CERATO-PLATANIN TREATED PLANE LEAVES RESTRICT *CERATOCYSTIS PLATANI* GROWTH AND OVEREXPRESS DEFENCE-RELATED GENES

F. Fontana[1], A. Santini[2], M. Salvini[3], L. Pazzagli[4], G. Cappugi[4], A. Scala[5], M. Durante[1], R. Bernardi[1]

[1] Dipartimento di Biologia delle Piante Agrarie, Sezione di Genetica, Università di Pisa, Via Matteotti 1/B, 56124 Pisa, Italy
[2] Istituto per la Protezione delle Piante del CNR, Sezione di Firenze, Via Madonna del Piano, Palazzo E, 50019 Sesto Fiorentino (FI), Italy
[3] Scuola Normale Superiore, Pisa, Italy
[4] Dipartimento di Scienze Biochimiche, Università degli Studi, Viale Morgagni 50, 50134 Firenze, Italy
[5] Dipartimento di Biotecnologie Agrarie, Laboratorio di Patologia Vegetale Molecolare, Università di Firenze, Via della Lastruccia 10, 50019 Sesto Fiorentino (FI), Italy

SUMMARY

Cerato-platanin (CP) is a small protein of 120 amino acids, produced by the ascomycete *Ceratocystis platani* (Walter) Engelbrecht et Harrington (*Cep*), the agent of plane canker stain. CP is located in the cell walls of the conidia, hyphae and ascospores of *Cep*, is able to self-aggregate, and is released into the culture medium in the first growth stages of *in vitro* culture. CP is the founder member of the CP family, a group of proteins involved in the interaction between the producing microbe and the host cell. Leaves of *Cep*-susceptible *Platanus acerifolia* react to treatments with CP in a resistance-like manner, producing extracellular and intracellular phenolic compounds and undergoing cell plasmolysis and/or death. The present study provides evidence that in plane leaves the CP-activated defence-related events are strictly correlated with the inhibition of *Cep* growth on the leaves and with a high level of transcripts of 78 differentially expressed genes. Of the transcripts, 19.2% regarded as genes coding for defence and/or stress related proteins. The other genes concerned DNA/RNA synthesis and metabolism, protein synthesis/turnover, energy, primary cellular metabolism and the signalling pathways. The results demonstrated that after treatment of the plane leaves with CP, a substantial network of regulatory interaction and coordination was formed, as well as other defence-related molecular events.

Key words: Platanus acerifolia, localized induced resistance, suppression subtractive hybridization.

INTRODUCTION

Cerato-platanin (CP) is a small protein of 120 amino acids, produced by the ascomycete *Ceratocystis platani* (Walter) Engelbrecht et Harrington (*Cep*) [formerly known as C. *fimbriata* (Ell. and Halst.) Davidson f. sp. *platani* Walter], the causal agent of plane canker stain (Walter *et al*., 1952; Sinclair *et al*., 1987; Engelbrecht and Harrington, 2005). *Cep* is a virulent pathogen of *Platanus orientalis*, *P. occidentalis*, and their hybrid *P. acerifolia*. Plane canker stain has had a dramatic impact on the plane trees native to many European and Mediterranean countries, where resistant host clones are not reported to exist (Anselmi *et al*., 1994; Panconesi, 1999; Vigouroux and Olivier, 2004). CP has a molecular weight of about 12.4 kDa and contains four cysteine residues at positions 20, 57, 60 and 115, forming two S-S bridges (Pazzagli *et al*., 1999). CP is located in the cell walls of conidia, hyphae and ascospores of *Cep*, is able to self-aggregate, and is released into the culture medium in the first growth stages of *in vitro* cultures (Boddi *et al*., 2004; Scala *et al*., 2004; Sbrana *et al*., 2007). The coding *cp* gene sequence was cloned in *Pichia pastoris*, and the structural and functional characterisation of the recombinant protein showed no significant differences with those of the native protein (Carresi *et al*., 2006; Pazzagli *et al*., 2006). Determination of the 3D solution structure of the recombinant CP is in progress (Oliveira *et al*., 2006). The CP 1-119 amino acid sequence is a new domain, called the CP domain (www.expasy.uniprot.org); CP thus became the founder member of the CP family. Fourteen proteins belonging to the CP family have so far been identified containing this domain. They are produced by various ascomycetes and by the basidiomycete *Antrodia camphorata*, and in some cases are involved in host cell-microbe interactions. When applied to host and non-host plant tissues, CP elicited the synthesis of phenolic compounds and/or phytoalexin, and caused intercellular and intracellular disorganisation of the spongy parenchyma cells, cell plasmolysis and/or necrosis (Pazzagli *et al*., 1999; Scala *et al*., 2004; Bennici *et al*., 2005, 2006).

Suppression subtractive hybridization (SSH) is a powerful technique that isolates expressed sequence tags (ESTs) representing genes that are differentially expressed in different mRNA populations. By combining the suppression PCR technique with the normalization and subtraction steps in a single reaction this technique can identify more weakly expressed genes (Diatchenko *et al*., 1996). Other advantages of SSH are that it detects low-abundance, differentially expressed transcripts and that it can isolate genes whose sequence or identity is

Corresponding author: Rodolfo Bernardi
Fax: +39050571556
E-mail: rbernard@agr.unipi.it

still unknown; moreover, SSH is carried out using common molecular biological techniques that do not require specialised equipment or analysis. SSH has already been used in various plant species to isolate genes involved in the response to biotic and abiotic stresses (Fernandez *et al*., 2004; Jiang *et al*., 2004; Ribeiro *et al*., 2004; Verica *et al*., 2004; Degenhardt *et al*., 2005; Kong *et al*., 2005; Ouziad *et al*., 2005; Rinaldi *et al*., 2007; Rizzo *et al*., 2007).

In the present study it is shown that when *Cep*-susceptible plane leaves are treated with CP concentrations lower that 10^{-4} M, *Cep* can no longer grow on them, and that the SSH expression profile of plane leaf genes modulated and/or activated by CP is consistent with the hypothesis that CP elicits defence-related proteins and metabolic pathways in plane trees.

MATERIALS AND METHODS

Production and purification of CP. Cerato-platanin was purified from culture filtrates of *Cep* (previously reported as *C. fimbriata* f. sp. *platani*) isolate Cf AF 100 following the procedure of Carresi *et al*. (2006).

