



Contents lists available at ScienceDirect



Plant Science

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

Review

Cerato-platanins: Elicitors and effectors

Luigia Pazzaglia^a, Verena Seidl-Seiboth^b, Mario Barsottini^c, Walter A. Vargas^d, Aniello Scala^e, Prasun K. Mukherjee^{f,*}

^a Department of Biomedical Experimental and Clinical Sciences, University of Florence, Morgagni Street, 50134 Florence, Italy

^b Biotechnology and Microbiology, Institute of Chemical Engineering, Vienna University of Technology, Gumpendorfer Strasse 1a, 1060 Vienna, Austria

^c Department of Genetics, Evolution and Bioagents/IB, State University of Campinas, Cidade Universitária Zeferino Vaz, 13083-970, Campinas, Brazil

^d Centro de EstudiosFotosintéticos y Bioquímicos (CEFOBI)-CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, Rosario 2000, Argentina

^e Department of Production Sciences Agri-Food and the Environment (DISPAA), University of Florence, Sesto Fiorentino, 50019 Florence, Italy

^f Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Trombay, Mumbai 400085, India

ARTICLE INFO

Article history:

Received 19 November 2013

Received in revised form 20 February 2014

Accepted 22 February 2014

Available online xxx

Keywords:

Cerato-platanin

Elicitor

Effector

Fungi

Self-assembly

Chitin-binding

ABSTRACT

Cerato-platanins are an interesting group of small, secreted, cysteine-rich proteins that have been implicated in virulence of certain plant pathogenic fungi. The relatively recent discovery of these proteins in plant beneficial fungi like *Trichoderma* spp., and their positive role in induction of defense in plants against invading pathogens has raised the question as to whether these proteins are effectors or elicitor molecules. Here we present a comprehensive review on the occurrence of these conserved proteins across the fungal kingdom, their structure–function relationships, and their physiological roles in plant pathogenic and symbiotic fungi. We also discuss the usefulness of these proteins in evolving strategies for crop protection through a transgenic approach or direct application as elicitors.

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* Corresponding author. Tel.: +91 2225590367; fax: +91 2225505151.

E-mail addresses: prasunmala@gmail.com, prasunmukherjee1@gmail.com (P.K. Mukherjee).

1. Introduction

Plant pathogenic fungi secrete a plethora of proteins that are involved in various aspects of parasitism and in the development of disease, such as dissemination by vectors, attachment to the surface of plant organs, expression of symptoms, and elicitation of defense responses. Among these proteins are various classes of enzymes, receptors and non-catalytic secreted fungal proteins that were defined by Templeton et al. [1] as cysteine-rich, secreted proteins. This large group of cysteine-rich proteins is highly diverse and the classification proposed by Templeton et al. [1] groups them based on various parameters, including the number of cysteines, their molecular weight, and role in pathogenic process, which, in many cases is not fully understood. Currently, on the basis of their primary sequence, seven non-catalytic fungal protein families have been identified, including cerato-platanin (CP) family (PF07249), class I hydrophobins (PF01185), class II hydrophobins (PF06766), elicitors (PF00964), the Pcf family (PF09461), and the NIP-1 family (PF08995). Some other fungal proteins for which a role in pathogenesis has been proposed contain a cysteine-rich domain belonging to the CFEM family (PF05730). Among these protein families, hydrophobins have received considerable attention in research and several functions have been described for them. They are uniquely found in fungi and self-assemble at hydrophobic-hydrophilic interfaces, thereby inverting the polarity of the surface and decreasing the surface tension of aqueous liquids [2]. When fungal hyphae emerge from aqueous growth medium to form aerial hyphae and produce conidia, these growth structures are covered with a layer of hydrophobins that render them hydrophobic [3]. Hydrophobins have been reported to be involved in several aspects of fungal development and in pathogen–host interactions [2].

Hall et al. [4] were the first to identify a CP-coding gene from a phytopathogenic fungus by describing the accumulation of transcripts of *snodprot1* from *Phaeosphaeria nodorum* (anamorph: *Stagonospora nodorum*) during infection of wheat leaves. Shortly after, Pazzaglia and colleagues [5,6] presented the first functional characterization of the type protein for this family – the CP from *Ceratocystis platani*, a necrotrophic fungus that infects plane trees (*Platanus* sp.). CP is secreted into the culture filtrate, and is also found in the cell wall of ascospores, conidia and hyphae [7]. An orthologous protein from *Ceratocystis populincola* (causal agent of a poplar disease), which was named Pop1, shows similar features [8].

CP proteins occur in filamentous fungi and most of the proteins have been shown to act either as virulence factors or as elicitors [9]. For example, Snodprot1 from *Phaeosphaeria nodorum* is required for virulence [10]. Sp1 from *Leptosphaeria maculans* induces auto-fluorescence in *Brassica napus* leaves [11] and MpCP1 from *Moniliophthora perniciosa* exhibits necrosis-inducing ability in tobacco and cacao leaves [12]. Sm1 and Epl1 proteins from *Trichoderma virens* and *Trichoderma atroviride* induce systemic resistance [13–16] and MgSM1 CP-like protein from *Magnaporthe oryzae* is able to induce enhanced disease resistance and HR (hypersensitive response) in leaves [17]. HR is also elicited by the recently identified BcSpl1, one of the most abundant proteins in the *Botrytis cinerea* secretome [18–20]. In addition, members of CP family play a major role in animal–fungus interactions, where they exhibit allergenic properties and induce strong immunological reactions. Examples are *Coccidioides*-specific antigen from *Coccidioides immitis* and AspF13 antigen from *Aspergillus fumigatus*. Both these fungi are causative agents of human lung diseases [21,22]. Another CP family member, Aca1 from the medicinal fungus *Antrodia camphorata*, acts as an immune modulatory protein by stimulating macrophage activation and eliciting pro-inflammatory responses [23].

CP proteins are found both in Ascomycete and Basidiomycete fungi. A phylogenetic analysis suggested that there were multiple

copies of cerato-platanins in the ancestor of Dikarya that got differentiated into Ascomycetes and Basidiomycetes [24] and were lost in some taxa, e.g., the Saccharomycotina and the Pucciniomycotina. No CP-like proteins are found in prokaryotes. CP proteins are expressed in organisms with a variety of life styles, ranging from plant pathogens, to endophytes, saprotrophs and nematophagous fungi. This might suggest that the primary biological role of CP proteins is related to a somewhat broader aspect of fungal development.

