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CERATO-PLATANIN, AN EARLY-PRODUCED PROTEIN BY *CERATOCYSTIS FIMBRIATA* F.SP. *PLATANI*, ELICITS PHYTOALEXIN SYNTHESIS IN HOST AND NON-HOST PLANTS

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SUMMARY

Cerato-platanin (CP), a previously purified and sequenced protein of about 12.4 kDa, has been studied with regard to the relationship between the fungal growth and the *in vitro* production by *Ceratocystis fimbriata* f.sp. *platani*, the causal agent of plane canker stain. Obtaining CP-specific polyclonal antibodies from rabbit allowed us to prepare a quantitative ELISA which indicated that CP was produced by *C. fimbriata* f.sp. *platani* in the first days of *in vitro* culture. Moreover, for the first time it was demonstrated that a small amount (<2 nmol) of CP elicited the synthesis of phytoalexins such as umbelliferone and glyceollin, in host plane leaves and non-host soybean cotyledons, respectively.

Key words: Cerato-platanin, elicitor, umbelliferone, glyceollin, ELISA.

INTRODUCTION

Cerato-platanin (CP) is a protein of about 12.4 kDa produced in culture by the Ascomycete *Ceratocystis fimbriata* (Ell. and Halst.) Davidson f.sp. *platani* Walter (*Cfp*) (Pazzagli *et al*., 1999). This fungus is responsible for canker stain of the plane tree, a severe disease showing a high incidence in *Platanus acerifolia* (Ait.) Willd. populations of southeastern Europe (Anselmi *et al*., 1994; Panconesi, 1999). The unavailability of effective curative treatments and the difficulties in implementing prophilaxy have made it impossible to counteract the spread of the pathogen and have lead to the loss of age-old planes in many European city avenues. The hybrid origin of *P. acerifolia* from *P. occidentalis* L. x *P. orientalis* L. seems to offer the opportunity to explore new strategies in genetic improvement of plane trees for resistance to the canker stain (Vigouroux *et al*., 1997, 1999), but new ideas are needed in order to prevent the dispersal of both the pathogen and the disease.

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The amino acid sequence of CP is highly homologous to the snodprot1 protein (Swiss Prot Accession N° O74238) produced by *Phaeosphaeria nodorum* during infection of wheat leaves, to the AspF13 allergen from *Aspergillus fumigatus* (Swiss Prot Accession N° O60022) and to an antigen of 19 kDa from *Coccidioides immitis* (Swiss Prot Accession N° Q00398). All these proteins are considered to belong to the new "cerato-platanin protein family". Recently, Wilson *et al*. (2002) characterized the gene *sp1* encoding a protein secreted by *Leptosphaeria maculans*, showing a high sequence homology with CP. Moreover, CP contains four cysteine residues at positions 20, 57, 60 and 115 involved in two disulphide bonds in the native protein, and shows some structural characteristics similar to cerato-ulmin, a Class II hydrophobin from *Ophiostoma* spp., and to other fungal hydrophobins: i) more than 40% of the N-terminal region of CP is homologous with that of cerato-ulmin; ii) 40% of the 120 amino acid residues of CP consist of hydrophobic amino acids; iii) the signature sequence Cys-Cys-Asn of hydrophobins is present with the conservative substitution Cys Æ Ser (Cys-Ser-Asn); and iv) CP is a structural component of the surface of hyphae, conidia and ascospores of *Cfp* (Ebbole, 1997; Wessels, 1997; Sereni *et al*., 2002). Pazzagli *et al*. (1999) suggested the potential role of CP as a signal molecule in the induction of plant defence mechanisms. CP elicited the release of fluorescent substances and/or cell death following both placements on *P. acerifolia* leaf lower surfaces and infiltration into tobacco leaf mesophyll.

In the present paper we report the production of CP-specific polyclonal antibodies and the preparation of a quantitative ELISA, a useful tool to demonstrate that *Cfp* releases CP early and abundantly when the fungus is grown in a liquid medium. Moreover, we examined the biological activity of CP as an elicitor of phytoalexins in host and non-host plants. The structural and functional similarities of CP with the cerato-platanin family and/or with the hydrophobins is also discussed.