Induction of localized resistance and plane leaf treatments. The capacity of CP to induce localized resistance was determined on the basis of its capacity to inhibit conidial germination and hyphal growth of the *Cep* strain Cf AF 100 inoculated on plane leaves that had been treated with CP or with distilled water. Plane leaves were harvested from a *P. acerifolia* tree growing in Pisa, Italy. Before CP treatment, the leaves were abundantly washed, their lower surface was carefully scraped and again washed with sterile distilled water. Conidia were harvested from fruiting cultures by gently touching the colony surface with a sterile wet Pasteur pipette, suspended in sterile distilled water and adjusted to about 2×10^5 conidia ml^{-1}. In a standard experiment the lower leaf surface was covered with a number of 5 µl water droplets (about 5 droplets per cm^2) containing 7.5×10^{-5} or 0 (control) M CP, and were then maintained in a moisture chamber at 23°C in the dark. Twenty four hours after CP treatment, 5 µl distilled water containing about 8×10^2 conidia was added to each droplet. To ensure that CP did not inhibit *Cep* growth directly, the same procedure was repeated with glass slides instead of leaves.

To determine how the CP concentration inhibited *Cep* growth, the eliciting treatment was performed with increasing concentrations of CP from 0.47 to 15×10^{-5} M.

To test the time interval required for leaf tissue to mount an effective defence against the fungus after elicitation with CP, the right part of the lower leaf surface was treated with a number of 5 µl water droplets of a 7.5×10^{-5} M CP solution, whereas the left part was treated with sterile distilled water (control). The position of the droplets was indicated on the leaves by a marker. Then the leaves were washed three times with sterile distilled water at 0, 24 and 48 h, before a further addition of 5 µl water droplets containing about 8×10^2 *Cep* conidia was made on the same spots where the CP droplets had previously been placed. Usually 5 to 10 droplets were utilised on the leaf surface for each CP concentration or treatment time.

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 10 min incubation, the treated leaves were inspected under the fluorescent microscope (Leitz Laborlux S). Induced resistance was evaluated as the degree of inhibition of conidial germination and mycelial growth according to a scale from 0 to 4, where 0 = no conidia germination; 1 = <10 % conidia germinated and <10% droplet area colonised by the mycelium; 2 = 10-40 % conidia germinated and 10-40% droplet area colonised by the mycelium; 3 = 41-70 % conidia germinated and 41-70% droplet area colonised by the mycelium; 4 = >70 % conidia germinated and >70% droplet area colonised by the mycelium.

In the experiments evaluating the relation between the residual CP concentration in the droplets added to the leaves and the release of the umbelliferone phytoalexin from plane, and studying the mRNA expression profile, numerous 10 µl droplets of CP 15×10^{-5} M were applied to the right part of the lower leaf surface, whereas the left part was treated with sterile distilled water (control). The leaves were maintained in a moist chamber at 23°C in the dark. Forty-eight hours after treatment the droplets were recovered, filtered through a 0.22 µm Millipore membrane and assayed for phytoalexin production as described by Carresi *et al*. (2006).

Phytoalexins were expressed as nmol/ml of umbelliferone equivalents. The CP concentration was evaluated by ELISA according to the procedure described in Scala *et al*. (2004) with a CP antiserum raised in rabbit against purified CP from culture filtrates of *C. platani* Cf AF 100. 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 leaf portions pre-treated with CP or with distilled water were recovered separately at -80°C before they were used for RNA isolation.

Statistical analysis. Data were analysed with ANOVA, fixed model; homogeneous groups were identified by means of Tukey's HDS test. The "t" test was used to compare the two groups of data. The analyses were performed using GraphPad InStat version 3.00.

Total RNA isolation. For total RNA isolation, samples of the treated and control leaves weighing approxi-

mately 2 g each were used. The leaves were ground in liquid nitrogen, and homogenised in CTAB extraction buffer (NaCl 1.4 M; EDTA 20 mM; Tris-HCl 100 mM; pH 8.0; CTAB 3% (w/v); 2-Mercaptoetanol 0.2% (v/v)) in a 6:1 ratio (v/w). The mixture was incubated for 20 min at 60°C, then extracted twice with isoamyl alcohol-chloroform. After adding isopropyl alcohol to the upper phase, the RNA was precipitated at 4°C for 1 h. The pellet obtained after centrifugation was washed with absolute ethanol and dissolved in 15 mM sodium citrate buffer, pH 7.0, containing 150 mM NaCl. The RNA was layered onto a cushion of 5.7 M CsCl in 25 mM sodium acetate, pH 6.4, and the gradient was centrifuged at 40,000 rpm with a Bekman Type 60 Ti rotor at 20°C for 24 h. The pellet obtained after centrifugation was dissolved in diethylenepyrocarbonate (DEPC)-treated water and precipitated by overnight incubation at -20°C after the addition of 0.1 vol (v/v) of 3 M sodium acetate pH 5.2 and 2.2 vol (v/v) of cold absolute ethanol. After washing the pellet with cold 70% DEPC ethanol, the RNA was dried, then dissolved in DEPC water. The samples were stored at -80°C.

SSH library construction. Poly (A)+ RNAs were isolated from the pools of the total RNAs extracted from leaves treated for 48 h with CP (T) or with sterile water (C) using the PolyATtract mRNA Isolation System (Promega, USA) following the the manufacturer's protocol.

Subtractive suppressive hybridization (SSH) was performed according to Diatchenko *et al.* (1996) using the BD PCR-Select cDNA Subtraction Kit (BD Biosciences,USA) after use of the BD SMART™ PCR cDNA Synthesis Kit (BD Biosciences Clontech,USA). The SMART procedure required 0.025–1µg of poly (A)+ mRNA to allow amplification of the complete mRNA population contained in each sample (treated and control). The cDNAs (T) were used as tester for forward subtraction and as driver for reverse subtraction according to the BD PCR-Select cDNA Subtraction kit. Amplified cDNA sequences from the forward and reverse subtractions were inserted directly into a T/A cloning vector using the TOPO TA Cloning Kit (Invitrogen, USA) according to manufacturer's instructions. The efficiency of subtraction was evaluated by PCR amplification using the 5.8S ribosomal gene (the subtraction was efficient if the 5.8S transcript was reduced); the primers were designed on the 5.8S rRNA gene and the internal transcribed spacers 1 and 2 of *Nicotiana tabacum* (accession No. AJ012363).

