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CP from *Ceratocystis platani*: primary role in the fungal life and functions in interaction with plants

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Abbreviations

CP – Cerato-platanin;

CPF – Cerato-platanin Family

CPs – Cerato-platanins

ECD – Extracellular Domain

ET – Ethylene

ETI – Effector Triggered Immunity

HR – Hypersensitive Response

JA – Jasmonic Acid

LRR – Leucine-Rich Repeat

MAPKs – Mitogen Associated Protein kinases

NB-LRR – Nucleotide-Binding-Leucine-Rich Repeat

NHR – NonHost Resistance

NO – Nitric Oxide

P/MAMP – Pathogen /Molecular -Associated Molecular Pattern

PCD – Programmed Cell Death

Pop1 – Cerato-populin;

PRRs – Pattern Recognition Receptors

PTI – PAMP-Triggered Immunity

RLKs – Receptor-Like kinases

RLPs – Receptor-Like Proteins

ROS – Reactive Oxygen Species

SA – Salicylic Acid

TM – Transmembrane domain.

Introduction

Plants through photosynthesis are able to convert the energy of sunlight into chemical energy stored in the bonds of sugars. Thus, plants are rich sources of nutrients and water that are, to no one's surprise, host to diverse microbial communities both above and below the ground. Microbes are likely to have accompanied the first plants that emigrated from water to land 400 to 500 hundred million years ago (Dangl et al 2013). Many of their descendant contemporary microbes are adapted to take advantage of the nutrient niches afforded to them by the huge diversity of plants all over the earth. In fact, many microbes, with different infection strategies and life styles, are able to live closely with plants and some of them are phytopathogenic.

Phytopathogens are broadly divided into those that kill the host and feed on the nutritive materials (necrotrophs), those that require a living host to continue their life cycle (biotrophs) and those which require a living host initially, but kill at later stage of infection (hemibiotrophs) (Hammond-Kossack and Jones 2000). Bacteria and fungi adopt either a biotrophic or necrotrophic mode of infection while viruses are ideal biotrophs although viral infection can consequently result in host cell death (Dangl and Jones 2001).

Plant pathogens cause devastating epidemics that have affected human civilizations since the dawn of agriculture (Agrios, 1988) therefore the study of the mechanisms involved in the interactions between plants and pathogens is important. For example the late blight Irish potato famine of the 1840s was caused by the oomycete *Phytophthora infestans* (Yoshida et al., 2013); the loss of the world's first mass-cultivated banana cultivar *Gros Michel* in the 1920s to Panama disease was caused by the fungus *Fusarium oxysporum* (Koeppel 2008); and the current wheat stem, leaf, and yellow stripe rust epidemics spreading from East Africa into the Indian subcontinent caused by rust fungi *Puccinia graminis* and *P. striiformis* (Singh et al., 2011) are all testament to the recurring impact of plant diseases. Plant pathogens can spread rapidly over great distances, vectored by water, wind, insects, and humans. Despite various cultural practices, crop protection chemicals, and available disease-resistant crop varieties, an estimated 15% of global crop production is lost to pre-harvest plant disease (Popp et al., 2011).

Currently, the main method of protecting plants against pathogens involves the use of pesticides, chemical compounds whose selectivity on target, unfortunately, is not very high. In fact, although the majority of plant protection products is active at low doses and characterized from a toxicological and eco-toxicological profile significantly better

than that the first families of pesticides, their impact on human health and environment is still controversial. In fact, it is known that most of these substances are water and soil pollutions. For this reason, research plays a central role in the development of alternative methods of crop protection to reduce the impact of pesticides on human health and the environment. In this context, it is of particular interest the use of elicitors, molecules of microbial origin, able to stimulate and strengthen the natural defenses of plants. These molecules are perceived by the plant and trigger a series of metabolic changes that limit the susceptibility to pathogens, both immediately and in the future (priming), therefore, the use of elicitors may decrease if not eliminate the use of pesticides.

Plant-pathogen interaction

Plants are exposed to a plethora of potential pathogens. Nevertheless, disease represents the exception in natural plant communities. The reason is that most plants are immune to the majority of would-be pathogens and susceptible to only a relatively small number of adapted microbes. Among the 7100 classified bacterial species, roughly 150 species cause diseases to plants (Buonaurio 2008), about 8000 species of fungi and fungal-like organisms are phytopathogens (Ellis et al. 2008) and there are 73 genera and 49 families of plant pathogenic viruses (Zaitlin and Palukaitis 2000).

The phenomenon that an entire plant species is resistant to all genetic variants of a non-adapted pathogen species (or bacterial pathovar [pv] or fungal forma specialis [f.sp.]) is termed nonhost resistance (NHR) (Thordal- Christensen, 2003; Mysore and Ryu, 2004; Nuernberger and Lipka, 2005). NHR therefore, is the most common form of disease resistance exhibited by plants. Such resistance contrasts with host resistance, which is expressed by plant genotypes within an otherwise susceptible host species. Host resistance is usually parasite-specific in that it is restricted to a particular pathogen species, and commonly is expressed against specific pathogen genotypes (Heath, 2000).

