergen Asp f 15 precursor (Asp f 13), from *Aspergillus fumigatus* (SwissProt O60022), and the heat-stable 19 kDa antigen (CS-Ag), from *Coccidioides immitis* (SwissProt Q00398). In addition, Wilson et al. [6] have recently characterized the gene *sp1* from *Leptosphaeria maculans*; this gene encoded a secreted protein closely related to the CP protein family. All these proteins are characterized by high sequence homology, but not always by clear functional similarities. However, they are all secreted, and in some cases seem to be involved in biological recognition phenomena. Snodprot1 is produced during infection of wheat leaves by *P. nodorum* [7]. The Asp f 15 precursor (Asp f 13) has been characterized together with many other allergens from *A. fumigatus* in order to find a serological procedure to diagnose allergic bronchopulmonary aspergillosis, a severe disease of the lungs in humans caused by *A. fumigatus* [8]. CS-Ag is one of a class of trypsin-like serine proteinases produced by both the saprophytic and the parasitic phases of *C. immitis*, causing San Joaquin

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Valley fever or coccidioidomycosis, a human respiratory disease, and was proposed as a *Coccidioides*-specific antigen for the diagnosis of this fungus [9]. The recombinant SP1 protein induced an autofluorescence response on host leaves [6].

Cerato-platanin shares some structural and functional characteristics with the hydrophobin family: (i) a low molecular mass; (ii) hydrophobicity; (iii) the sequence Cys–Ser–Asn is aligned with the signature sequence of the hydrophobins (Cys–Cys–Asn), except for the conservative substitution Cys → Ser; (iiii) the high homology of the N-terminal region of CP with that of cerato-ulmin, a hydrophobin from *Ophiostoma* spp. [10–12]. However, CP differs from the hydrophobins in having only four cysteines, all involved in two disulphide bonds, and in having a distinctive hydropathicity profile, unlike that of both class I and class II hydrophobins.

In the present paper we show that CP is located in the mycelial cell walls of *Cfp* by three different methods, all based on the immunorelationship between CP and a CP-specific rabbit antiserum.

2. Materials and methods

2.1. Fungal cultures

The origin of the reference *Cfp* strain Cf AF 100 has been previously described [1]. Details of the other *Cfp* strains (CF 3, CF 5, CF 6, CF 7, CF 8, CF 9, CF 11, CF 12, CF 15, CF 16, CF 17, CF 18, CF 22, CF 23, CF 24, CF 25, CF 27, CF 42) are given in Santini and Capretti [13]. The strains were routinely cultured on potato dextrose agar (PDA) (Difco, Detroit, MI, USA). For long-term storage, conidia and mycelial fragments collected from 4-day-old liquid shaken mini cultures (3 ml) in potato dextrose broth (PDB) were resuspended in 10% (v/v) glycerol and stored at −70 °C.

In the experiments described below, mycelial fractions including aerial hyphae and hyaline and cylindrical conidia were carefully collected from the surface of a 6-day-old culture, centrifuged at 5000*g* for 10 min, washed three times with 0.9% (w/v) NaCl and twice with distilled water, and then resuspended to obtain the appropriate concentrations of hyphae and conidia. The ascospores were collected from mature perithecia produced on the surface of 2–3-week-old cultures on PDA, and diluted in octane.

2.2. Purification of CP and antiserum preparation

Cerato-platanin was purified from culture filtrate of *Cfp* strain Cf AF 100 according to the procedure in Pazzagli et al. [1]. Rabbit CP-specific antiserum was prepared according to Scala et al. [9], divided into 1 ml aliquots and stored at −20 °C. Pre-immune serum was collected from a rabbit before the first injection and stored at −20 °C to be used as a negative control.