Differential screening of the subtracted cDNA libraries. The recovered clones were cultured in LB medium with 10 mg/ml ampicillin in 96-well plates at 37°C. The cDNA inserts were amplified by PCR with nested PCR primers 1 and 2R, which were complementary to the adaptors, to check the presence and size of the individual inserts.

PCR reactions (25 µl) contained 18.5 µl sterile water, 0.6 µl of each primer (10 µM each), 2.5 µl 10x reaction buffer (Euroclone, Italy), 1 µl $MgCl_2$ (Euroclone), 0.6 µl of dNTP mix (2.5 mM each), 0.25 µl Euro Taq polymerase (Euroclone) and 1 µl bacterial culture.

Samples were denatured at 94°C for 10 min, followed by 30 cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 1 min 30 sec, with a final extension at 72°C for 10 min. All PCR products were analysed by agarose-gel electrophoresis.

Two identical blots were created by spotting heated-denatured PCR products (1 µl) from each of the clones of the subtractive libraries onto nylon membranes positively charged (Roche, Germany) and cross-linked by UV.

The membranes were pre-hybridized for 3 h. in pre-hybridization buffer (DIG easy Hyb, Roche), then incubated overnight at 42°C in hybridization buffer (DIG easy Hyb containing the labelled cDNAs obtained by DIG-DNA Labelling Kit Nonradioactive, Roche).

The fragments that hybridized only with the labelled forward cDNA or that showed at least higher signals than the signals obtained with the reverse labelled cDNA were subjected to sequencing analysis.

Clone sequencing and analysis. Differentially expressed clones were sequenced by automated sequencing (MWG Biotech, Germany). Homology searches of all sequences were done in the GenBank EMBL-EBI database using the BLAST algorithm.

Semiquantitative determination of transcript levels by RT-PCR. Reverse transcriptions were carried out with total RNA (2 µg) isolated from CP-treated and control leaves of *P. acerifolia* with the iScript cDNA Synthesis kit (Bio-Rad, USA) according to manufacturer's instructions.

One µl of the first-strand cDNAs was used for each PCR amplification, and PCRs were done using gene-specific primers for chlorophyll a/b-binding protein for PSII, clone L2B (right, 5'-ACGAAGTTGGTGGCATAGGC-3'; left, 5'-GAGATCAAGAACGGGAGACTG-3'); for the selenium binding protein, clone L1B (right, 5'-TCTGAGGGTTCCTTCTTTGC-3'; left, 5'-ACTATGGCCTGGTTTCTCCC-3'); for the tubby-like protein, clone Q7H (right, 5'-CTCATGCTGGAGCCACAGT-3'; left, 5'-GCATCCATGACACACTGCAT-3'); for the RARI protein, clone L3A (right, 5'-GCCGGTCAATAGGAATCAGA-3'; left, 5'-ATCCTGGCCCTGCTATTTTC-3'); for Ferredoxin A, clone L2D (right, 5'-CAGGTTCTTGCTCTTCCTGC-3'; left, 5'-AGGTCCTCTTCCTTGTGGGT- 3'), and for elongation factor 1α (EF-1), clone Q11H (right, 5'-TCTCCACGCTCTTGATGACTCC-3'; left, 5'- CGGTGATGCTGGGTTTGTGAAG-3'). Housekeeping gene was the 18S ribosomal gene from Ambion's Competimer™

Technology (QuantumRNA™ Universal 18S Internal Standard) to modulate the efficiency of amplification without affecting the performance of other targets in multiplex PCR. The 18S competimers were modified at their 3' ends to block extension by DNA polymerase. By mixing the 18S primers with increasing amounts of 18S competimers, the overall PCR amplification efficiency of 18S cDNA could be reduced without primers becoming limiting and without loss of relative quantification. For each analysed gene we identified the cycles of the exponential phase and we did the same thing with the 18S rRNA using different mixes of primer-competitors and testing which mix was in the exponential phase in the same range of cycles as the analysed gene. The relative amounts of each PCR product were readily quantified by direct scanning with a densitometer of ethidium bromide-stained 2% agarose gel electrophoresis with Quantity One® Software (Bio-Rad, USA). To standardise the total RNA and the efficiency of cDNA synthesis from various tissue samples, the band intensities were standardised with the average intensity of the 18S product across the samples investigated. The ratio between the value of the analysed gene product level and the 18S product level of each sample was calculated from three independent experiments performed for each gene.

RESULTS

Localized resistance induced by CP. Figure 1 shows *Cep* grown for two days on a plane leaf surface treated with CP 15×10^{-5} M (b) or with sterile distilled water (a). The conidia were added 24 h after treatment with CP. When the leaves had been treated with CP, no hyphal growth occurred since conidia germination was completely inhibited. In similar experiments on glass slides, however, conidia germinated whether or not CP was present, with comparable mycelial growth rates (data not shown).

The inhibiting effects of treatment with CP at concentrations varying from 0.47 to 15×10^{-5} M on *Cep* after 2 days of growth on the leaves are shown in Fig. 2. Fungal growth, evaluated with an arbitrary scale considering the percentages of conidia germination and *Cep*-colonisation (as described in Materials and Methods), exhibited a linear inverse correlation with CP concentration from 0 to 7.5×10^{-5} M ($y= -0.4854x+3.885$, $r^2 = 0.9896$, $P < 0.0001$). The minimum concentration of CP that reliably (more than 30% of maximum inhibition) inhibited conidia germination and *Cep* colonisation was about 2×10^{-5} M. Fungal growth was restricted by more than 60% of the maximum with about 4×10^{-5} M CP and was only slightly more than nil when the leaves had been treated with 7.5×10^{-5} M CP.

Fig. 3 shows the time, starting from treatment with 7.5×10^{-5} M CP, that was necessary for plane leaves to block germination of *Cep* conidia and hyphal growth. CP treatment for 24 h, and still more for 48 h, drastically reduced fungal growth compared with the mycelial growth that occurred when the leaves were only treated with distilled water.

When 10 µl droplets of 15×10^{-5} M CP were placed on the lower leaf surface of *P. acerifolia*, the CP concentration of the droplets diminished between 12 and 24 h post treatment (HPT) (Fig. 4). The residual CP amount was about 50% of the maximum 24 HPT, and was almost nil 48 HPT. The residual CP on the leaves remained stable without any degradation as determined by RP-HPLC according to Carresi *et al.* (2006) (data not shown). The accumulation of umbelliferone equivalents in the droplets was positively correlated with the time after treatment; phytoalexin levels began to rise between 12 and 24 HPT almost at the same time as the concentration of CP in the droplets of the leaves first started to show signs of decline.