MATERIALS AND METHODS

A **Fungal cultures.** The origin of the *Cfp* strain Cf AF 100 has been previously described (Pazzagli *et al*.,

1999). Details of the other cultures of *Cfp* were described by Santini and Capretti (2000). The fungus was routinely cultured on Potato Dextrose Agar (PDA) or Potato Dextrose Broth (PDB) (Difco, Detroit, MI, USA). For long-term storage, conidia and mycelial fragments collected from 4-day-old liquid shaken mini-cultures (3 ml) in PDB were resuspended in 20% (v/v) glycerol and stored at -70°C.

Isolates of *Eutypa lata*, *Phellinus ignarius*, *Verticillium dahliae*, *Ophiostoma ulmi* and *O. novo-ulmi*, to be used as putative negative controls, were from the Culture Collection of the Dipartimento di Biotecnologie Agrarie, Sezione di Patologia Vegetale, University of Florence. *E. lata*, *P. ignarius* and *V. dahliae* were grown in PDB, while *O. ulmi* and *O. novo-ulmi* were grown in the modified Takay medium (Scala *et al*., 1994). Culture filtrates were obtained by filtering the fungal liquid culture through Whatman 41 filter paper in order to separate the mycelium from the substrate, and subsequently through a 0.45 mm Millipore membrane. The filter paper was previously dried for 24 h at 140°C, weighed, used as described above, and then placed again in the drying oven at 140°C for a further 24 h. After this time, the filters were weighed again. The difference between the final and initial weights was assumed to be the dry weight of the mycelium.

To determine the relationship between fungal growth and CP production, thirty-six Erlenmeyer flasks (100 ml) containing 50 ml PDB were inoculated with a 10^5 ml^{-1} conidial suspension of *Cfp* isolate Cf AF 100 and incubated in the dark at 23°C on a rotary shaker (Gerhardt, Germany) at 100 rpm. After 1, 2, 3, 4, 5, 7, 10, 14 and 17 days of culture, 4 flasks were harvested for each time. Aliquots of 1 ml from each flask were dialyzed against distilled water at 4°C, dried using a vacuum spinning evaporator Speed Vac SC110 (Savant, Hicksville, NY, USA), and stored at -30°C.

Antiserum preparation. A 4-month-old female rabbit was injected four times (at 7 days intervals) with 1 mg of homogeneous CP purified from the culture filtrate of *Cfp* isolate Cf AF 100 (Pazzagli *et al*., 1999), dissolved in 1 ml Phosphate Buffered Saline (PBS) 0.15 M, pH 7.2, containing 0.83% NaCl, and emulsified with an equal volume of Freund's adjuvant (Sigma, St. Louis, MO, USA) immediately before use. Blood samples were collected from the marginal vein of the ear of the rabbit 8 days after the last injection. The serum was named anti-CP antiserum, divided into 1 ml aliquots and stored at -20°C. Before the first injection, a pre-immune serum was collected and stored at -20°C to be used as a negative control.

In some experiments, the rabbit anti-cerato-ulmin (anti-CU) polyclonal antiserum, obtained as described by Scala *et al.* (1994), was utilized.

Western blot analysis. Ten microliters of the culture filtrate were applied to a 15% SDS-PAGE gel according to Laemmli (1970) using a Mini Protean II (Bio-Rad Instruments, Hercules, CA, USA). An aliquot of 5 mg of purified CP was used as a positive control. Protein blotting on the nitro-cellulose membrane was performed using a Mini Trans Blot Electrophoretic Transfer Cell (Bio-Rad Instruments, Hercules, CA, USA) according to Towbin *et al*. (1979) at 100V for 1 h. Nitrocellulose membranes were then thoroughly washed in TBS (Tris 10 mM, NaCl 150 mM) supplemented with 0.05% Tween 20, pH 7.4, blocked with 3% albumin and probed with anti-CP antiserum 1:1000. Immunocomplexes were finally detected with horseradish conjugated species-specific secondary antibodies (HPR-conjugated anti-rabbit IgG; Chemicon, Temecula, CA, USA) followed by enhanced chemioluminescence reaction (Pierce, Rockford, IL, USA).