Because host and nonhost pathogens can be recognized by similar plant perception mechanisms, nonhost resistance overlaps with host resistance.

Plants defend themselves from pathogens by implementing defensive strategies that may be different depending on the pathogen (fungi, bacteria, viruses). In general they are opposed to pathogens by combining structural features which act as physical barriers against the invasion of the pathogen, and biochemical reactions which lead, in most cases, to the production of toxic substances to the pathogen. Recently Senthil-Kumar and Mysore (2013) have proposed a model for plants nonhost resistance against foliar pathogens attack, especially bacterial pathogens, which involves three main steps (Figure 1).

The first plant defense layer restricts pathogen entry. Pathogens that land on a plant surface are exposed to a wide range of preformed plant defenses, such as a wax layer (Heath, 2000; Huckelhoven, 2007), and to be pathogenic, most microbes must access the plant interior, either by penetrating the leaf or root surface directly or by entering through wounds or natural openings such as stomata, pores in the underside of the leaf used for gas exchange (Chisholm et al., 2006). In addition, already at this first level of defense, there are inducible defenses, such as stomatal closure (Grimmer et al., 2012; Ham et al., 2007; Melotto et al., 2006). The second layer of defense occurs after the bacterial pathogen reaches the apoplastic region of photosynthetically active mesophyll cells. After gaining access to the apoplastic fluid, a successful pathogen manipulates

plant cells to release more nutrients so that it can multiply and eventually cause disease. This step also involves preformed defenses, such as the presence of antimicrobial compounds, coupled with overall apoplastic physiological incompatibility and induced defenses, such as phytoalexin production (Dixon et al., 2002; Ham et al., 2007; Hann et al., 2007; Huckelhoven, 2007)(Figure 1).

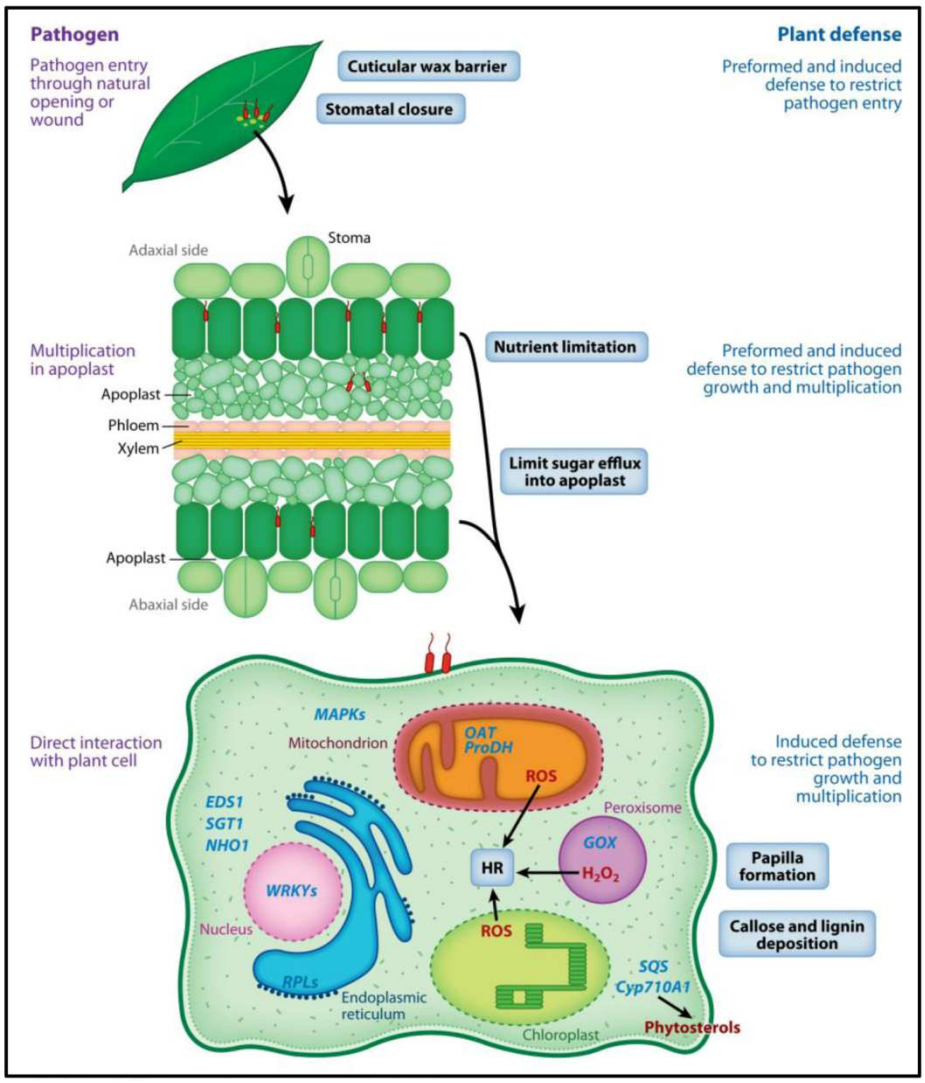

Figure 1: *Different layers of resistance of plants against pathogens. The first layer of plant defense on the leaf surface and in the apoplast involves the presence of preformed or induced structural barriers, such as wax, and defense by chemical molecules, such as antibacterial compounds. Inducible defense responses occur first due PAMP perception, and, in the advanced stages, by recognition of effector molecules. The direct interaction between pathogen and plant cell usually initiates either the hypersensitive response (HR) or other defense pathways, leading to an arrest of pathogen growth. Some of the recently identified genes contributing to resistance are shown (Senthil-Kumar and Mysore, 2013)*