Construction of SSH libraries and identification of overexpressed genes. Tester and driver cDNAs were reverse transcribed from the mRNAs obtained from leaves treated for 48 h (T) with CP or with sterile water (C). SSH was performed using as tester the mRNAs isolated from the treated leaves, and as driver the mRNAs isolated from the control leaves, and vice versa. In this way we obtained a forward library, containing new or increased transcription products of genes induced by CP treatment and a reverse library, containing the prod-

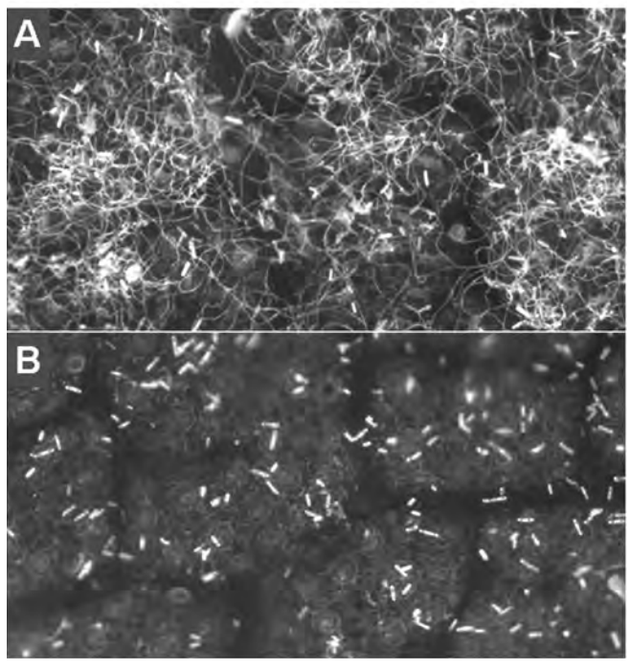

Fig. 1. *Ceratocystis platani* (*Cep*) grown for two days on plane leaves that had been treated 24 h earlier with (A) distilled water or (B) 15×10^{-5} M cerato-platanin. (Initial *Cep* conidial concentration *ca.* 8×10^2).

ucts of genes whose expression was suppressed or diminished by CP treatment. Forward and reverse libraries showed several bands with differential complexities ranging from 300 bp to 1200 bp (data not shown), but most of which were longer than 600 bp. The efficiency of subtraction was evaluated by PCR amplification using the 5.8S ribosomal gene (the subtraction was efficient if the 5.8S transcript was reduced). The 5.8S gene was detectable as a faint band in the unsubtracted forward (T–C) sample after only 8 amplification cycles of PCR, while in the unsubtracted reverse (C-T) sample it was detected after 15 cycles. No amplification was detected in the subtracted libraries after an equal number of PCR cycles (data not shown). After this result, the forward and reverse cDNAs were cloned and about 1600 independent clones were picked out from the forward and reverse subtractive libraries.

A cDNA select differential screening was carried out to determine the relative expression levels of the cloned cDNAs and to eliminate any false positive clones. Two identical blots were created by spotting PCR products and they were hybridized with the cDNAs from the forward and reverse subtracted libraries (Fig. 5). The differentially expressed genes were ascertained when their signals were detected or were more intense in the forward-subtracted pool than in the reverse subtracted one. We analysed sequences from the forward library by sequencing 78 differential clones selected from 576 clones of the forward subtractive libraries screened as described above. Analysis of the 78 clones with FASTA, BLASTN and BLASTX identified 72 putative upregulated genes, whereas the function of the other six clones was unknown (Table 1). Relative PCR analysis for the clones confirmed that they were overexpressed in the plane leaves 48 h after treatment with CP. Fig. 6 shows the results of 6 randomly selected clones: the chlorophyll a/b-binding protein for PSII (clone L2B), the selenium binding protein (clone L1B), the RarI protein (clone L3A), the elongation factor 1 alpha (clone Q11H), ferredoxin (clone L2D), and the tubby-like proteins (clone Q7H). In this study the primers for the studied gene were used

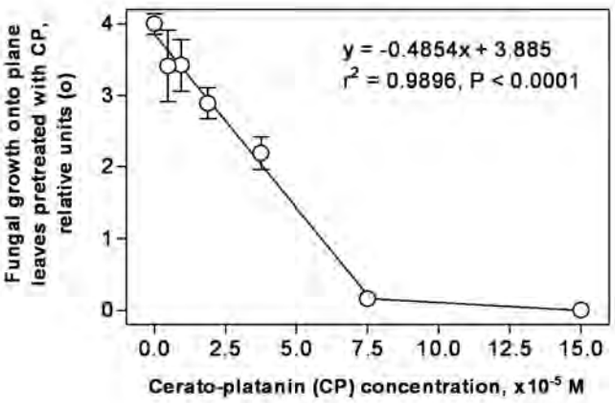

Fig. 2. *Ceratocystis platani* grown for two days on plane leaves treated with cerato-platanin at different concentrations. Fungal growth was scored according to the following scale: 0 = no conidia germination; 1 = <10 % conidia germination and <10% droplet area colonised by mycelium; 2 = 10-40 % conidia germination and 10-40% droplet area colonised by mycelium; 3 = 41-70 % conidia germination and 41-70% droplet area colonised by mycelium; 4 = >70 % conidia germination and >70% droplet area colonised by mycelium. A linear correlation was found between the relative fungal growth and CP concentrations between 0 and 7.5×10^{-5} M. Values are the means of 14 data from two independent experiments ± SEM. Error bars that are not visible are included in the symbols.