Quantitative ELISA. Aliquots of 500 ml of culture filtrates obtained at various days after inoculation were dried; the residue was solubilized in 500 ml PBS 0.1 M, pH 7.2, and used for coating the wells (50 ml per well) (Falcon 3911 Microtest flexibles plates; Becton Dickinson Labware, Oxnar, CA, USA). The culture filtrates were serially twofold diluted, from 1:1 to 1:8; 50 ml samples for each dilution were added in triplicate to wells and maintained for 3 h at 37°C. 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 ml per well of 0.5% (w/v) gelatine in PBS for 2 h at 37°C. After saturation, 50 ml of anti-CP antiserum diluted 1:1000 in PBS, containing 0.25% (w/v) gelatine and 0.2% (v/v) Tween 20, were added. Rabbit pre-immune serum was used in 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 ml per well of goat anti-rabbit IgG/peroxidase (Sigma, St. Louis, MO, USA) 1:2000 in PBS containing 0.25% (w/v) gelatine and 0.2% (v/v) Tween 20. Wells were washed three times with cold PBS, and then a 150 ml substrate solution (0.4 mg ml^{-1} o-phenylenediammine dihydrochloride, 0.012% (v/v) H_2O_2 in 0.1 M citrate-phosphate buffer, pH 5.0) was added. After 30 min incubation in the dark at room temperature, the A_{492} was measured with a Model 550 Microplate Reader (Bio-Rad). The concentration of CP in the samples was determined using a standard curve. The standard calibration curves of A_{492} vs. the log of purified CP concentration had a linear correlation coefficient >0.95 using purified CP over a concentration ranging from 3 ng to 1 mg per well. The concentration of 1 mg per well gave an A_{492} of approximately 0.700. Negative samples always yielded an $A_{492} < 0.030$. In some experiments, the rabbit anti-CU polyclonal antiserum was utilized in order to check positive cross-reactions.

Eliciting of phytoalexins. Phytoalexins from plane tree leaves were determined as described by El Modafar

et al. (1995) with minor modifications. Leaves of *P. acerifolia* were removed from trees and placed directly into previously sterilized 30x40 cm boxes containing moist filter paper. Twenty eight 10 µl droplets (subdivided in 7 groups, each containing 0.031, 0.062, 0.125, 0.250, 0.5, 1.0 and 2.0 nmoles of pure CP) were added to the left half of the plane leaf lower surface, whereas the right half was inoculated with sterile distilled water droplets. Samples were maintained at 23°C under continuous light. After 36 h, droplets were separately collected and filtered through a 0.22 µm Millipore membrane. The samples were assayed by a DyNA Quant 200 Fluorometer (Amersham-Pharmacia Biosciences AB, Uppsala, Sweden) at 365 and 460 nm, corresponding to excitation and emission wavelength, respectively. The standard calibration curve had a linear correlation coefficient >0.99 using 0.1–10 µM pure umbelliferone (Sigma, St. Louis, MO, USA). A solution of 1 µM umbelliferone gave a value of 6.505 relative fluorescence intensity units. Phytoalexin concentration in samples was determined in comparison to the umbelliferone standard curve and the results were expressed as nmoles ml^{-1}.