The third layer involves preformed and inducible defense responses triggered in the plant cell and executed in the cell wall region or apoplast. When the plant interior has been breached, microbes are faced first with the plant cell wall, a rigid cellulose-based support surrounding every cell, and second to the cell membrane, where they encounter

extracellular surface receptors that recognize Pathogen-Associated Molecular Patterns (PAMPs or MAMPs, Molecular-Associated Molecular Patterns, a class of elicitors). PAMPs are protein or non-protein pathogen-derived compounds that are essential for pathogens but are usually recognized by the host. Recognitions of PAMPs initiate PAMP-Triggered Immunity (PTI), which usually arrest infection before the microbe gains a hold in the plant. Additionally pathogens can secrete inside the plant cell, effectors that are able to suppress the PTI and restore the susceptibility (Jones e Dangl, 2006). On the other hand plants possess a more specialized mechanism to detect microbes, the Effector Triggered Immunity (ETI). ETI involves the direct or indirect recognition of the effector proteins by plant resistance proteins and is an amplified PTI which generally leads to an apoptotic cell death (PCD, Programmed Cell Death) localized at the site of infection, which is called Hypersensitive Response (HR) (Figure 1).
The induced plant defense responses in all of the above mentioned steps involve perception of PAMPs, effectors, and other molecular responses (Li et al., 2005; Nicaise et al., 2009; Sohn et al., 2012). Similar steps can also occur during plant interactions with root and xylem/phloem-invading pathogens (Senthil-Kumar and Mysore, 2013).
However, the separation between PTI and ETI is a model that greatly simplifies the real situation in which there is a continuum among the two process. Also there isn't a clear separation between PAMPs and effectors (Thomma et al., 2011; Uma et al., 2011). In fact, some groups of effectors present the typical characteristics of PAMPs, instead effectors of being specific molecules are very conserved molecules, as commonly happens to PAMPs.

Preformed defenses

Preformed defenses are among the most important components of resistance (Heath, 2000). They include both physical restriction and chemical inhibition of pathogens. Most of them are present in all the plant species like the cuticle layer, the epidermis and the cell wall. In addition, the low frequency of stomata on leaf surfaces is a further obstacle to the penetration of the pathogen (Gottwald and Graham, 1992; Ramos et al., 1987; Ramos et al., 1992; Xiao et al., 2004).
The cuticle is a continuous layer connected with the epidermal plant cells. It consists in pectin, cutin and wax layers. Pectin is located in the inner side of the cuticle while waxes are concentrated outside. The composition and the thickness of the cuticle are variable; it changes according to the tissue, the environmental conditions (humidity, temperature and light) and to the development phase. Waxes comprise a mixture of very long-chain fatty acids (24–36 carbon atoms) that seem ubiquitously present in most plant species. In addition, triterpenes, β-diketones as well as phenylpropanoids are associated with the

wax fraction (Samuels et al., 2008; Kunst and Samuels, 2009; L'Haridon et al., 2011). The cutin consists primarily of esterized C16 and C18 fatty acids, with numerous minor components. With the long carbon chains of the wax fractions on the outside of the cuticle, it forms a highly hydrophobic structure. This makes it suitable to retain water and prevent the loss of nutrients.

It is obvious that a successful pathogen has to overcome the plant cuticle in order to invade the host tissue, therefore different pathogens have developed different strategies (Schafer, 1998):

- Bacterial and some fungal pathogens circumvent the cuticle and enter through stomata, lenti cells or wounds.
- Other fungal pathogens penetrate through the appressorium, an organ able to penetrate in the host by turgor pressure.
- Another group of fungal pathogens drill their way directly through the cuticle, but without forming the appressorium.

In addition most pathogens that directly penetrate through the cell wall produce cutinase, an enzyme capable of degrading cutin in its monomers (L'Haridon et al., 2011). It has been demonstrated that components of the cuticle might function as important developmental cues perceived by invading microorganisms (Kolattukudy et al., 1995). For example, cutin monomers can induce the expression of cutinase (Lin and Kolattukudy 1978; Woloshuk and Kolattukudy 1986) or act as a plant signal for the induction of germination and appressorium in fungal pathogens (Lin and Kolattukudy 1978; Gilbert et al., 1996). Similarly, surface waxes can also affect the development of fungi at the plant surface (Podila et al.,1993).