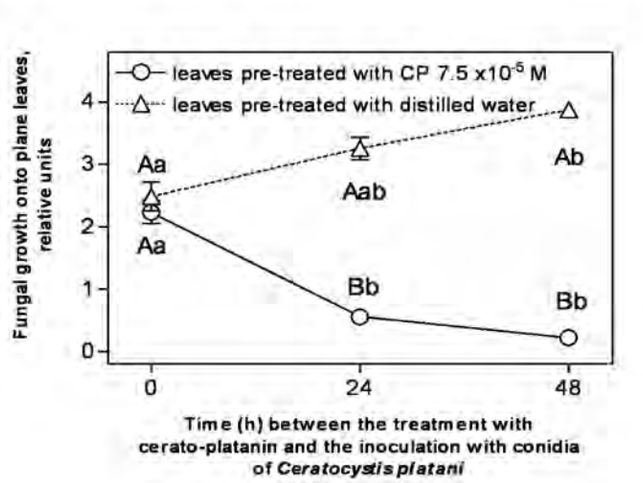

Fig. 3. *Ceratocystis platani* (*Cep*) grown for two days on plane leaves treated with distilled water droplets containing $7.5\text{x}10^{-5}$ M CP (○) or no CP (Δ) with various lengths of time (0, 24, 48 h) elapsing between treatment with CP and inoculation with *Cep* conidia. Values are the means of 20 measurements from two independent experiments ± SEM. For each length of time, the values marked with different capital letters are significantly different (at $P \leq 0.05$, according to the t-Student test). For each CP pre-treatment time, values marked with a different small letter were significantly different (at $P \leq 0.05$, according to the Tukey test).

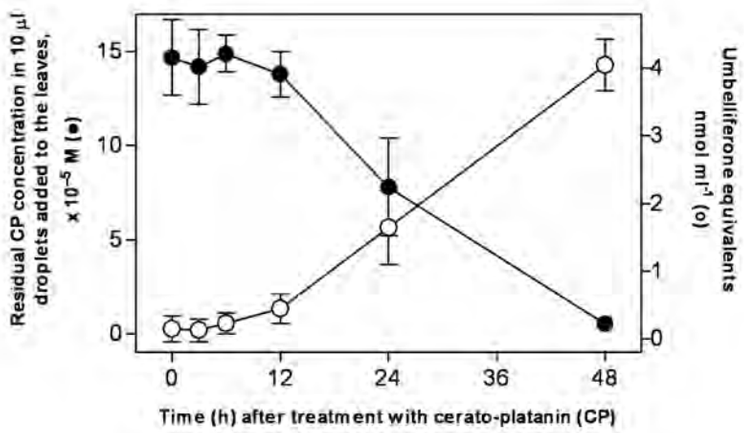

Fig. 4. Time course relations between the disappearance of CP from 10 µl droplets added to plane leaves and production of umbelliferone equivalents. Values are the means of 20 measurements from two independent experiments ± SEM.

Table 1. Identification of upregulated cDNA sequences from plane leaves treated with 15×10^{-4} M cerato-platanin.

Clone	Accession No[(a).]	Accession No. of matching sequence[(b)]	E value[(c)]	Putative identification[(d)]
DNA/RNA synthesis and metabolism				
L1C	AM260493	AAM19887.1	1e-79	Histone deacetylase
R1B	AM293618	BAA97391	1e-142	DEAD box helicase protein
A10D	AM260513	AAM61323	1e-81	Nucleotide sugar epimerase
C3D	AM286242	AAF22455	1e-11	MADS box protein
C3F	AM286254	NP_197031	1e-81	Putative nucleic acid binding protein
L4A	AM293609	NP_193524	2e-87	Zinc-binding family protein
R5C	AM260507	CAB75429	1e-117	Oligouridylate binding protein
R7A	AM260511	U9SA34	1e-136	Inosine monophosphate dehydrogenase
L9B	AM293617	BAB83610	5e-09	Putative transcription factor
Q1E	AM397239	AAF64454	3e-45	DnaJ-like protein
Q1A	AM397240	ABE87461	9e-05	Putative transcription factor
Protein synthesis/turnover				
F1G	AM260500	AAG51475	5e-74	Serine carboxypeptidase II
F5G	AM293611	CAB81384	3e-17	Putative ribosomal protein S10
L4F	AM286233	AAM64318	6e-70	Plastid ribosomal protein PRPL5
L5A	AM260506	XP_468840	2e-61	Hydrolase
Q11H	AM293616	AAC39447	1e-100	Elongation factor 1 alpha
A5H	AM260517	CAI11456	1e-45	Glycosyltransferase
C3C	AM286146	AAK15322	2e-106	FtsH protease, chloroplast precursor
C6G	AM286248	ABD66517	5e-122	Translation elongation factor 1 alpha
R4B	AM260495	NP_198638.2	3e-57	Catalytic protein with α/β hydrolase domain
R1F	AM260496	AAZ43369.1	1e-96	Alanine aminotransferase
F10F	AM260499	BAD81372	7e-168	Histidinol dehydrogenase
R2F	AM260502	S30145	9e-46	Ketol-acid reductoisomerase
Q6G	AM293614	NP_564815	7e-61	D-alanyl-D-alanine endopeptidase/ peptidase
C6F	AM397234	Q8L7H3	2e-05	Putative xyloglucanendotransglucosylase /hydrolase
Energy				
L2B	AM286231	AATO8647	1e-117	Chloroplast chlorophyll a/b-binding protein, PSII
F10C	AM286234	CAA45523	2e-67	Chloroplast chlorophyll a/b binding protein, PS I
L2D	AM286232	P09911	4e-25	Ferredoxin A
Q1B	AM293623	X60008	2e-42	Photosystem I reaction center subunit II
Q2D	AM293608	BAB71853	8e-30	Phosphoenolpiruvate carboxylase kinase
C5G	AM286245	AAW21667	4e-33	Ribulose-bisphosphate carboxylase
A4H	AM260516	AAM6833	5e-54	Phospho-glycerate dehydrogenase
Cellular metabolism				
A11H	AM293622	AY162465	1e-122	Glutamine synthetase
F4C	AM286255	CAA63981	7e-108	Cytosolic Glutamine synthetase
A11A	AM260515	AAL69511	3e-85	AMP-binding protein
Q2B	AM260498	T52308	7e-61	Fatty acid condensing enzyme CUT1
F9E	AM286237	AAL49750	2e-28	Aquaporin
L5B	AM293612	CAA04451	1e-55	Potassium channel beta subunit
F9A	AM293607	T07808	2e-91	Inorganic phosphate transporter
C6H	AM286250	CAC84545	4e-93	Dicarboxylate/tricarboxylate carrier
F9B	AM260510	BAA88226.1	1e-66	Thiamine biosynthetic enzyme
F4F	AM293615	XM_479463	2e-140	Arginine N-methyltransferase protein
L1B	AM260504	CAC65501	2e-92	Selenium binding protein
L5G	AM260508	AAS17751	5e-105	Beta-xylosidase
Q4G and Q7H	AM2604505	AAM20254	1e-102	TUBBY-like proteins (TULPs)
R12D	AM260497	XT479475.1	3e-97	TGF-beta inducible protein
F4D	AM293613	AAK11299	5e-88	ADPglucose pyrophosphorylase
Signalling				
C7H	AM286251	CAA54803	5e-47	Shaggy/glycogen synthase kinase-3 like protein
Defence and/or stress related proteins				
L3A	AM286235	AAM62409	1e-54	Rar1 protein
L8B	AM260509	AAS44667	1e-80	β-1,3 glucanase