The ability of CP to elicit the synthesis of glyceollin phytoalexin in soybean cotyledons was assayed according to the method of Ayers *et al.* (1976) with minor modifications. Seeds of commercial soybean were surface-sterilized by soaking in 1% sodium hypochlorite for 5 min and washed three times with sterile distilled water. The surface-sterilized seeds were put onto a layer of sterile moist hydrophylic cotton wool on the bottom of 1-liter beaker, and maintained at 23°C in the dark. After germination, the seedlings were maintained in daylight. Cotyledons, detached from 8-day-old seedlings, were soaked for 2 min in 1% sodium hypochlorite and extensively rinsed with sterile distilled water. A section of approximately 1 mm thick and 6 mm in diameter was cut from the upper surface of each cotyledon. Twelve 10 µl droplets (subdivided in 4 groups, each containing 0.31, 0.62, 1.25 and 2.5 nmoles of pure CP) were applied to each cotyledon. One group of five cotyledons per concentration was used; distilled water was used as a control. The treated cotyledons were placed on a moist filter paper disk in a 90-mm diameter Petri dish and incubated for 20 h at 23°C in the dark. After this time, each group of 5 cotyledons was transferred to 10 ml distilled water to rinse off the droplets retained on the wounded surface; the solution thus obtained was named "Droplets Solution" (DS). The A_{285} of DS was assayed in a UV-VIS recording spectrophotometer (model UV-160; Shimadzu, Japan) against the DS obtained by treating cotyledons with distilled water. All measurements of glyceollin concentration, reported in the present paper, refer to DS. Since Ayers *et al.* (1976) demonstrated that an A_{285} of 0.200 corresponded to a concentration of 3 µM glyceollin, we transformed the values of A_{285} for each DS into µM glyceollin. For each experiment three groups of cotyledons were used for each concentration of CP.

RESULTS

Extracellular production of cerato-platanin. Western blot analysis of the culture filtrate obtained from a 6-day-old culture of *Cfp* isolate Cf AF 100 is shown in Fig. 1. One band was observed reacting with the anti-CP antiserum; this band had a molecular mass similar to that obtained when pure CP (approx. 12.4 kDa) was used.

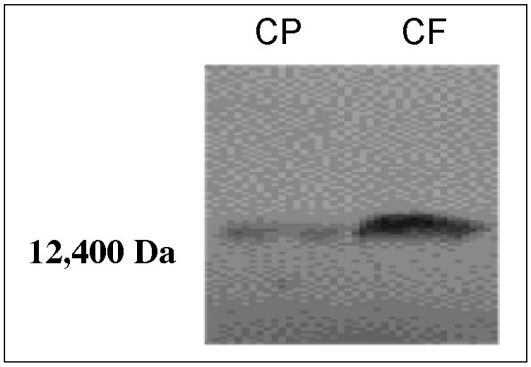

Fig. 1. Western blot analysis of pure cerato-platanin (CP) and of *Ceratocystis fimbriata* f.sp. *platani* culture filtrate (CF) after 6 days of liquid culture, probed with anti-CP rabbit polyclonal antiserum.

Fig. 2 shows the fungal growth rate and time course of CP produced in liquid shake culture by *Cfp* isolate Cf AF 100. CP was determined by ELISA and expressed as mg ml^{-1}. During the first 3 days the growth rate was approx. 23 mg day^{-1}, and decreased to a value of about 7 mg day^{-1} from day 4 to 7, when it reached a plateau that was maintained until day 17. *Cfp* isolate Cf AF 100 produced CP from the beginning of the logarithmic phase of growth. On the first day, a production of 60 mg ml^{-1} was obtained, while the peak was reached between the third and the seventh day of growth, when it was of 150-170 mg ml^{-1}. Subsequently, the concentration decreased to 70 mg ml^{-1} (day 17).

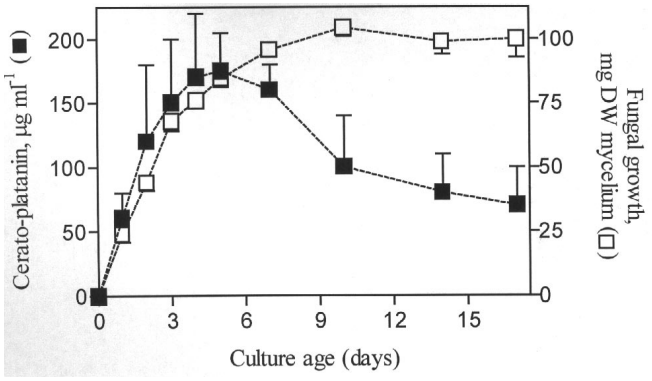

Fig. 2. Time-course production of cerato-platanin, determined by ELISA and expressed as mg ml^{-1}, in culture filtrates of *Ceratocystis fimbriata* f.sp. *platani* at various days from inoculation; fungal growth is expressed as mg d.wt mycelium. Values are the means of 4 replicate culture flasks ± SEM.