2.3. Quantitative ELISA

The amount of CP in *Cfp* mycelium was quantified by ELISA. A freeze-dried aliquot (10 mg) of each sample was extracted for 2 min with 500 μl of 60% (v/v) ethanol and centrifuged for 5 min at 13000*g*. The supernatant was dried under vacuum. The residue was solubilized in 500 μl phosphate buffered saline (PBS), 0.1 M, pH 7.2, and used for coating the wells (50 μl per well) (Falcon 3911 Microtest flexible plates, Becton Dickinson Labware, Oxnar, CA, USA) in the ELISA. The culture filtrates were serially twofold diluted, from 1:1 to 1:8; 50 μl samples of each dilution were added in triplicate to the wells and maintained at 37 °C for 3 h. Serial dilutions of CP and uninoculated PDB were used as positive and negative controls respectively. The wells were washed three times with cold PBS and any remaining binding sites were blocked with 50 μl per well of 0.5% (w/v) gelatin in PBS for 2 h at 37 °C. After saturation, 50 μl of CP antiserum, diluted 1:1000 in PBS containing 0.25% (w/v) gelatin and 0.2% (v/v) Tween 20, was added. Rabbit pre-immune serum was used in the control wells. After overnight incubation, the multiwell plates were washed with cold PBS and incubated at 37 °C for 2 h in the presence of 50 μl per well of goat anti-rabbit IgG/peroxidase (Sigma–Aldrich, St. Louis, MO, USA) diluted 1:2000 in PBS containing 0.25% (w/v) gelatin and 0.2% (v/v) Tween 20. The wells were washed three times with cold PBS, after which a 150 μl substrate solution (0.4 mg ml^{-1} *o*-phenylenediamine dihydrochloride, 0.012% (v/v) H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0) was added; after 30 min of incubation at room temperature in the dark, the optical density at 492 nm (OD_{492}) was measured with a Model 550 Microplate reader (Bio-Rad, Hercules, CA, USA). The concentration of CP in the samples was determined using a standard curve. The standard linear calibration curves at 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. The concentration of 1 μg per well gave an OD_{492} of about 0.700. Negative samples always yielded an $OD_{492} < 0.030$.

2.4. Immunofluorescence assay

Aliquots (20 μl) of hyphal, conidial and ascospore suspensions at a concentration of about 10^5 cells ml^{-1} were dispensed to 12-well Multitest slides (ICN Biomedicals, Aurora, OH, USA) and dried at 60 °C. The wells were washed with PBS, filled with 20 μl of CP antiserum diluted 1:100 in PBS and incubated for 2 h at room temperature. Control wells were filled with pre-

immune serum diluted 1:100. After washing three times with PBS, the wells were filled with 20 μl each of a 1:40 dilution of goat antirabbit IG-fluorescein isothiocyanate conjugate (Sigma–Aldrich) in PBS and incubated for 2 h at room temperature. The slides were washed with distilled water and examined under a Leitz Phloemopack 2.1 microscope with an incidence light excitation system, equipped with UV filters and a 75-W Leitz 100Z Xenon lamp.

2.5. Immunogold labelling

Hyphae, conidia and ascospores were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.2) overnight at 4 °C. After washing in the same buffer the material was postfixed in 1% OsO_4 buffer for 1 h, washed three times with buffer, and dehydrated in an ethanol series of 30, 50, 70, 90 and 100% (v/v), at room temperature. The samples were infiltrated in 2:1 (v/v) ethanol:LR White (Sigma) for 1 h; 1:2 (v/v) ethanol:LR White for 2 h; 100% LR White overnight at 4 °C and finally embedded in LR White resin according to the method of Tagu et al. [14] with modifications. Ultrathin sections were cut with an LKB ultramicrotome, collected on formvar-coated gold grids and processed for immunogold labelling to localize the CP. The sections, collected on formvar-coated gold grids, were first incubated in PBS with 1% bovine serum albumin (BSA, British BioCell International, Cardiff, UK) (pH 8.2) for 20 min. They were then incubated for 1–2 h in CP antiserum (primary antibody) diluted 1:100 in the first buffer. The grids were washed five times for 5 min with the buffer, then incubated for 30 min at room temperature on a drop of goat anti-rabbit IgG antibodies conjugated to 10 nm gold particles (Sigma) diluted 1:20 in the buffer [15] (modified). After rinsing with the buffer and distilled water, the grids were stained with uranyl acetate and lead citrate. Examination was in a Philips EM 201C transmission electron microscope operating at 80 kV.

Control reactions were performed under the same conditions using pre-immune serum instead of the CP antiserum, or the gold-conjugated secondary antibody (goat anti-rabbit IgG) alone.

2.6. Statistical analysis

ELISA data of the CP content of *Cfp* isolate mycelium were analysed by ANOVA, fixed model. Homogeneous groups were identified by means of the Tukey HSD test.

3. Results and discussion

The cellular content of CP in *Cfp* mycelium, as quantified by ELISA, was 16.61 ± 0.42 ng mg^{-1} fresh weight mycelium for the reference isolate CF AF 100. Results with the other isolates did not differ statistically from that of the reference isolate.