R1C	AM293619	AAM44961*	9e-15	Thaumatin protein
A11B	AM286253	NP_849614	5e-33	Aldehyde lyase (threonine aldolase activity)
A12D	AM293606	AAK72616	2e-59	Actin-depolymerizing factor
C4G	AM286244	CAB51533	8e-32	Galactinol synthase
C5H	AM286249	AAL27855	3e-40	Lipid transfer protein
A6H	AM260514	AAB81996	6e-04	Translation initiation factor eIF-1A
C3E	AM286243	AAS80139	3e-17	Arachidonic acid-induced DEA1-like
A9B	AM286239	XP_470175	1e-19	Putative ubiquitin protein
L1E	AM260494	AAV31238	4e-110	26S proteasome non-ATPase regulatory subunit 1
A12B	AM286240	AAC24708	1e-40	Ribulose-phosphate 3-epimerase
C8H	AM286252	AAOO33154	9e-121	Transketolase
C7G	AM293605	CAB61246	1e-77	Putative serine/threonine kinase
L8A	AM260492	O22432	1.7e-25	Putative cell wall protein (glycine-rich protein)
Other proteins				
C6E	AM397241			Putative Ty1-copia reverse transcriptase and partial DfRedu pseudogene
A10C	AM286247	CAB79665	2e-56	Hypothetical protein
A12F	AM286241	BAB02703	1e-103	Hypothetical protein
F3G	AM286238	AAU44392	2e-58	Hypothetical protein
FC9	CAL25343	AAC79135	1e-06	Hypothetical protein
Q8E	CAL25354	AY057515	8e-29	Hypothetical protein
L1A	AM286236	AAL76137	1e-36	Hypothetical protein
L4G	AM260503	XP_464048	7e-35	Hypothetical protein
F3F	AM397237			EST
F3H	AM397238			EST
L4B	AM397236			EST
F9F	AM293624			EST
C7F	AM397235			EST
F9H	AM397242			EST

[a]Accession number of the clones; [b]Accession number of the best match sequence; [c]E value of the best match sequence, calculated by BLAST analysis; [d]Functional assignment based on sequence similarity.

together with those for the housekeeping gene. The internal control expression was the same in all the analysed samples so as to standardise the data.

Many of the over-expressed clones could be classified into more than one category on the basis of the metabolic processes they were associated with. For this reason the up-regulated clones listed in Table 1 were classified into 6 putative macro groups taking into account the functional categories established for *Arabidopsis* (The Arabidopsis Genome Initiative, 2000). The macrogroups related to: DNA/RNA synthesis and metabolism (14.1%), protein synthesis/turnover (18.0%), energy (9.0%), cellular primary metabolism (19.2%), the signalling pathways (1.3%) and defence and/or stress related proteins (19.2%). Clones matching genes encoding hypothetical proteins and EST were included in the group "Other proteins" (18.0%).

The percentage of over-transcribed genes involved in defence responses was very high and contained genes known to be involved in the resistance reaction of many plant-microbe interactions. This group included genes encoding, for example, the Rar1 protein, a β-1,3 glucanase, thaumatin, a lipid transfer protein, the translation initiation factor eIF-1A, a serine/threonine kinase, and the enzymes ribulose-phosphate 3-epimerase and transketolase, which are involved in the pentose phosphate pathway. An important proportion of genes was involved in DNA synthesis (histone deacetylase, DEAD box helicase, and various transcription factors), in RNA processing (oligouridylate binding protein) and in protein synthesis/turnover (putative ribosomal protein S10, elongation factor 1 alpha, FtsH protease chloroplast precursor, catalytic protein with α, β hydrolase domain). Other over-expressed genes in plane leaves in response to CP were involved in the primary metabolic pathways, like photosynthesis (ribulose-biphosphate carboxylase, chloroplast chlorophyll a/b-binding protein, PSII and PSI and photosystem I reaction centre subunit II), nitrogen metabolism (glutamine synthetase and cytosolic glutamine synthetase), lipid metabolism (AMP-binding protein and fatty acid condensing enzyme CUT1) and starch synthesis (ADPglucose pyrophosphorylase, a key enzyme in starch synthesis).

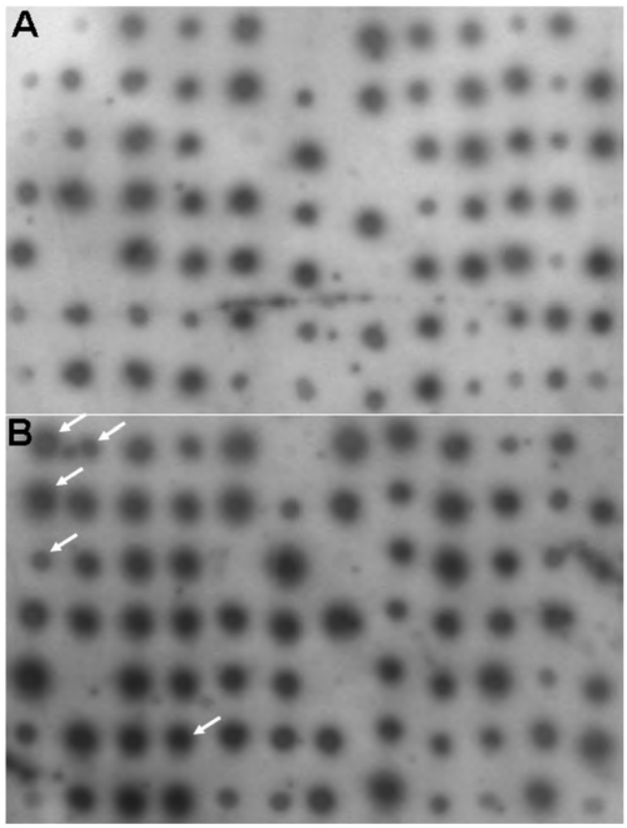

Fig. 5. Differential screening of clones from forward library. Duplicate membranes with Dot blot PCR products from forward subtractive clones were hybridized with cDNAs derived from the reverse library (B) and with cDNAs derived from the forward library (B). Arrows indicate candidate clones differently expressed in the treated leaf samples.