In a similar manner, it has been shown that fragments of the cuticle may be perceived from the plant itself and lead to activation of inducible defenses. For instance, Synthetic cutin monomers applied on leaves of barley or rice increased resistance to *Erysiphe graminis* and *Magnaporthe grisea* respectively (Schweizer et al., 1994; Schweizer et al., 1996). Therefore the cuticle is not a simple static system that acts as a barrier against water loss or pathogens entry, but is a dynamic system which is in contact with the underlying cell wall and can interact with the cell interior.

The other main preformed defense present in all the plant cells is the cell wall that is a tough, flexible but sometimes fairly rigid layer. It is located outside the cell membrane and provides these cells with structural support and protection, also to acting as a filtering mechanism. A major function of the cell wall is to act as a pressure vessel, preventing over-expansion when water enters the cell. In addition, forming the interface between adjacent cells, plant cell walls often play important roles in intercellular communication. Finally, because of their surface location play an important role in plant-microbe interactions, including defense responses against potential pathogens.

The most characteristic component found in all plant cell walls is cellulose. It consists of a collection of β-1,4-linked glucan chains that interact with each other via hydrogen

bonds to form a crystalline microfibril (Somerville, 2006). In addition to cellulose, plant cell walls contain several matrix polysaccharides that are grouped into two general categories:

1. the pectic polysaccharides include homogalacturonan, and rhamnogalacturonan I and II (Harholt et al., 2010)
2. the hemicellulosic polysaccharides include xyloglucans, glucomannans, xylans, and mixed-linkage glucans (Scheller and Ulvskov, 2010).

Plant cell walls also contain many proteins and glycoproteins, including various enzymes and structural proteins (Rose and Lee, 2010) (Figure 2).

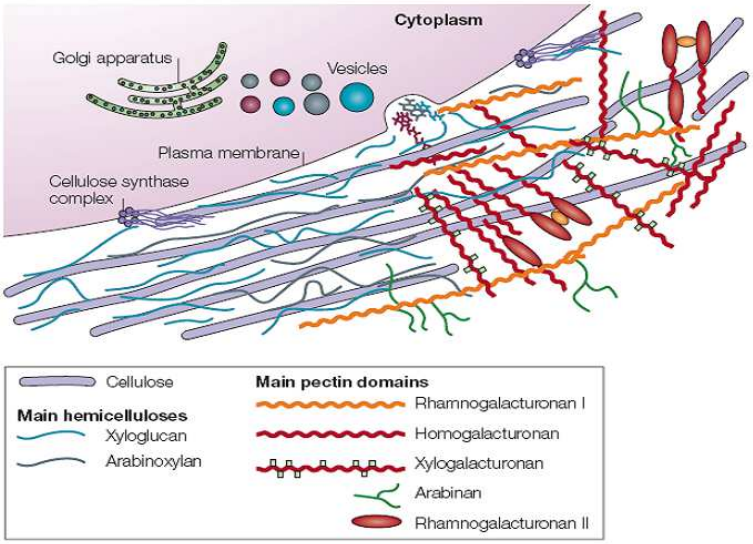

Figure 2: *Cellulose microfibrils are synthesized by large hexameric complexes in the plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the Golgi apparatus and are deposited to the wall surface by vesicles. For clarity, the hemicellulose–cellulose network is shown on the left part of the cell wall without pectins, which are emphasized on the right part of the figure. In most plant species the main hemicellulose is xyloglucan, while hemicelluloses such as arabinoxylans and mannans (not shown) are found in lesser amounts. The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I (not shown) and rhamnogalacturonan II (Cosgrove, 2005).*

Growth and cell shape are largely determined by the balance between expansion driven by turgor and constraint provided by the plant cell wall. Therefore the cell wall is a highly dynamic structure that is constantly remodelled during growth and development and in response to environmental cues.

Pathogens use mechanical force or release cell wall degrading enzymes to break down this barrier. Necrotrophs release copious amounts of cell wall degrading enzymes, presumably in an attempt to lyse plant cells before they can mount an effective defence. Biotrophs, however, appear to operate by stealth, minimizing damage to the host cell wall (Hematy et al., 2009). This difference in lifestyle is partially reflected in the repertoire of cell wall degrading enzymes encoded by the genomes of the two types of pathogens. The biotroph *Ustilago maydis* encodes relatively few cell wall degrading enzymes (Kamper et al., 2006); while a necrotroph like *Erwinia spp.*, which causes soft

rot diseases, produces a broad spectrum of lipases and cutinases to degrade the host cuticle, pectinases to increase accessibility for other enzymes like cellulases and xylanases and several other hydrolases to break down the hemicellulose chains (Toth et al., 2005). Interestingly, the nematode roundworm, *Globodera rostochiensis*, produces expansin proteins to loosen the cell wall (Qin et al., 2004).

At the cell wall, pathogens also release PAMPs either inadvertently or as a consequence of plant degradative enzymes (e.g. the release of chitin oligomers by plant chitinases). Plants, in turn, appear to sense these PAMPs and damage to their cell walls and activate a variety of induced plant defense responses.