However, growing evidence indicates that the various CP proteins do not always share the same biochemical and biological properties. Their biological significance can diverge even among paralogs. Here we will discuss what is currently known about CP protein family and their possible roles during fungal development in different scenarios with a particular attention to their role in fungus–plant interactions.

2. Understanding the structure of cerato-platanins

2.1. General structural features

CP proteins constitute a well conserved family with a 70% similarity at some conserved motifs [24]. Fig. 1 shows the alignment of some representative members of the CP family whose sequences have been verified at protein level. Among these, the sequence identity is 13% and the sequence similarity is about 40%, an unusually high value if compared with other secreted fungal protein families. All CP proteins possess a signal peptide of about 16–19 amino acids and the mature proteins are 120–134 amino acids long. Many of the conserved amino acids are hydrophobic and all these proteins contain four cysteines forming two disulfide bridges [25]. Moreover, an amino acid signature sequence has recently been highlighted: 63% of the cerato-platanin proteins contain the CSD signature and 19% contain CSN [24]. Most of the CPs lack post-translational modifications, but some proteins such as Sm1 from *Trichoderma virens* present the motif DNGRS which is glycosylated. Glycosylation has been reported to prevent the formation of a dimer, which is a less active form of the protein with respect to the induction of plant defense responses [16]. On the contrary, other non-glycosylated proteins such as CP and Pop1 show higher biological activity when these are in a polymeric, aggregate form [8,25]. Therefore, the presence of a glycosylation motif in some CPs can be a structural feature responsible for a specific mechanism of action as reported for dimerization of the glycosylated Sm1 and aggregation of CP and Pop1 [16,25].

Nevertheless, on the basis of sequence similarity, the PFAM database has defined the 1–119 sequence of the mature CP as the “cerato-platanin domain” which is widespread in filamentous fungi (more than 130 sequences are identified in Ascomycetes and Basidiomycetes) [24].

2.2. The 3D structure of cerato-platanins

The first 3D structure was obtained for the founder member of the cerato-platanin family (CP, Protein Data Bank (PDB) ID2kqa)) by NMR techniques [26]. Afterwards, other structures were determined using crystallography methods: Sm1 from *T. virens* (PDB 3m3g) and MpCP1, MpCP2, MpCP3 and MpCP5 from *M. perniciosa* (PDB 3suj, 3sul, 3sul and 3sum, respectively) [27]. The structures elucidated till date show a high level of homology even at the 3D-level and particularly the comparison of CP, Sm1 and MpCP3 highlights the conservation of main structural features as discussed here. All of them comprise a single domain containing the double- $\psi\beta$ -barrel fold remarkably similar to those found in plant and bacterial expansins, lytic transglycosylases (LTs), endoglucanases,