DISCUSSION

Leaves from *Cep*-susceptible resistant clones of *P. acerifolia* and *P. occidentalis* were differently colonised when *Cep* conidia were applied to their surface (El Modafar *et al*., 1995). *Cep* grew abundantly only on the leaves from susceptible clones, whereas its growth was partially or completely inhibited on the leaves from resistant clones. The lower fungal growth on resistant clones was correlated with higher levels of phytoalexin. The same leaves also exhibited higher levels of phytoalexin after treatments with jasmonic acid or a glycoprotein elicitor produced by *Cep* (Clérivet and Alami, 1999). The leaves were therefore a good choice to study the pathogenic interaction between plane and *Cep*, even though *Cep* colonises the bark and wood rather than the leaves of plane trees. More recently *Cep*-susceptible *P. acerifolia* leaves have been found to react to CP treatment in a resistance-like manner, producing extracellular and intracellular phenolic compounds and undergoing structural disorganisation of the cells (Scala *et al*., 2004; Bennici *et al*., 2005, 2006). The levels of phytoalexins in CP-treated droplets were the same as or higher than those reported by Clérivet and Alami (1999) and El Modafar *et al*. (1995), when they utilised resistant *P. occidentalis* clone-derived leaves. This 'resistance' response, including for example phytoalexin synthesis, induced by a non-host specific elicitor in a susceptible plant, has been known for a long time in other elicitor-treated plants (Buiatti *et al*., 1985; Storti *et al*., 1988; Schaffrath *et al*., 1995; Tavernier *et al*., 1995). Here other factors in the pathogen and/or the plant determine the degree of com-

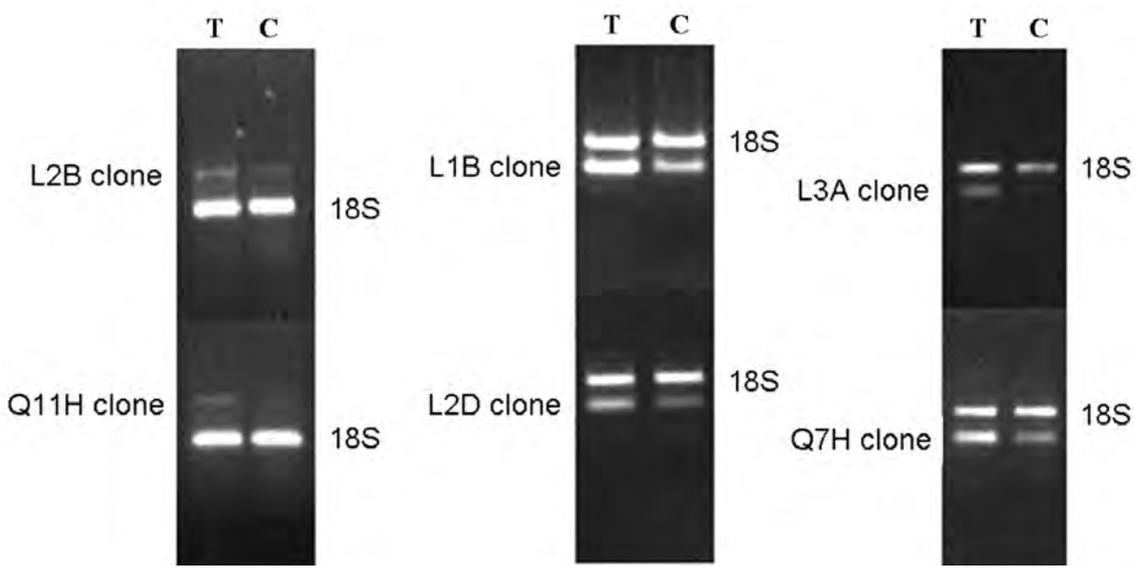

Fig. 6. RT-PCR analysis of selected clones: clone L2B (chlorophyll a/b-binding protein for PSII); clone L1B (selenium binding protein); clone 3A (RARI protein); clone Q11H (EF-1 alpha); clone L2D (ferredoxin), and clone Q7H (tubby-like proteins). The 18S rRNA gene was used as the internal control gene. T = total RNA isolated from CP-treated leaves; C = total RNA isolated from control leaves.

patibility during the process of natural infection.

In the present work we showed that when the CP protein was applied to the lower surface of *P. acerifolia* leaves, its concentration decreased from an initial concentration of 15×10^{-5} M to slightly more than 0 after 48 h (an evident decrease was already found after 24 h), but this was not due to the proteolytic degradation of protein. Almost at the same time levels of phytoalexin rose and there was a decrease in the antimycotic activity of CP itself, since *in vitro* there were no significant differences between conidial germination and hyphal length of *Cep* cultured with CP and *Cep* cultured without. CP was active at a minimum concentration of about 2×10^{-5} M, while maximum mycelial growth inhibition already occurred at a concentration of about 7.5×10^{-5} M. Therefore CP acted at low concentrations like some other very active substances that elicit defence responses in plants, such as, for example, the α-elicitins and the oligogalacturonides (Le Berre *et al*., 1994; Huet *et al*., 1995; Ferrari *et al*., 2003).

The overall conclusion that emerged from the findings was that CP was absorbed from the leaf surface and/or into the leaf tissues which recognised it as a non-self substance, triggering a defence response against *Cep*. The hypothesis that CP was an effective defence response elicitor was largely confirmed by SSH, which revealed that many defence-related genes were over-transcribed in plane leaves following CP treatment. Since one half of the leaf was treated with CP, and the other half was used as the control, it could not be excluded that CP had an eliciting action with systemic effect even in the untreated half-leaf. In that case the extent of over-expression of some of the genes may have been underestimated.

SSH is an effective method to isolate genes that are specifically and differentially transcribed under various conditions or in response to various biotic and abiotic stresses. SSH has not frequently been used to study plant interactions with pathogen or elicitors; one of the first was by Dellagi *et al.* (2000) who characterised the gene *St-WRKY1* that was up-regulated in potato leaves after inoculation with *Erwinia carotovora* subsp. *atroseptica*. WRKYs are elicitor-induced proteins that bind the sequence TGAC, or W box, in the promoters of PR-proteins genes and appear to be responsible for the up-regulation of these genes.