In addition to the preformed barriers such as the cuticle and the cell wall, there are also several chemical compounds between the preformed defenses. In fact plants constitutively produce antimicrobial compounds that inhibit host- and non-host pathogen growth (Che et al., 2011; Lee et al., 2008). Several secondary metabolites have antimicrobial properties and are directly involved in restricting the growth of invading pathogens (Aires et al., 2009; Fan et al., 2011; Senthil-Kumar and Mysore, 2013). Phytoanticipins are major secondary metabolites present in their active form in plants before pathogen infection (Dixon et al., 2002, Van Etten et al., 1994). For example, *Arabidopsis* plant extracts have been shown to inhibit the in vitro growth of most nonhost *P. syringae* pathovars (Fan et al., 2011). In a similar manner fragarin, a phytoanticipin isolated from the cytosolic fraction of *Fragaria ananassa* leaf tissues, inhibited the growth of many bacterial pathogens (Filippone et al., 1999).

Induced defenses

The inducible defense responses include all the processes that occur within the plant cell once the recognition of pathogen. They represent the major part of resistance of plants against pathogens and arrest pathogen growth by producing structural barriers, inducing de novo biosynthesis of antimicrobial chemicals and/or proteins, and activating several defense pathways at the molecular level.

Clearly, the beginning of the inducible responses depends on the recognition of the pathogen. Plants lack circulating cells specialized in microbe recognition such as macrophages of vertebrates. Instead, each cell is able to recognize and respond to pathogens autonomously. As we have already mentioned at the beginning, the molecules that indicate to the plant cell the presence of the pathogen are PAMPs that are recognized by pattern recognition receptors (PRRs), which are localized on the surface of every cells. In addition, some PRRs recognize host-derived ‘danger’ signals (damage-associated molecular patterns; DAMPs, another class of elicitors), such as plant peptides or cell wall fragments released during infection or wounding. PAMP-binding or DAMP-

binding activates PRRs and triggers profound and tractable physiological changes in plant cells resulting in PTI (Monaghan and Zipfel 2012).

All known plant PRRs are plasma membrane-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) with modular functional domains. RLKs contain an extracellular domain (ECD) characterized by the presence of many leucine (leucine-rich repeat, LRR) and capable of binding PAMPs, a single pass transmembrane (TM) domain, and an intracellular kinase domain. RLPs contain an ECD and a TM but have only a short cytosolic domain without an obvious signaling domain. Notably, in contrast to mammals, no intracellular nucleotide-binding-leucine-rich repeat (NB-LRR) protein recognizing a PAMP has yet been identified in plants (Maekawa et al., 2011) (Figure 3).

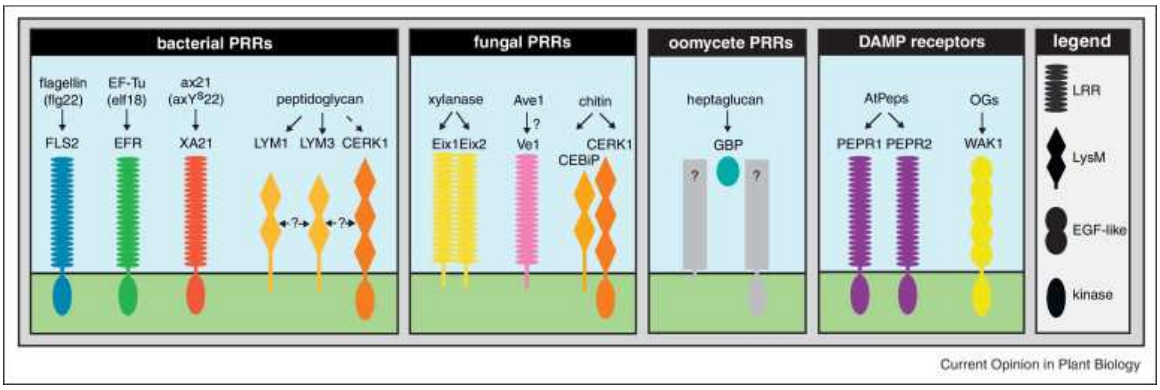

Figure 3: *Pattern recognition receptors with known ligands. The major types of ligand are fungal and bacterial PAMPs and DAMPs (Monaghan and Zipfel 2012).*

PAMPs are highly conserved molecules in a wide range of pathogens and play, sometimes, important structural or enzymatic functions. In fact, are classified as PAMPs enzymatic proteins of the life cycle of pathogens, products of bacterial metabolism, cell wall fragments of fungi and degrading enzymes such as polygalacturonases, lyases and a large variety of cellulases.

Has been demonstrated that the LRR-RLK FLS2 recognizes bacterial flagellin (or the active epitope flg22) in *Arabidopsis thaliana*, tomato, *N. benthamiana* and rice (Boller and Felix 2009). Comparatively, EFR is a Brassicaceae specific PRR that recognizes bacterial elongation factor Tu (EF-Tu or its 18 amino-acid epitope elf18) that is another well known bacterial PAMPs (Figure 3). Likewise, the tomato LRR-RLPs Eix1 and Eix2 are PRRs that bind fungal xylanase (Ron and Avni 2004). The well known fungal PAMPs are chitin and β-glucan that are recognize by CERK1 (in collaboration with CEBiP) and GBP (β-glucanbinding protein) respectively (Umemoto et al., 1997). Finally, the *Arabidopsis* LRR-RLKs PEPR1 and PEPR2 recognize AtPeps, a family of peptide DAMPs that may amplify PTI signalling (Krol et al., 2010), while the RLK WAK1 is a PRR for oligogalacturonides, which are released from plant cell walls during fungal infections or wounding (Brutus et al., 2010) (Figure 3).