Aca1_tr Q6J935 Q6J935	-----VNVIYDPPFDNPNNLSLYVA <u>CSDSTNGLLTK</u> -GYTTLGSILPDEPYIGGAYA <u>JACWNS</u> -PSCGGTCWEL--TYNNV-----SINILGID--TAAG--ENIALTAMNVLTNNA <u>AVDLGEVD</u> -AAAIVQDVSSVCGL-----
Acpf15_sp O60022 AL15	-----I PVSVDYDPYRDNAGTSMDVCS <u>NCNGVNLVTK</u> --WPTFGSVPGFARI <u>GGA</u> PTPGWNS-PNCGGCKYKL--QYEON-----TIVYTAIDAAPOG--ENIATSAMD <u>QLTNGM</u> AVELGRVQ-A [*] TYEEADP <u>SHCAG</u> -----
BcSp1_gi 154320365	-----ITVSYDVGYYDASR <u>SLAVVCS</u> CDGS <u>NGNL</u> LTK--GTTQ <u>GS</u> LN <u>PI</u> IG <u>GA</u> SVV <u>VAG</u> W <u>N</u> D-ANC <u>GG</u> Y <u>CL</u> U <u>SY</u> GR--SINVLVIDHAGAG-ENI <u>GR</u> QAL <u>NT</u> LTG <u>Q</u> AA <u>AL</u> GRID-ASYTQVD <u>KSA</u> CG <u>L</u> -----
CP_sp P81702	-----VSI <u>SY</u> DP <u>Y</u> IA-ADLM <u>SGMSV</u> AC <u>SG</u> H <u>GL</u> MA <u>O</u> --YPT <u>LE</u> W <u>GP</u> E <u>GP</u> V <u>GI</u> P <u>DI</u> FAGW <u>D</u> -PSCGGTCW <u>V</u> T <u>I</u> PN <u>G</u> NS-----IPIRGV <u>D</u> -SGRG <u>GN</u> VNP <u>TA</u> FTK <u>L</u> V--STEAGRV <u>D</u> NVNYQ <u>Q</u> VD <u>L</u> NC <u>ING</u> AN-----
CS-AG_tr Q1E8D2 AG19	-----TPL <u>AST</u> TT <u>D</u> YD <u>PS</u> 1 <u>AL</u> S <u>LG</u> PS <u>CS</u> GD <u>GN</u> MT <u>TK</u> --YNT <u>AGE</u> D <u>2</u> IP <u>N</u> Y <u>P</u> H <u>G</u> G <u>A</u> F <u>T</u> WT <u>W</u> N <u>S</u> -PNC <u>GG</u> Y <u>KK</u> V--TYN-----AKT <u>TP</u> I <u>D</u> AI <u>H</u> SN <u>S</u> -EN <u>IA</u> K <u>KK</u> SMD <u>V</u> LN <u>TR</u> GA <u>EL</u> GR <u>K</u> -VIVYBE <u>V</u> ASS <u>L</u> CG <u>L</u> K-----
Epl1_tr G9IS53 G9IS53	-----DT <u>V</u> S <u>Y</u> DT <u>G</u> DD <u>G</u> RS <u>SL</u> TA <u>V</u> S <u>C</u> SD <u>G</u> EN <u>GL</u> LT <u>Y</u> W <u>T</u> O <u>G</u> E <u>V</u> S <u>N</u> P <u>Y</u> I <u>G</u> G <u>V</u> G <u>O</u> Y <u>W</u> N <u>S</u> -T <u>Q</u> CG <u>T</u> CH <u>R</u> -----BY <u>GR</u> --SIN <u>IL</u> LA <u>D</u> AA <u>Y</u> NG <u>G</u> NI <u>AL</u> K <u>AL</u> DT <u>L</u> TD <u>G</u> H <u>A</u> VE <u>W</u> GH <u>Y</u> D- <u>AV</u> AT <u>OV</u> S <u>V</u> NE <u>C</u> GL <u>FT</u> V <u>Y</u> -----
Epl2_tr G9MXR6 G9MXR6	-----TW <u>G</u> SP <u>D</u> IT <u>G</u> DD <u>P</u> S <u>R</u> SM <u>T</u> Q <u>V</u> A <u>C</u> SD <u>G</u> V <u>GN</u> LT <u>I</u> TKY <u>W</u> T <u>O</u> G <u>E</u> Y <u>V</u> S <u>N</u> P <u>Y</u> I <u>G</u> G <u>V</u> G <u>O</u> Y <u>W</u> N <u>S</u> -T <u>Q</u> CG <u>T</u> CH <u>R</u> -----BY <u>GR</u> --SIN <u>IL</u> LA <u>D</u> AA <u>Y</u> NG <u>G</u> NI <u>AL</u> K <u>AL</u> DT <u>L</u> TD <u>G</u> H <u>A</u> VE <u>W</u> GH <u>Y</u> D- <u>AV</u> AT <u>OV</u> S <u>V</u> NE <u>C</u> GL <u>FT</u> V <u>Y</u> -----
MpCP1_tr B2C3H7 B2C3H7	-----AG <u>AV</u> K <u>L</u> S <u>Y</u> EA <u>D</u> DN <u>P</u> S <u>U</u> SS <u>L</u> S <u>V</u> T <u>C</u> SD <u>G</u> V <u>GN</u> LT <u>I</u> TKY <u>W</u> T <u>O</u> G <u>E</u> Y <u>V</u> S <u>N</u> P <u>Y</u> I <u>G</u> G <u>V</u> G <u>O</u> Y <u>W</u> N <u>S</u> -T <u>Q</u> CG <u>T</u> CH <u>R</u> -----BY <u>GR</u> --SIN <u>IL</u> LA <u>D</u> AA <u>Y</u> NG <u>G</u> NI <u>AL</u> K <u>AL</u> DT <u>L</u> TD <u>G</u> H <u>A</u> VE <u>W</u> GH <u>Y</u> D- <u>AV</u> AT <u>OV</u> S <u>V</u> NE <u>C</u> GL <u>FT</u> V <u>Y</u> -----
MpCP2_tr B2C3H9 B2C3H9	-----T <u>G</u> AV <u>Q</u> L <u>R</u> F <u>Y</u> NT <u>I</u> DN <u>A</u> GS <u>M</u> NT <u>V</u> AC <u>ST</u> G <u>AN</u> GL <u>QR</u> --P <u>F</u> PT <u>G</u> S <u>V</u> TF <u>P</u> H <u>I</u> G <u>A</u> SS <u>D</u> GG <u>F</u> NS-PAC <u>CC</u> NC <u>Y</u> TI <u>S</u> TF <u>Q</u> GV <u>T</u> --RS <u>IN</u> LV <u>A</u> DI <u>H</u> AG <u>NG</u> -EN <u>V</u> QA <u>Q</u> AM <u>DE</u> LT <u>N</u> GA <u>VL</u> AG <u>GT</u> -----V <u>Q</u> S <u>Q</u> VAR <u>S</u> VG <u>G</u> -----
MpCP3_tr B2C3H11 B2C3H11	-----VA <u>V</u> Q <u>L</u> Q <u>Y</u> D <u>U</u> AD <u>Q</u> S <u>F</u> GT <u>S</u> X--GA <u>V</u> D <u>T</u> GT <u>W</u> N <u>S</u> -ES <u>CG</u> TC <u>Y</u> Q <u>I</u> --T <u>W</u> ST <u>G</u> --K <u>I</u> TH <u>V</u> G <u>V</u> D <u>V</u> AG <u>NG</u> -EN <u>V</u> GU <u>R</u> AM <u>D</u> LT <u>N</u> Q <u>U</u> AV <u>AL</u> GN <u>I</u> D- <u>V</u> TA <u>T</u> LD <u>K</u> SA <u>CR</u> L-----
Pop1_gi 121624694	-----VS <u>T</u> S <u>Y</u> D <u>P</u> V <u>V</u> G <u>N</u> P <u>Q</u> SM <u>U</u> NT <u>V</u> AC <u>C</u> SN <u>G</u> K <u>PG</u> LA <u>AK</u> --YPT <u>L</u> G <u>D</u> LP <u>A</u> P <u>P</u> N <u>V</u> G <u>I</u> P <u>D</u> PD <u>W</u> ND <u>D</u> NC <u>G</u> T <u>W</u> V <u>T</u> V <u>P</u> N <u>R</u> N-----I <u>P</u> IL <u>G</u> V <u>D</u> -SG <u>K</u> G <u>N</u> V <u>N</u> S <u>P</u> Q <u>A</u> FT <u>L</u> T <u>G</u> --STD <u>A</u> GR <u>V</u> ND <u>I</u> Q <u>Y</u> KE <u>V</u> S <u>A</u> D <u>N</u> CK <u>M</u> PV-----
Sm1_tr Q0R411 Q0R411	-----DT <u>V</u> S <u>Y</u> DT <u>G</u> DD <u>P</u> S <u>R</u> SL <u>ND</u> V <u>S</u> C <u>SD</u> G <u>EN</u> LT <u>Y</u> W <u>T</u> O <u>G</u> E <u>V</u> S <u>N</u> P <u>Y</u> I <u>G</u> G <u>V</u> G <u>O</u> Y <u>W</u> N <u>S</u> -T <u>Q</u> CG <u>T</u> CH <u>R</u> -----BY <u>GR</u> --SIN <u>IL</u> LA <u>D</u> AA <u>Y</u> NG <u>G</u> NI <u>AL</u> K <u>AL</u> DT <u>L</u> TD <u>G</u> H <u>A</u> VE <u>W</u> GH <u>Y</u> D- <u>AV</u> AT <u>OV</u> S <u>V</u> NE <u>C</u> GL <u>FT</u> V <u>Y</u> -----
Snodprot1_sp 074238	-----IS <u>V</u> S <u>Y</u> Q <u>U</u> D <u>U</u> GG <u>A</u> RS <u>SL</u> TS <u>V</u> C <u>SD</u> G <u>EN</u> LT <u>I</u> TKY <u>W</u> Q <u>N</u> Q <u>G</u> A <u>V</u> G <u>E</u> P <u>R</u> IG <u>Y</u> S <u>G</u> E <u>A</u> W <u>N</u> S-A <u>Q</u> CG <u>T</u> Y <u>I</u> --TY <u>GN</u> -----I <u>P</u> IL <u>G</u> V <u>D</u> -SG <u>K</u> G <u>N</u> V <u>N</u> S <u>P</u> Q <u>A</u> FT <u>L</u> T <u>G</u> --STD <u>A</u> GR <u>V</u> ND <u>I</u> Q <u>Y</u> KE <u>V</u> S <u>A</u> D <u>N</u> CK <u>M</u> PV-----