SSH has provided new and interesting information about the genes involved in the defence reaction over the last few years. Kong *et al.* (2005) inoculated *Triticum aestivum* 'Ning7840', one of the few wheat cultivars with resistance to *Fusarium graminearum*, with this pathogen, and demonstrated that some defence related genes, like chitinase, were involved in resistance. Degenhardt *et al.* (2005) reported that many genes were differentially transcribed in apple leaves depending on whether the leaves were resistant or susceptible to *Venturia inequalis*. Some typical defence genes, such as β-1,3 glucanase, cysteine protease inhibitor and superoxide dismutase, were over-transcribed only in the resistant cultivar. Recently, Lotan-Pompan *et al*. (2007), combined SSH and cDNA-amplified fragment-length polymorphism (cDNA-AFLP) and isolated a significant number of clones similar to genes that were formerly described as stress- or defence-related. All these authors suggested that the over-expressed genes identified by SSH were the result of a primary induction rather than of subsequent secondary effects such as tissue degradation due to pathogen colonization or chemical treatment.

In our study CP altered the expression pathways of many genes at various regulation steps relating to gene accessibility, the initiation of transcription and the synthesis of RNA. Important transcriptional variations were observed at the level of protein synthesis/turnover and primary metabolism. A total of 78 new *P. acerifolia* gene-expressed sequences were identified, the majority of which were more than 600 bp long. A number of these sequences belonged to genes involved in the defence reaction. For example, clone L3A corresponded to the *Rar1* gene that codes for protein that regulates R-gene mediated resistance in various plant species (Freialdenhoven *et al*., 1994; Liu *et al*., 2002). The genes coding for 26S proteasome non-ATPase regulatory subunit 1 and ubiquitin were also found to be involved in the resistance response of plane leaves elicited by CP.

The ubiquitin/26S proteasome-dependent proteolytic pathway has emerged as a powerful regulatory mechanism in a wide range of cellular processes (Ciechanover, 1998; Zeng *et al*., 2006). A number of findings have recently suggested that ubiquitin-mediated protein degradation may also act as a regulatory mechanism in plant defence by removing the negative regulators of the resistance response (Muskett and Parker, 2003). Other ESTs corresponded to pathogenesis-related (PR) proteins, which are induced in host plants and accumulate as a result of pathogen infection or abiotic stress conditions (Kim and Hwang, 2000).

Thaumatin-like proteins (TLPs) belong to the 5 (PR-5) group: they are proteins commonly found in both monocotyledonous and dicotyledonous species in response to pathogen infection, and they have an antifungal activity (Morris *et al*., 1998; Selitrennikoff, 2001). Another PR protein was β-1,3 glucanase (PR-2), which is an endoglycohydrolase enzyme widely distributed in the higher plants and which has been known for a long time to be involved in resistance against invading fungi (Neuhaus *et al*., 1992; Buchanan *et al*., 2000; Agrios, 2005). Moreover, ribulose-phosphate 3-epimerase and transketolase are two key enzymes of the pentose-phosphate pathway, which is the main source of phenolic compounds in plants and has also long been known to be associated with defence mechanisms (Buchanan *et al*., 2000; Agrios, 2005). Cells under stress change their

primary metabolism in important ways, not only to provide building blocks and energy for the biosynthesis of defence compounds but also to enable cross talk to occur between the defence responses and other signalling pathways (Schenk *et al*., 2000).

Even when they were not included in the macrogroup 'Defence and/or stress related proteins', many other genes were over-expressed after elicititation by CP, and were indirectly involved in pathogen- and abiotic stress-responses. These genes include those encoding the histone deacetylase, the DEAD box helicase protein, the chloroplast chlorophyll a/b-binding protein, PSII, and other genes that are photosynthesis-related. For example, the histone acetylation is involved in the plant response to abiotic stresses (Kim *et al*., 2004; Lee *et al*., 2005; Song *et al*., 2005; Zhou *et al*., 2005; Sridha and Wu, 2006); and the overexpression of the HDA19 gene in *Arabidopsis* induces ethylene and jasmonate-regulated PR-proteins production and increases resistance to a plant pathogen. The first report of a stress-induced helicase gene in plants was in a cDNA microarray analysis of 1300 *Arabidopsis* genes in which a DEAD-box helicase gene was found to be a cold stress-inducible gene, suggesting that helicases had a role in stress signalling (Seki *et al*., 2001).

It has been suggested that there is cross talk between defence responses and other signalling pathways; the induction of genes encoding chlorophyll a/b-binding proteins by the salicylic acid treatment is one of the best examples of such a coordinate response (Schenk *et al*., 2000). Genoud *et al*. (2002) examining variegated mutants that form chloroplast-deficient leaf zones near intact green areas found that a signal coming from both the salicylic acid and the phytochrome pathways regulated the hypersensitive response (HR) induced by an avirulent pathogen, whereas the expression of PR proteins was not changed when the active chloroplasts were reduced. It has also been reported that photosynthesis-related proteins, such as the rubisco small subunit, several photosystem subunits, and chlorophyll a/b binding proteins were significantly down-regulated during proteasome-mediated programmed cell death, but up-regulated during hypersensitive cell death (Kim *et al*., 2006).

In conclusion, earlier studies by our team focused on the capacity of CP to synthesise phenolic compounds and/or phytoalexin when it was applied to host and non host plant tissues, and to cause the intercellular and intracellular disorganisation of plane spongy parenchyma cells, and plant cell plasmolysis and/or necrosis. The present study presents evidence that in CP-treated plane leaves such synthesis was correlated with the inhibition of *Cep* growth on those leaves and a high level of transcripts of numerous defence-associated and regulatory genes. Following treatment with CP there was a substantial network of regulatory interaction and coordination among different plane defence pathways, which so far are not completely known. This result opens up the prospect of examining how CP interacts with the host cells (the hydrophobic surface of the leaf and/or a putative cell receptor). The availability of the 3D solution structure of recombinant CP (Oliveira *et al*., 2006; A. Spisni *et al.*, personal communication) will also make it possible to carry out new studies aimed at finding out which part of the protein is active as an elicitor of a defence response in plane.

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