The binding of PAMPs by PRRs leads to the activation of the kinase domain of the receptor and thus brings to the activation of the inducible defenses through metabolic events within the cell. These include the production of reactive oxygen species (ROS, also called the oxidative burst), production of reactive nitrogen species such as nitric oxide (NO), the activation of mitogen- associated and calcium-dependent protein kinases (MAPKs and CDPKs), variation in the levels of phytohormones such as ethylene (ET), jasmonic acid (JA) and salicylic acid (SA), alterations in the plant cell wall, induction of antimicrobial compounds and the synthesis of pathogenesis-related (PR) proteins (Nicaise et al., 2009; Tena et al., 2011) (Figure 4).

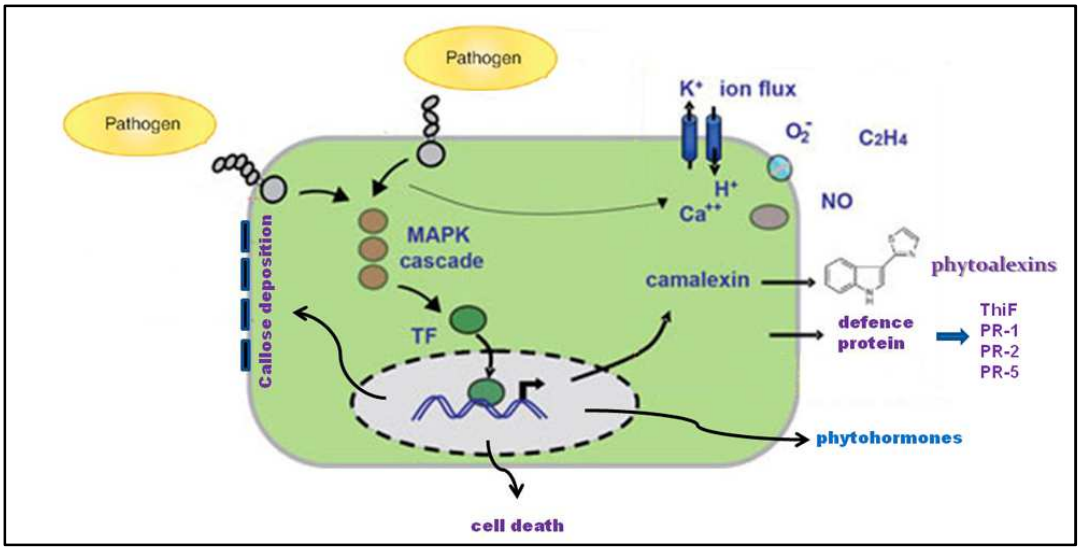

Figure 4: *Schematization of plant defense response against pathogen after the recognition of PAMPs.*

ROS, primarily superoxide and hydrogen peroxide, and nitrous oxide production, is one of the earliest events that occurs in response to the recognition of pathogen. During this response, ROS are produced by plant cells via the enhanced enzymatic activity of plasma-membrane bound NADPH-oxidases, cell wall bound peroxidases and amine oxidases in the apoplast (Hammond-Kosack and Jones 2000).

In addition during plant-pathogen interactions, the activity and levels of the ROS detoxifying enzymes ascorbate peroxidase (APX) and catalase (CAT) are suppressed by SA and NO (Klessig et al., 2000). Because during the plant pathogen defense response the plant simultaneously produces more ROS while decreasing its ROS scavenging capacities, accumulation of ROS occurs.

ROS play a dual role, both are toxic, thus contributing to the elimination of the pathogen, both act as second messengers. H_2O_2 can diffuse into cells and activate many of the plant defenses, including PCD (Dangl and Jones 2001) and the systemic expression of various defense-related genes (Orozco-Cardenas et al., 2001). Also, it has been demonstrated that ROS can activate pathways that lead to callose deposition (Rojas et al., 2012) and drive oxidative cross-linking of polymers in the plant cell wall to strengthen it against degradation, which may restrict pathogen spread.

Finally, it has been demonstrated that ROS can interact with MAPK cascade. Via a phosphorelay mechanism these cascades, minimally composed of a MAPKKK (MAPK kinase kinase), a MAPKK (MAPK kinase) and a MAPK, link upstream receptors to downstream targets. MAPK pathways are involved in the regulation of development, growth, programmed cell death and in responses to a diversity of environmental stimuli including cold, heat, reactive oxygen species, UV, drought and pathogen attack (Pitzschke et al., 2009).