Table 1 summarizes the results of quantitative ELISA detecting the production of CP by numerous isolates of *Cfp* after 2, 5 and 7 days of growth in liquid culture. All the isolates produced CP in quantities comparable to that of the reference isolate Cf AF 100. The putative negative control isolates *E. lata*, *P. ignarius*, *V. dahliae*, *O. ulmi* and *O. novo-ulmi* did not show CP production. Negative results were also obtained when the culture filtrates of *Cfp* were treated with both the rabbit pre-immune serum. The treatment with the anti-cerato-ulmin polyclonal antiserum gave positive results only when *O. ulmi* and *O. novo-ulmi* were tested (data not shown).

Eliciting activity of cerato-platanin. Synthesis of 0.06–4.66 nmoles ml^{-1} umbelliferone equivalents was elicited 36 h after various quantities of CP had been added to the lower surface of plane leaves (Fig. 3). The accumulation of umbelliferone in droplets was positively correlated with the CP quantities used. The minimum quantity of CP able to elicit an appreciable quantity of umbelliferone (1 nmole ml^{-1}) was about 0.25 nmoles.

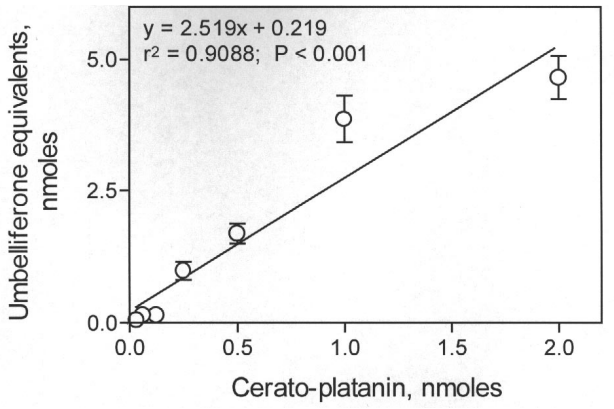

Fig. 3. Elicitation of umbelliferone after treatment of plane leaves with various amounts of cerato-platanin. Values are the means of 8 data from two independent experiments ± SEM.

Table 1. Production of cerato-platanin in culture filtrates of various isolates of *Ceratocystis fimbriata* f.sp. *platani* (*Cfp*) after 2, 5 and 7 days of growth in Potato Dextrose Broth.

Fungal isolate	Cerato-platanin[b] at different days of culture age µg ml^{-1}		
	2	5	7
Cfp CF AF100	120±60	175±30	160±20
Cfp CF 3	65±21	87±18	112±22
Cfp CF 5	92±10	105±15	116±19
Cfp CF 6	126±22	130±28	103±12
Cfp CF 7	99±26	116±32	131±19
Cfp CF 8	122±15	133±13	125±26
Cfp CF 11	79±17	104±22	120±18
Cfp CF 12	87±11	110±15	119±25
Cfp CF 15	141±13	158±32	152±36
Cfp CF 16	81±22	99±19	85±21
Cfp CF 17	95± 8	101±15	123±27
Cfp CF 18	111±12	125±32	117±29
Cfp CF 19	144± 9	93±25	93±10
Cfp CF 22	66±12	81±18	75±14
Cfp CF 23	82±10	130±17	128±22
Cfp CF 24	62±15	83± 8	110±12
Cfp CF 25	129±23	135±12	112±22
Cfp CF 27	74±12	103± 9	109± 5
Cfp CF 42	68±11	98±10	80± 6
Eutypa lata [a]	-	-	-
O. novo-ulmi [a]	-	-	-
Ophiostoma ulmi [a]	-	-	-
Phellinus ignarius [a]	-	-	-
Verticillium dahliae [a]	-	-	-

[a] Used as putative negative controls, grown as described in Materials and Methods.
[b] Detected by ELISA.