Fig. 1. Alignment of some well characterized CP proteins that are cited in this paper. Alignment was performed with ClustalW by MEGA 5.2.1 [75]. Invariable residues are shaded in light purple and marked with asterisks. Conserved residues are marked in light blue and marked with colons (:). The N-terminal secretion signal sequences of all proteins were removed to optimize the alignment. CP proteins were from: *Trichoderma atroviride* Epl1 (G9IS53) and Epl2 (G9MXR6); *Botrytis cinerea* (BcSp1, 154320365); *Trichoderma virens* (Sm1, Q0R411); *Phaeosphaeria nodorum* (Snodprot1, 074238); *Coccidioides immitis* (CS-AG; Q1E8D2); *Taiwanofungus camphoratus* (Aca1; Q6J935); *Moniliophthora perniciosa* MpCP1 (B2C3H7), MpCP3 (B2C3H11), MpCP2 (B2C3H9); *Neosartorya fumigata* (Aspf15, O60022); *Ceratocystis platani* (CP, P71802), *Ceratocystis populicola* (Pop1, 121624694).

formate dehydrogenase H, dimethyl-sulfoxide reductase, aspartic proteinases and in the plant defense protein barwin. Endoglucanases hydrolyze polysaccharides, while expansins loosen the plant cell wall via a non-enzymatic way. Apart from other CP homologs, the most similar fold is observed between CP and the Domain 1 from EXLX1, a two-domain expansin from *Bacillus subtilis* that binds to plant cell walls and to carbohydrates [28]. Expansins have two sugar-binding domains, and it is believed that they reduce the interaction between cellulose and hemi-cellulose fibers in the plant cell wall, allowing these fibers to slide against each other [29,30]. As generally observed for expansins, CP and MpCP2 did not display any detectable cellulase, endo-1,3-β-glucanase, polygalacturonase or chitinase activity, even when used at a high protein concentration [26,27], and the lack of glycoside hydrolase activity can be explained by the absence of residues involved in hydrolytic catalysis in endoglucanases and LTs [26].

Expansins have a high affinity for plant cell wall components, while the CP proteins described so far bind exclusively to chitin. In this sense, CP proteins are more closely related to barwin proteins, which belong to the plant pathogenesis-related (PR)-4 protein family and present antifungal activity. Like the CP proteins, some barwin proteins contain only a single domain [31]. In fact, like barwin, CP is able to bind oligosaccharides (dimers, trimers, tetramers, pentamers, and hexamers of N-acetylglucosamine (GlcNAc)) and the region involved in GlcNAc binding forms a flat and shallow groove on one face of the barrel, which is rich in polar and aromatic residues suitable for sugar/carbohydrate binding (Fig. 2). Furthermore, all residues involved in GlcNAc-binding are among the most conserved ones in CP family, strongly supporting the hypothesis that one of the primary biological functions of proteins belonging to CP family may be related to binding with chitin and chitin-like materials [26,32,33].

Although the available CP structures belong to proteins with distinct effects in plants and are produced by fungi with diverse life styles, CP proteins are remarkably similar. For example, given the available structural data, there is a Cα r.m.s.d. (root mean square deviation of carbon alpha) of about 1.3 Å between the CP and Sm1, MpCP1, MpCP2 and MpCP3, as calculated by Coot [34], indicative of similar 3D structures. The only known exception so far is MpCP5, and possibly MpCP4 and MpCP11, which present a different carbohydrate binding site than the one described for the CP [27]. On the other hand, this implies that the diversity of the biological properties of CP proteins is due to subtle differences in their amino acid compositions. Studies on Sm1/Epl1 proteins revealed that there is a negative correlation between the glycosylation of a specific amino acid residue (Asn29 in Sm1) and the capability of the protein to dimerize. CP homologs from the Epl1/2/3 clusters also present a conserved Trp residue that is oxidized in the Epl1 dimer. As the dimeric forms of the Sm1/Epl1 proteins do not display elicitor activity in plants, the dimerization process may be a mechanism to