The IF assay showed that CP occurred on the surface of the conidia, hyphae and ascospores *of Cfp* isolate CF AF 100 (Fig. 1). The fluorescence reaction remained intense even after numerous washes with 80% ethanol, suggesting that the CP was a stable component of the

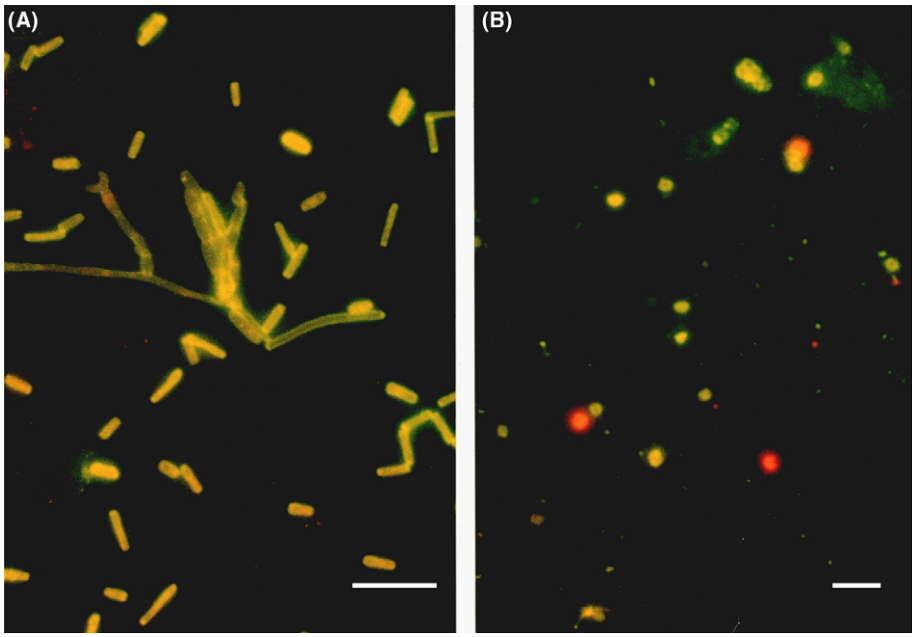

Fig. 1. Immunofluorescence labelling of hyphae and conidia (A, scale bar = 50 μm) and ascospores (B, scale bar = 10 μm) with CP antiserum.

fungal surface. No fluorescence was observed in samples treated with rabbit pre-immune serum (data not shown).

Immunoelectron microscopy showed unequivocally that CP was a component of the *Cfp* cell walls. The positive reaction was located on the walls of hyphae (Fig. 2(A) and (B)), conidia (Fig. 2(C)) and ascospores (Fig. 2(D) and (E)) of *Cfp*. CP was more abundant on the ascospore and conidial wall than on the hyphal wall (Fig. 2(C)–(E)). Labelling on the conidia was distributed across the entire thickness of the wall, while on the ascospore wall the gold granules were located more densely on the electron-transparent layer near the plasmamembrane than on the more outward layer. There was labelling also on the fibrillar material at the surface of ascospores (Fig. 2(D) and (E)). No labelling was found on control sections where pre-immune serum was used or where the first antibody was omitted (data not shown).

All the experimental evidence demonstrated that CP was a significant component of the cell walls of *Cfp* conidia, hyphae and ascospores and of the fibrillar material at the surface of *Cfp* ascospores. This finding suggests that this protein may have some new structural functions, apart from the already known fact that it is secreted early in culture and elicits phytoalexin synthesis and/or cell death in both host and non-host plants [1,4,5]. CP resembles the hydrophobins, known to be located on the surface of the conidial and hyphal walls, where they form insoluble complexes that have a structural role in forming aerial hyphae [16–19]. Hydrophobins are also often excreted abundantly in the form of monomeric proteins into the culture medium by submerged feeding hyphae, and/or they are involved in