Representative soybean cotyledons treated with various quantities of CP are shown in Fig. 4. Faint-to-intense browning was observed on the treated surface of soybean cotyledons in the presence of CP concentrations varying from 0.31 to 2.50 nmoles cotyledon^{-1}. The concentration of glyceollin elicited and assayed in the droplet solution (DP) at 285 nm is shown in Fig. 5. The minimum quantity of CP able to induce a detecteble concentration of glyceollin (about 2.5 mM) corresponded to 0.62 nmoles.

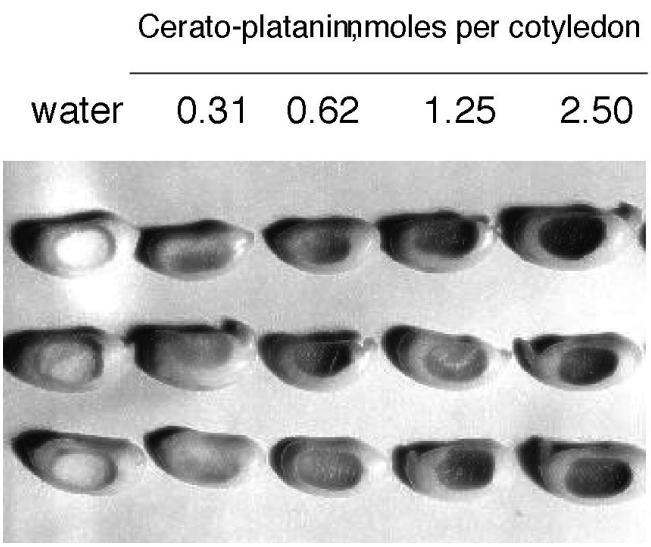

Fig. 4. Browning of soybean cotyledons after treatment with various amounts of cerato-platanin.

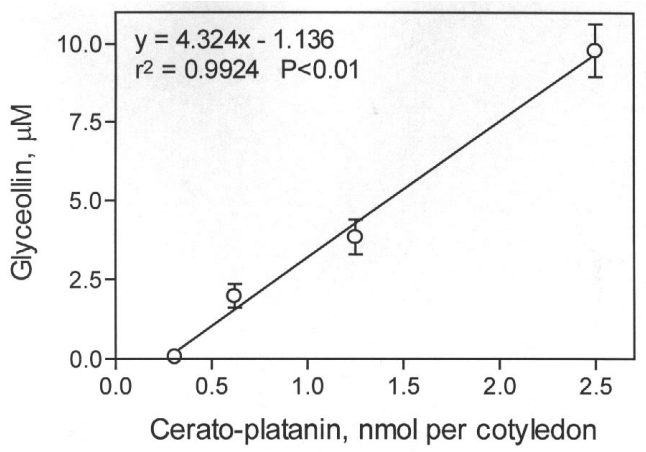

Fig. 5. Elicitation of glyceollin phytoalexin after treatment of soybean cotyledons with various amounts of cerato-platanin. Values are the means of 6 data from two independent experiments ± SEM.

DISCUSSION

The present study has demonstrated the production of polyclonal antibodies for the specific detection of CP, a small protein previously purified from culture filtrates of *Cfp*. Moreover, we described their application in a quantitative ELISA, a useful tool in detecting CP in culture filtrates. The specificity of the anti-CP antiserum was ascertained by examining its reactivity in Western blot experiments using both purified CP and the culture filtrate of our *Cfp* reference isolate Cf AF 100, grown in standard conditions. Moreover, ELISA always gave positive results when culture filtrates of numerous other *Cfp* isolates were examined, whereas negative responses were obtained when five phytopathogenic fungi belonging to other genera were used. All the *Cfp* assayed isolates secreted CP abundantly and at comparable concentrations, confirming the high homogeneity found for the same isolates when analysed at the molecular level by PCR techniques (Santini and Capretti, 2000). Importantly, our reference isolate Cf AF100 secreted large amounts of CP (about 60 mg ml^{-1}) on the first day of the logarithmic phase of growth; similarly, the most tested *Cfp* isolates produced CP abundantly during the first days of culture.