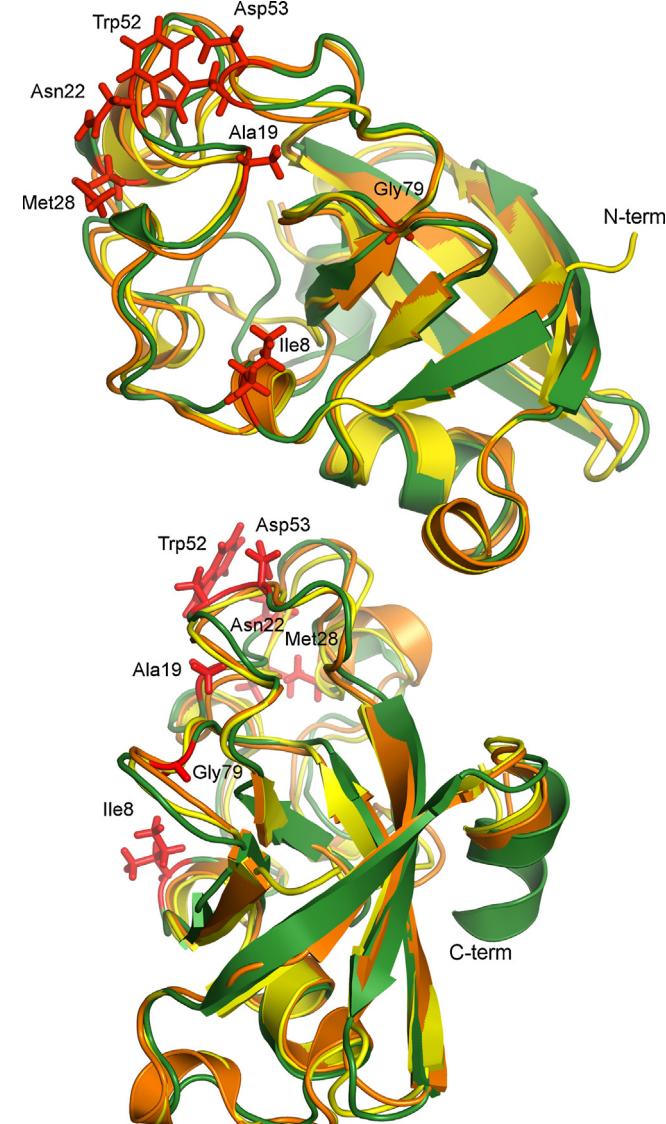


Fig. 2. 3D structure alignment of cerato-platanin (CP, PDB id 2KQA, green), cerato-platanin 3 (MpCP3, PDB id 3SUL, yellow), and Sm1 (PDB id 3M3G, orange) observed from two different points of view. The side chains of residues important for binding of GlcNAcs in CP are shown in red and labeled. Alignment was done with MultiProt [76], structure manipulation was performed with PyMol (The PyMOL Molecular Graphics System, Version 1.4.1, Schrödinger, LLC), and final assembly was done with Gimp v 2.6.12. Sm1 structure was taken from <http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=88487> (Krieger, I., Vargas, W., Kenerley, C.M. and Sacchettini, J., deposited 2010).

regulate the cross-talk between fungi and their hosts: glycosylation prevents dimer formation and thus, increases the eliciting activity of Sm1 and Epl1 by increasing the fraction of elicitor capable of interacting with the cell interface and activating defense responses [16]. Other CP proteins that contain a putative glycosylation site in this region, as predicted with the aid of the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>), are Snodprot1 from *N. crassa*, SP1 and some MpCPs, but there is no study on the effects of dimerization, if it does occur, on their biological activity. On the contrary, the proteins that do not present a putative glycosylation site are CP, BcSpl1, MSP1 and Asp f13 [5,10,19]. CP shows a higher biological activity when in a polymeric, aggregated structure than in the soluble form [24]. Also, aggregated MpCP2 from *M. perniciosa* shows higher activity compared to the soluble one [27]. The mechanism of interaction with cell wall or membrane is not yet determined for any CPs and the reason for a high activity of polymeric CP and a low activity of dimeric Epl1 is not known. Apart from Asp f13 from the opportunistic human pathogen *A. fumigatus*, the other proteins are produced by necrotrophic plant pathogens.

3. The primary functions of cerato-platanins

High level of homology between cerato-platanins from different species and the presence of some of them in the fungal cell wall suggest that these proteins can have an important role in the lifestyle of fungi. However, until now, little data are available dealing with the primary function of CPs. It has been shown that stress conditions such as low temperature or osmotic stress, which reduce the growth of *C. platani*, also reduce the level of *cp* transcript. In addition, a positive correlation between *cp* gene expression and chlamydospore formation was found, suggesting an important role of CP in fungal development [35]. Likewise, the expression of CP homologue from the edible mushroom *Pleurotus ostreatus* and MpCP1 from *M. perniciosa* were detected in their basidiocarps [27,36]. Structural data of CP highlighted the presence of a polar pocket on the molecule that is able to bind N-acetylglucosamine oligomers [26]. It has been recently shown that CP, the orthologous Pop1 from *C. populincola*, some MpCPs and Epl1, are able to bind chitin and colloidal chitin with high affinity, which has been measured for CP bound to colloidal chitin ($B_{max} = 2.07 \pm 0.19 \mu\text{mol/g}$ of chitin, $K_d = 40.56 \pm 9.50 \mu\text{M}$). The ability to bind chitin and the localization in the cell wall suggests a structural role in the fungal cell wall. Moreover, an involvement of CPs in the remodeling and enlargement of the cell wall during the hyphal growth and the formation of spores cannot be excluded [32]. Other data arise from the observation that the expression of the CP homologue from *N. crassa* is regulated by the circadian rhythm and by the GRH (grainy head) transcription factor. *N. crassa grhl* (grainy head-like) mutants fail to remodel their cell wall during conidial separation [37,38]. In animals, GRH controls epidermal barrier development and other morphogenetic processes. The ability to bind chitin had already been reported for expansin-like proteins from fungi, although it is not clear whether these proteins cause changes in the chitin structure [39,40]. As discussed in the following sections, the similarity between CPs and expansins is opening up new perspectives in understanding the role of CPs in host interactions. Finally, based on the presence of CP from *C. platani* on cell wall and its aggregation propensity, it was suggested that a parallelism exists between CP proteins and hydrophobins, which facilitate hyphal growth, adhesion, host cell penetration and sporulation [41,42]. As stated before, the expression of *cp* from *C. platani* is up-regulated during rapid hyphal proliferation and spore formation [35]; MpCP2 and MpCP3 are also expressed during fast growth of *M. perniciosa* [27,43].

MSP1, in turn, is not detected in the cell wall of *M. grisea* and is dispensable for appressorium formation [10]. The *epl1* from *T.*

atroviride is expressed during hyphal growth, and *epl2* expression was only observed during spore maturation and in cultivations that are accompanied by sporulation, e.g. growth on chitin [33]. However, no difference in growth and overall phenotype between the wild-type and the *epl1* and *epl2* knockout mutants was observed, suggesting a non-essential role of these proteins in fungal growth and development in *T. atroviride* [33]. The role of CP homologs in *Trichoderma* spp. will be discussed in Section 5.

4. The role of cerato-platanins in plant interactions

4.1. General features

Since most of the CPs are both secreted and localized in the fungal cell wall, these proteins are likely to play an active role in interaction between fungi and their host plants. As mentioned earlier, CP, Pop1 and MpCP2 have an expansin-like activity in vitro, loosening cellulosic materials, even though not binding to cellulose with a high affinity [27,32]. Although no direct evidence exists, it is possible that the role of CPs in plant interactions is due to this expansin-like activity. Expansins unlock the network of cell wall polysaccharides through a non-enzymatic mechanism, permitting turgor-driven cell enlargement in plant cell walls. Other expansin-like proteins can also be found in fungi, e.g., swollenin (SWO1), which, similarly to many plant expansins, has a cellulose-binding domain, and has a function in cellulose degradation in *Trichoderma reesei* [44,45]. However, it should be noted that SWO1 does not belong to the CP protein family. Acting in a similar way, CPs may facilitate the growth/infection of pathogenic fungi inside their hosts, either by promoting the access of enzymes and effectors into the plant cell or cell wall, or by enabling the acquisition of nutrients after the death of the affected tissue, by virtue of loosening the cellulosic materials. CP, Pop1 and MpCP2 are representative of the CP family (Fig. 1), and thus there are substantial reasons to believe that the activity on cellulose without binding to it, although with some differences, is a common feature of CPs.