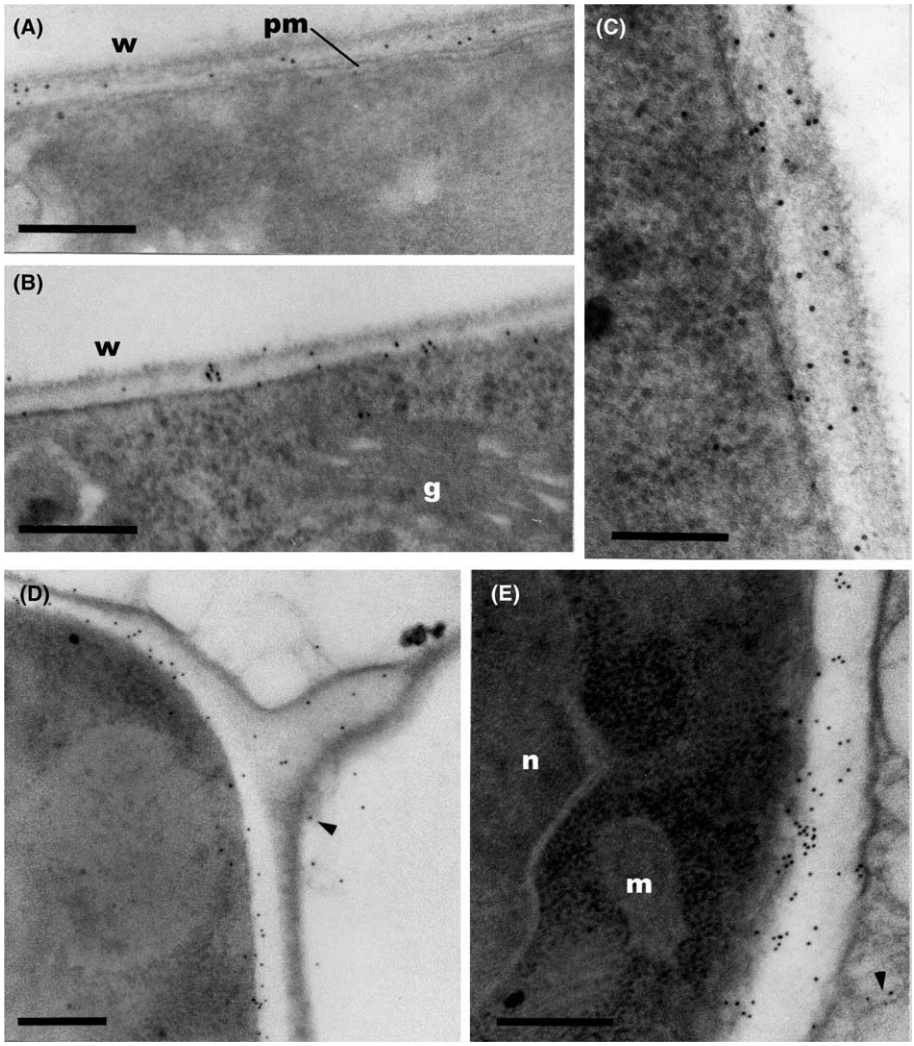

Fig. 2. Transmission electron microscopy of immunogold labelling of *Ceratocystis fimbriata* f. sp. *platani*. A, B: Hyphae with gold granules on the wall. Scale bar = 0.3 μm. C: labelling distributed across the conidial wall. Scale bar = 0.2 μm. D, E: Gold granules on the ascospore wall and on the fibrillar material on the surface of ascospores (arrowhead). Scale bar = 0.3 μm. Abbreviations: g, golgi body; m, mitochondrion; n, nucleus; pm, plasmamembrane; w, wall.

ensuring contact and communication between the fungus and its environment. In the outermost layer of the fungal cell walls the hydrophobins self-assemble into polymeric and amphipatic monolayers with a higher content of beta-sheets than the monomeric proteins, as has been demonstrated in the case of amyloid fibrils [20,21]. Other hydrophobic cell wall proteins make the cell walls hydrophobic as well and have a role in the formation of aerial hyphal structures, or in securing the adhesion of the hyphae to the hydrophobic surface of the host [22,23]. However, the capacity of being secreted into the environment and of being involved in biological recognition phenomena with other organisms, is a characteristic common only to some of the hydrophobins and CP family members. The phytopathogenic fungi are reported to produce proteins a morphologic and/or pathogenic role. In all these cases elucidation of the protein functions was facilited through our obtaining fungal mutants with traditional mutagenesis methods, and was made more effective by targeted gene disruption. Thus cerato-ulmin hydrophobin from the *Ophiostoma* species was shown to be involved in causing the hydrophobicity of the mycelium grown on solid culture, its adhesion to vector insects, and elm wilting symptom expression [24–27]. Disruption of the gene codifing unsecreted MPG1 hydrophobin produced mutants of *Magnaporthe grisea* with lower levels of virulence, conidiation and appressorium formation [28,29], while in the case of the *SC3* gene of *Schizophyllum commune* the composition of the fungal cell wall was affected by its disruption [30]. The HCf-1 hydrophobin of *Cladosporium fulvum* was demonstrated to be required for efficient water-mediated dispersal of conidia [31]. In other cases the targeted deletion of genes did not lead to differences in the morphology and hydrophobicity of spores, or in virulence on the host plant, as was the case with the *cpph1* hydrophobin gene of *Claviceps purpurea* [32], and the *sp1* CP family member protein gene from *Leptosphaeria maculans* [6].

At the moment, the role of CP presence in the fungal cell wall is unclear, but our research group, having sequenced the *cp* gene (NCBI accession number AJ311644), is now focusing on targeted mutants unable to produce CP. These will help to elucidate the role of CP in the pathogenesis of plane canker stain and in the structural integrity of cell walls and/or their morphogenesis. Studies are also under way to determine whether CP self-assembles at the interface between the fungal cells and the air, and whether this modifies the beta-structure content.

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