We also reported the ability of purified CP to elicit phytoalexin synthesis in the host plane and in a non-host plant. A small amount (<2.0 nmol) of CP induced the synthesis of umbelliferone, the major reference phytoalexin from plane, and of glyceollin, a phytoalexin from soybean, a non-host plant for *Cfp*. In tobacco leaves, cell necrosis and fluorescent phenolic compound synthesis have been demonstrated (Pazzagli *et al.,* 1999).

Recently, Alami *et al.* (1998, 1999) isolated another protein from germinating *Cfp* spores, glycoprotein GP66, able to elicit the phenolic metabolism and the synthesis of umbelliferone, scopoletin and xanthoarnol in cell suspension cultures derived from the susceptible plane, *P. acerifolia*.

It is known that in most plant-microorganism interactions, plant defense mechanisms are activated at the initial phase of the pathological process (Agrios, 1997; Jackson and Taylor, 1996). This activation needs perception by the host of an external, physical and/or chemical stimulus, and must be rapid for plant defenses to be effective against microbial attack. Our results seem to suggest that, after fungus-plane physical contact has occurred, CP could be one of the fungal proteins involved in the first molecular events of plane canker stain pathogenesis: the recognition process and phytoalexin elicitation. Even if the species of *Platanus* utilized in worldwide urban areas and parks (*P. acerifolia*, *P. orientalis*, *P. occidentalis*) are all susceptible to the canker stain disease (with the partial exception of some genotypes of *P. occidentalis*), this does not necessarily mean that under natural conditions this hypothetical phytoalexin-based defense mechanism is uneffective, because other plant and/or fungal factors could interfere with this potential resistance mechanism. In order to elucidate the real involvement of this CP-induced mechanism in *Cfp*-plane interaction, it needs to further investigate whether and when CP (and/or GP66) is

(are) released during pathogenesis under natural conditions, and whether and how they are involved in the differential activation of phytoalexin synthesis and/or in other plant defense-related events in resistant or susceptible plane genotypes. These findings would give useful information related to the genetic improvement of plane trees, able to show earlier and more effective resistance responses to *Cfp* attacks.

According to various databases (Swiss-Prot, EMBL and GenBank™), CP was highly homologous with three other proteins produced by different Ascomycota: the snodprot1 protein of *Phaeosphaeria nodorum*, the AspF13 allergen of *A. fumigatus* and the antigen of *C. immitis*. All these proteins belong to the "cerato-platanin protein family". The functional similarities in the "cerato-platanin family" are not clear, whereas some structural characteristics make CP similar to cerato-ulmin and, in general, to the hydrophobin family (Ebbole, 1997; Wessels, 1994, 1997; Wosten *et al.*, 1999), not least the early and abundant release in culture fluids and the ability of CP to interact with the host plant. It is known that hydrophobins often play a role in morphogenesis and/or pathogenicity and host specificity of fungal producers. Hydrophobins are products of genes abundantly expressed and accumulate in cell walls or are secreted into the culture medium. They are involved in active adhesion of fungal spores to host surfaces, as for *Magnaporte grisea*, and in pathogenesis and parasitic fitness, such as in the case of *O. novo-ulmi* (Del Sorbo *et al*., 2000, 2002; Scala *et al*., 1997; Takai, 1974; Temple *et al.,* 1997). Other hydrophobic proteins are found on the surface of cell walls of phytopathogenic fungi. In the Basidiomycete *Ustilago maydis*, cause of the smut disease of maize, an abundant class of small cell wall proteins, named "repellents", has been identified during the filamentous dimorphic stage of the pathogen (Kershaw and Talbot, 1998; Wosten *et al*., 1996). In general, all these fungal surface-proteins were indicated as playing a structural role in the formation of aerial hyphae, where they show a remarkable ability to self-assemble into polymeric and amphipatic monolayers. These polymeric structures show a higher content of beta-sheets in comparison with monomeric proteins and are similar to amyloid fibrils (Wosten and Willey, 2000; Mackay *et al*., 2001).

At the moment, CP seems to be a surface protein suitable for release by *Cfp* in the extracellular environment; this finding is in agreement with the hypothesis that CP is one of the first fungal substances able to interact with the host plane. Our future goal is to investigate this working hypothesis.

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