Another mechanism by which CPs can act on plant cells may be derived from the ability of CPs to bind chitin and N-acetylglucosamine (GlcNac) oligomers via amino acid residues that are conserved in all members of the family. Chitin is a major component of fungal cell walls and serves as a molecular pattern for the recognition of potential pathogens by the innate immune systems in plants, inducing various defense responses, such as over-expression of cell wall degrading enzymes, which in turn would produce chitin oligomers [46]. Binding to chitin has also been demonstrated for CP from *C. platani* [47], for Epl1 from *T. atroviride* and for MpCP1, MpCP2, MpCP3 and MpCP5 from *M. perniciosa* [27,33]. MpCP3 and MpCP5 from *M. perniciosa* also bind to (GlcNac) oligomers, and while MpCP3 binds to these ligands through the conserved region, MpCP5 shows a completely different interaction surface, nevertheless indicating that there is a selective pressure to maintain the ability of MpCP5 to interact with chitin [26,27,32]. Distinct binding affinities of each CP protein to chitin and chitin-derived oligomers can explain the different cellular responses observed in host and non-host plants: the chitin fragments released by the fungus as a consequence of plant-microbe interaction would be (more or less) sequestered by CPs according to their affinity (further discussion in Section 4.4).

4.2. The phytotoxic cerato-platanins: role in virulence

It is apparent that some of the CP proteins enhance the virulence of necrotrophic fungi, as many of the proteins are expressed during plant-pathogen interactions and induce death of the affected tissue. BcSpl1 induces necrosis on tobacco leaves just one hour after

application, and *B. cinerea* knock-out mutants for BcSpl1 showed reduced virulence in tobacco and tomato plants [19]. BcSpl1 was suggested to bind to the plant plasma membrane causing cell shrinkage and chloroplast disorganization [48]. Furthermore, *M. oryzae* knock-out mutants for MSP1 also displayed reduced virulence against rice and barley, although exogenously injected, recombinant MSP1 did not induce any detectable necrosis [10]. Necrosis-inducing ability was also observed for MpCP1 from *M. perniciosa*, a pathogen from cocoa [12], although this toxic effect could not be reproduced [27]. Phytotoxicity of some CPs could be due to their ability to bind chitin, at least in part. Like CPs, most fungal effectors are secreted, cysteine-rich proteins, and a role in virulence has been shown for a few of them that also have a high affinity for chitin and chitin-oligomers [49,50]. For instance, Avr4 from *Cladosporium fulvum* protects fungal cell wall against plant chitinases [51]. LysM effectors, such as Ecp6 from *C. fulvum* and Slp1 from *M. oryzae* contribute to pathogen virulence by sequestering chitin oligosaccharides released by the fungus during infection, thereby blocking the activation of host chitin receptors [52]. As a result, the host immune response is extinguished as it was shown for the virulence effector Ecp6, that is able to sequester (1,4)-poly-N-acetyl-D-glucosamine released from the hyphal cell wall during infection, thus preventing the elicitation of host immunity [53–55]. Therefore, the virulence-promoting activity shown by some CPs in consequence of fungal attack could be driven by a strong affinity for chitin as discussed above.

4.3. Cerato-platanins as elicitors of defense responses

Cerato-platanins seem to be one of a few known examples of isolated fungal molecules which are able to induce systemic defense response by themselves, as has been proven previously for bacterial flagellin and lipopolysaccharides [56,57]. Plant immune system is composed of surveillance systems that perceive several general microbial elicitors, which allow plants to switch from growth and development into a defense mode, rejecting most potentially harmful microbes. Elicitors of primary defense response or MAMPs/PAMPs (microbe/pathogen-associated molecular patterns) are essential structures for pathogen survival, are conserved among pathogens, and initiate a signaling cascade leading to the activation of defense to prevent penetration and to restrict the growth of pathogens [57,58]. PAMPs recognition activates a network of signal transduction pathways, which involves a number of signaling molecules including ROS, NO, salicylic acid, jasmonic acid and ethylene. In fact, soon after CP was shown to induce necrosis when infiltrated in tobacco leaves, it was demonstrated that CP and the ortholog Pop1 are able to induce the synthesis of defense phenolic compounds, generally known as phytoalexins, when applied on the lower surface of host as well as non-host leaves [5,8,59]. Subsequent studies revealed that both CP and Pop1 can trigger accumulation of starch, nitric oxide (NO) and reactive oxygen species (ROS), all markers of plant defense. Moreover, these proteins are able to induce mitogen-activated protein kinase (MAPK) cascades by phosphorylation of two kinases of 44 and 47 kDa, probably the homologs of MAPK3 and MAPK6 from *Arabidopsis*, and the characteristic DNA fragmentation and cytological features typical of programmed cell death [6,59,60]. Finally, several genes involved in defense are over-expressed upon treatment of leaves with CP and Pop1, like the WRKY transcription factor, the PR1 and PR5 defense proteins and the alkaline alpha galactosidase (AGA), uridine diphosphate glycosyltransferase (UGT) and the 14-3-3 proteins, all of which are involved in defense and a hypersensitive response [47,61].

Another protein reported to be involved in induction of plant defense response is the MgSM1, a CP-like protein from *M. oryzae* that is able to induce hypersensitive response when expressed in

Arabidopsis by Agrobacterium-mediated transient expression, and restricting the spread of *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato* (Pst) DC3000, accompanied by up-regulation of defense genes PR-1, PR-5 and PDF1.2 [17]. The findings obtained with CP are further supported by the fact that *A. thaliana* lacking a gene from the PAMP signaling pathway had reduced sensitivity to BcSpl1 [19]. It is noteworthy that the *A. thaliana* plants still displayed necrotic symptoms at high BcSpl1 concentrations [19], which suggests that there may be an alternative mechanism by which plants recognize and respond to CP proteins (further discussion in Section 4.4).

In agreement with the results mentioned above, there are at least some instances in which the induction of defense responses by CP proteins is unfavorable to the pathogens themselves. In fact, the growth and infection rate of *C. platani*, *C. populicola* and *B. cinerea* are reduced in plants pre-treated with their respective CP proteins, either by exogenous application, or *in planta* transient expression [8,17,20,61]. However, it is widely accepted that the pathogenicity of a microorganism is not an unambiguous index of the toxicity of its external proteins, and these findings imply that pathogens must circumvent the otherwise adverse side effects of the plant's general defense responses induced by CP proteins during the infection.