MAPK activation is one of the earliest signaling events after the plant senses invading pathogens (Pedley et al., 2005) and several PRRs have been shown to trigger MAPK signaling upon perception of PAMPs. Both flg22 and elf18 can trigger a strong but transient activation of MAPKs in *Arabidopsis* (Asai et al., 2002; Roux et al., 2011) and upon stimulation by these PAMPs, both FLS2 and EFR form heterodimers with BAK1, a receptor-like protein kinases (Chinchilla et al., 2007). Loss of BAK1 results in marked reduction of flg22- and elf18-triggered activation of MAPK (Heese et al., 2007).

The *Arabidopsis* genome contains about 110 genes coding for putative MAPK pathway components: 20 MAPKs, 10 MAPKKs and more than 80 MAPKKKs (MAPK Group 2002). The best-characterized *Arabidopsis* MAPKs are MPK3, MPK4 and MPK6, all of which are activated by a diversity of stimuli including pathogens and oxidative stress. While MPK4 negatively regulates biotic stress signalling, MPK3 and MPK6 act as positive mediators of defense responses.

It has been demonstrated that H_2O_2 activates MPK3 and MPK6 and that these two kinases are necessary to induce defence (Kovtun et al., 2000). Although exogenously H_2O_2 can activate the MAPK cascade, recent evidence suggests that PAMP-triggered MAPK activation is independent of the NADPH oxidase mediated oxidative burst. Silencing of *RbohB*, a gene required for ROS accumulation, in *Nicotiana benthamiana* blocks the ROS burst upon flg22 or chitin treatment but does not affect MAPK activation induced by these PAMPs (Segonzac et al., 2011).

During the interaction with the pathogen, the plant produces defense molecules; in fact, the healthy cells adjacent to the damaged ones, in response to material released by them, produce the phytoalexins that are lipophilic compounds with low molecular weight and antimicrobial activity. They belong to structurally different chemical groups such as flavonoids, furanocoumarins, terpenoids, stilbenes and polyacetylenes. Most of phytoalexins are produced in response to a fungal attack but there are also produced against bacteria, viruses, nematodes. These compounds are toxic and inhibit the growth implementing changes in physiological and biochemical processes that alter the membranes permeability (Morrisey e Osburn, 1999). The phytoalexins are not normally detectable in the plant before infection, but are synthesized rapidly after recognition of pathogen through the activation of new biosynthetic pathways.

Camalexin (3-thiazol-20-yl-indole) is the major phytoalexins produced in *Arabidopsis* and related Brassicaceae species (Ahuja et al., 2012). Emerging evidence suggests that

MAPKs play key roles in the regulation of camalexin production. In fact, it has been demonstrated that activation of MPK3/MPK6, through the WRKY33 transcription factor, leads to camalexin induction and up regulation of multiple genes, including *pad2*, *cyp71a13*, and *pad3*, that encode enzymes in the camalexin biosynthetic pathway (Mao et al., 2011; Ren et al., 2008). In vivo binding of WRKY33 to the *pad3* promoter suggests that WRKY33 directly activates the expression of camalexin biosynthetic genes and biochemical data demonstrate that WRKY33 is a substrate of MPK3/MPK6 (Mao et al., 2011). Interestingly, MPK3/MPK6 also control the pathogen-inducible expression of the *wrky33* gene, and WRKY33 can bind to its own promoter, suggesting a potential MPK3/MPK6-mediated positive feedback regulatory loop that controls *wrky33* expression (Mao et al., 2011). As a result, dual-level regulations of WRKY33 by MPK3/MPK6 at post-translational and transcriptional levels play an important role in regulating camalexin biosynthesis in *Arabidopsis* (Meng and Zhang 2013).

The activation of MAPK, in addition to the production of phytoalexins, leads also to other types of responses. In general, the activation of the MAPK cascade leads to a response at genetic-level, which can be of two types: MAPKs can activate defense gene expression through direct phosphorylation of downstream transcription factors as in the case of WRKY33 or via modulating hormone biosynthesis and signaling. For example, in *Arabidopsis*, MPK3/MPK6 interacts with and phosphorylates ERF104 and ERF6, which activates defensin genes *pdf1.1, pdf1.2a, pdf1.2b*, *ChiB* and *hel* (Bethke et al., 2009; Meng et al., 2013). Also, the transcription factor VIP1, is phosphorylated by MPK3, which consequently leads to the relocalization of VIP1 from the cytoplasm to the nucleus to activate the *pr1* pathogenesis-related gene (Djamei et al., 2007). PR1 is an antifungal protein that belongs to the Pathogenesis-Related (PR) proteins. PR proteins are proteins specialized in response to pathogens and comprise a number of families that include enzymes. Most PR proteins in the plant species are acid-soluble, low molecular weight, and protease-resistant proteins (Leubner-Metzger et al., 1999). Acidic PR proteins are located in the intercellular spaces, whereas, basic PR proteins are predominantly located in the vacuole. Currently PR-proteins were categorized into 17 families according to their properties and functions, including β-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, ribosome-inactivating proteins, defenses, thionins, nonspecific lipid transfer proteins, oxalate oxidase, andoxalate-oxidase-like proteins (Van Loon et al., 1999) (Table 1). Among these PR proteins chitinases and β-1,3-glucanases are two important hydrolytic enzymes that are abundant in many plant species after infection by different type of pathogens. The amount of them significantly increase and play main role of defense reaction against fungal pathogen by degrading cell wall, because chitin and β-1,3-glucan is also a major structural component of the cell walls of many pathogenic fungi (Saboki et al., 2011).