4.4. Non-specific and overlapping functions

Until now, no PRRs (pattern recognition receptors) have been identified to explain the elicitation mechanism of CP and Pop1 in the host, although, the involvement of the BAK1 kinase in the cascade signaling of BcSpl1 protein in *Arabidopsis* has been proposed [19]. Interestingly, CP and Pop1 are able to interact with hydrophobic components (such as cuticle) of the host without penetrating the cell wall, suggesting an unusual mechanism of signal transduction, which has not been well explained so far [62]. On the other hand, SP1 from *L. maculans* does not induce plant cell death and the sp1 knock-out strains showed no difference in host infection capability [11]. One can, however, reason that *L. maculans* may secrete other proteins that compensate for the lack of SP1. Also, expression of CP proteins from hemi-biotrophic pathogens *M. perniciosa* (MpCP) and *Colletotrichum truncatum* (CtCP) was detected during the biotrophic phase of fungal growth, when host death is not expected. *In planta* heterologous expression of CtCP did not induce detectable defense responses in tobacco, and the same (lack of) response was observed after injection of four different MpCPs from *M. perniciosa* into tobacco leaves [27,63].

These findings indicate that, even among pathogens, there may be more to the CP proteins than just killing their hosts. It is well-known that chitin fragments, released upon fungal attack to plant, are strong elicitors of plant immune system [64], such that preventing their perception by the plant is the key to the success of the fungal infection. One way to accomplish this is by scavenging the chitin fragments with the aid of proteins with high affinity to these ligands, as discussed above. Therefore, it is possible (even though not yet demonstrated) that the plethora of effects induced by cerato-platanins in plants may be the results of different affinities for chitin and chitin-oligomers: a strong affinity for chitin could sequester this elicitor and, as a consequence, induce phytotoxic effect during pathogenesis. On the contrary, a low affinity for chitin could enable the induction of defense. The expansin-like activity of cerato-platanins could also be taken into account as a further mechanism by which these fungal proteins can affect defense responses, as plants can recognize structural changes in their cell wall as a danger signal.

5. Cerato-platanins from biocontrol fungi

In *Trichoderma* spp. CP proteins act as elicitors of plant defense, as was shown for Sm1 from *T. virens* and Epl1 from *T. atroviride*

[14–16]. Epl1 was, in addition to its role in fungal–plant interactions, found to be expressed on a variety of carbon sources and was the major secreted protein in the secretome of submerged *T. atroviride* cultivated with glucose as carbon source [13]. It is also one of the most abundantly expressed proteins in *T. reesei* under many different growth conditions (C.P. Kubicek and B. Seibold, pers. comm.). As noted earlier, the amino acid sequence of Epl1-orthologues is strongly conserved among several filamentous fungi with different life-styles ranging from saprotrophs to parasites and pathogens [33]. Some differences in amino acid sequence between these proteins from *Trichoderma* and from *Ceratocystis* species are present and they can support the differences in biological activity on plants (Fig. 1). Analysis of the biochemical properties of the protein Epl1 from *T. atroviride* showed that it binds strongly to various forms of chitin, but not to cellulose. Further, a number of other intriguing properties were detected for Epl1. It readily self-assembles at air/water interface and forms protein layers that can be re-dissolved in water [33]. These properties are reminiscent of hydrophobins. However, surface contact angle measurements yielded the opposite of what is usually observed for hydrophobins, since Epl1 increased the polarity of solutions and surfaces. This also has implications on the potential biological function of these CP proteins. Hydrophobins render fungal structures hydrophobic, but the results for Epl1 point to the contrary—Epl1 increases the polarity of solutions and surfaces. This could lead to a possible increase in the wetting properties of hyphae, enabling them to grow in aqueous environments and covering their hyphae in a water layer or protecting them from desiccation. Thus, these are rather ‘hydrophilins’ than hydrophobin-like proteins. AFM (atomic force microscopy) imaging showed that Epl1 assembles into irregular meshwork-like sub-structures at an air/water interface (Fig. 3).

Expression analysis of three *epl* genes of *T. atroviride* showed that they are not co-regulated, but are expressed during different growth stages [33]. While *epl1* was predominantly expressed during hyphal growth, *epl2* was mainly expressed during spore formation, suggesting that the respective proteins are involved



Fig. 3. Formation of protein layers of *T. atroviride* Epl1 at air/water interfaces. Epl1 forms protein layers on the surface of water droplets. 25 μl of an Epl1 solution (0.5 mg/ml) were incubated for ca. 12 h at 25 °C on microscope cover slips in petridishes sealed with parafilm (to prevent evaporation). Subsequently they were imaged on an inverted Nikon TE300 microscope. Upon shrinkage of the water droplets due to the heat of the microscope lamp, wrinkles and folds of the EPL1 protein layer become readily visible.

in different biological processes. For *epl3* no gene expression was detected under most growth conditions. Unlike in *C. platani*, where the expression levels of *cp* are elevated during chlamydospore formation, expression of *epl1* is not induced during chlamydosporogenesis in *T. atroviride* [33] or *T. virens* (R. Gaderer and V. Seidl-Seibold, unpublished results). In *T. virens*, the expression of Sm1 is enhanced in the presence of plant roots or after sucrose degradation [14,65]. These observations are evidence of the specific regulation of the *sm1* gene expression during plant root colonization.

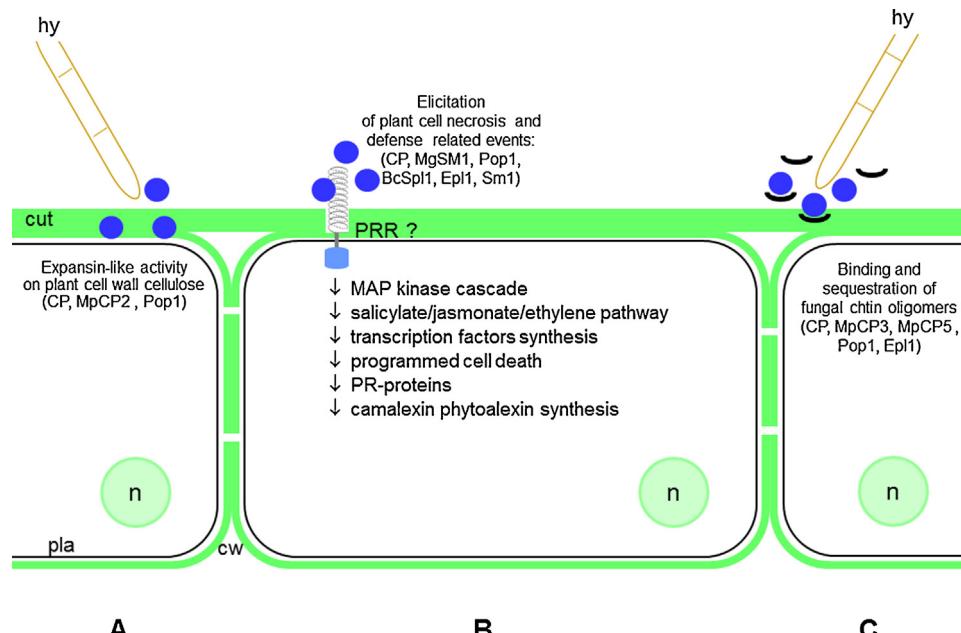


Fig. 4. Schematic representation of the possible interaction scenarios between CP proteins and plant. (A) Expansin-like activity on cell wall; (B) PAMP activity of CP proteins upon interaction with a putative receptor; (C) Effector-like activity as a consequence of chitin oligomers sequestration. Examples of CP family proteins involved are indicated in parentheses.

[● - CP-like protein; — - fungal oligo-N-acetylglucosamine produced by hypha after plant interaction; ? - hypothetical, hy- fungal hyphae, cut- plant cuticle; cw- plant cell wall; pla- plant cell plasma membrane; n- plant cell nucleus; PRR- plant pattern-recognition receptor].

Analysis by gene knockout showed that gene expression of *epl1/sm1* and *epl2/sm2* was not altered in $\Delta epl2/sm2$ and $\Delta epl1/sm1$ strains, respectively, indicating that the regulation of these genes is not connected in such a way that they compensate for each other in single knockout strains [33; R. Gaderer and V. Seidl-Seiboth, unpublished results]. The phenotypes of the strains were tested with respect to the following properties: growth rate on agar plates, formation of aerial hyphae, growth along (moist) surfaces, bridging of gaps between two agar blocks and transition of hyphae between solid/liquid interfaces. Different types of desiccation stress, e.g. drying of water droplets and drying of thin agar plates were also examined. Additionally, we analyzed conidiation, biomass formation in shake flask cultivations, germination efficiency, hydrophobicity of the mycelium, chlamydospore formation, osmotic stress and cell wall stress. No differences between the wild-type and the single or double knockout strains were detected under any of these conditions. Further, the mycoparasitic potential against *Rhizoctonia solani* and *B. cinerea* was unaltered in the knockout strains. Taken together, these findings indicate that, despite their intriguing biochemical properties, these proteins do not have any essential function in fungal growth and development of *Trichoderma*.

Infiltration of Sm1 to maize and cotton leaves activated plant immune system and induced systemic resistance (ISR) mechanisms that aid in preventing pathogenic development of *Colletotrichum* sp., but no phytotoxic activity was detected [14,16]. In maize, it was demonstrated that Sm1 activates defense mechanisms through jasmonic acid (JA) and green leafy volatile (GLV) signaling pathways, as evidenced by the expression profiles of the marker genes *aos*, *hpl*, *opr7* and *pal* [15,16]. In the nematophagous fungus *Dactylellina cionopaga*, a Snodprot changed the chemotaxis and increased the body-bend frequency of *Caenorhabditis elegans* in a concentration-dependent manner. The corresponding gene was induced in *D. cionopaga* during development of traps and conidia [66]. A Snodprot orthologue (Sit-1) have also been suggested to be involved in entomotoxicity of *Metarhizium anisoplae* (E. Quesada-Moraga, personal communication).

6. Conclusion and outlook

Until recently, the best characterized roles of CP, Pop, Epl1, BcSpl1 and other cerato-platanins happened to be the induction of localized and systemic defense responses in plants, and phyto-toxicity (Fig. 4). However, these effects (virulence or defense) are not mutually exclusive because the boundary between the effects induced by PAMPs and by effectors is faint and it is due more to the intensity of the response rather than to the kind of downstream molecular events [67]. Therefore, cerato-platanins could behave as elicitors of defense responses and inducers of resistance if the appropriate conditions (e.g. mode of application and concentration) can be defined.

Induced resistance offers the hope to devise a durable and broad-spectrum disease control that exploits the plant's own defense mechanisms. Induced resistance is also expected to contribute to overcoming fungicide insensitivity and breakdown of host resistance, problems that are worsened by the specter of global climate change and by the ever-increasing human population. Even though no CP-based product has so far been developed for applications in plant health management, probably due to the fact that the responses provoked in plants by a single purified protein are not strong enough to warrant economical disease management, the ability of CPs in inducing ROS synthesis, MAPK activation, phytoalexins production, and overexpression of defense related genes could be enhanced by engineering such proteins to encompass active domains from various elicitor molecules in a putative synthetic protein or in peptides derived from it. Engineered CP proteins or derived peptides could also complement and integrate

the use of chitosan and other induced resistance activators in the treatment of crop plants [68,69]. Since these proteins can be easily produced in active form in heterologous hosts, for example yeasts [70], it should be possible to use a cocktail of such recombinant proteins as elicitors for improving plant defense. It is also possible to express these proteins in transgenic plants for enhancing resistance [17]. Cerato-platanins have been over-expressed in the plant pathogens *Colletotrichum coccodes* and *Fusarium oxysporum* in order to enhance the virulence of these mycoherbicides [71]. Overexpression of Sm1 in *T. virens* enhanced resistance-inducing properties of this biocontrol fungus [15], thus opening-up the possibility of engineering biocontrol agents with enhanced ability to invoke plant defense. Furthermore, these compounds may be beneficial for the control of viral diseases that, nowadays, is based only on preventive actions. Elicitors are routinely used to enhance the production of plant secondary metabolites of pharmaceutical significance [72–74]. The potential of CP proteins to enhance the production of these secondary metabolites, either added to the culture media or expressed *in planta*, needs to be explored.

Acknowledgments

P.K. Mukherjee is grateful to the Head, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, for encouragements and support. We also acknowledge Dr. Matteo Ramazzotti for his essential assistance in the elaboration of 3D structure comparison. M. Barsottini acknowledges the financial support in the form of a FAPESP fellowship (grant no. 2010/14504-2).

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