Families	Type member	Properties
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase type I,II, IV,V,VI,VII
PR-4	Tobacco 'R'	Chitinase type I,II
PR-5	Tobacco S	Thaumatin- like
PR-6	Tomato Inhibitor I	Proteinase- inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	Ribonuclease like
PR-11	Tobacco 'class V' chitinase	Chitinase, type I
PR-12	Radish Rs- AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid- transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

Table 1: *Classification of pathogenesis related proteins*

MAPK directly influence the activity of phytohormones. The plant hormones ethylene, Jasmonic acid, and salicylic acid are important components of plant defense signaling (Broekaert et al., 2006; Browse et al., 2009; Spoel et al., 2012). Pathogen-triggered biosynthesis and signaling of these defense hormones induce the activation of a complex array of defense related genes, leading to diverse defense responses. Plant MAPK cascades have been implicated in both the regulation of defense hormone biosynthesis and the signaling events downstream of hormone sensing (Meng and Zhang 2013).

Plants under pathogen attack produce high levels of ethylene. Recent studies indicate that a subset of MAPKs in plants, represented by tobacco SIPK/Ntf4/WIPK and *Arabidopsis* MPK3/MPK6, plays key roles in regulating pathogen-induced ET biosynthesis (Han et al., 2010; Liu and Zhang, 2004). Two enzymatic steps are unique to ethylene biosynthesis: conversion of S-adenosyl-methionine (SAM), a common metabolic precursor, to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS) and oxidative cleavage of ACC to form ethylene by ACC oxidase (ACO). ACS is the rate-limiting enzyme in ethylene biosynthesis, and its activity is very low in unstressed tissues. Phosphorylation of ACS2/ACS6, two type I ACS isoforms, by MPK3 and MPK6

stabilizes the ACS protein in vivo, resulting in increases in cellular ACS activity and in ethylene production (Han et al., 2010; Liu and Zhang, 2004). In addition to post translational regulation, MPK3 and MPK6 also regulate the expression of *acs2* and *acs6* genes. WRKY33 directly binds to the promoters of *ACS2* and *ACS6* in vivo and is required for MPK3/MPK6-induced *acs2/acs6* gene expression (Li et al.,2012).

Similar to ET, JA and SA are also regulated by the activity of MAPK. SA is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens as well as the establishment of systemic acquired resistance(SAR) (Grant and Lamb 2006). Mutants that are affected in the accumulation of SA or are insensitive to SA show enhanced susceptibility to biotrophic and hemi-biotrophic pathogens. Recently, it has been shown that, methyl salicylate, which is induced upon pathogen infection, acts as a mobile inducer of SAR in tobacco (Park et al. 2007). SA levels increase in pathogen-challenged tissues of plants and exogenous applications result in the induction of pathogenesis related genes and enhanced resistance to a broad range of pathogens.

By contrast, JA and ET are usually associated with defense against necrotrophic pathogens and herbivorous insects. Although, SA and JA/ET defense pathways are mutually antagonistic, evidences of synergistic interactions have also been reported (Schenk et al. 2000; Kunkel and Brooks 2002; Beckers and Spoel 2006; Mur et al. 2006). This suggests that the defense signaling network activated and utilized by the plant is dependent on the nature of the pathogen and its mode of pathogenicity. In addition, the lifestyles of different pathogens are not often readily classifiable as purely biotrophic or necrotrophic. Therefore, the positive or negative cross talk between SA and JA/ET pathways may be regulated depending on the specific pathogen (Adie et al. 2007). However, in natural environments, plants often cope with multiple attackers and therefore plants employ complex regulatory mechanisms to trigger effective defense responses against various pathogens and pests (Bari and Jones 2009).

Among the inducible defense responses due to the presence of a pathogen, there is also the stomata closure. Plants are capable of closing stomata rapidly after sensing PAMPs (Melotto et al., 2006) and MAPK are also involved in this type of response (Meng and Zhang 2013). Stomatal closure during abiotic stresses (for example drought), requires a signaling pathway consisting of abscisic acid (ABA), H_2O_2, and NO (Neill et al., 2008). It has been shown that Guard cell–specific silencing of *mpk3* compromises PAMP -induced stomatal closure but does not affect the stomatal closure induced by ABA. On the basis of the fact that the promotion of stomatal closure by exogenous H_2O_2 is also abolished in *mpk3*-silenced plants, it is speculated that MPK3 might function downstream of PAMP/pathogen-induced ROS burst in promoting stomatal closure (Gudesblat et al., 2009).

By amplifying and transducing pathogen-derived signals perceived at membrane receptors and transducing these signals into altered gene expression, plant MAPK modules play a key role in the induction of defense mechanisms (Figure 5).

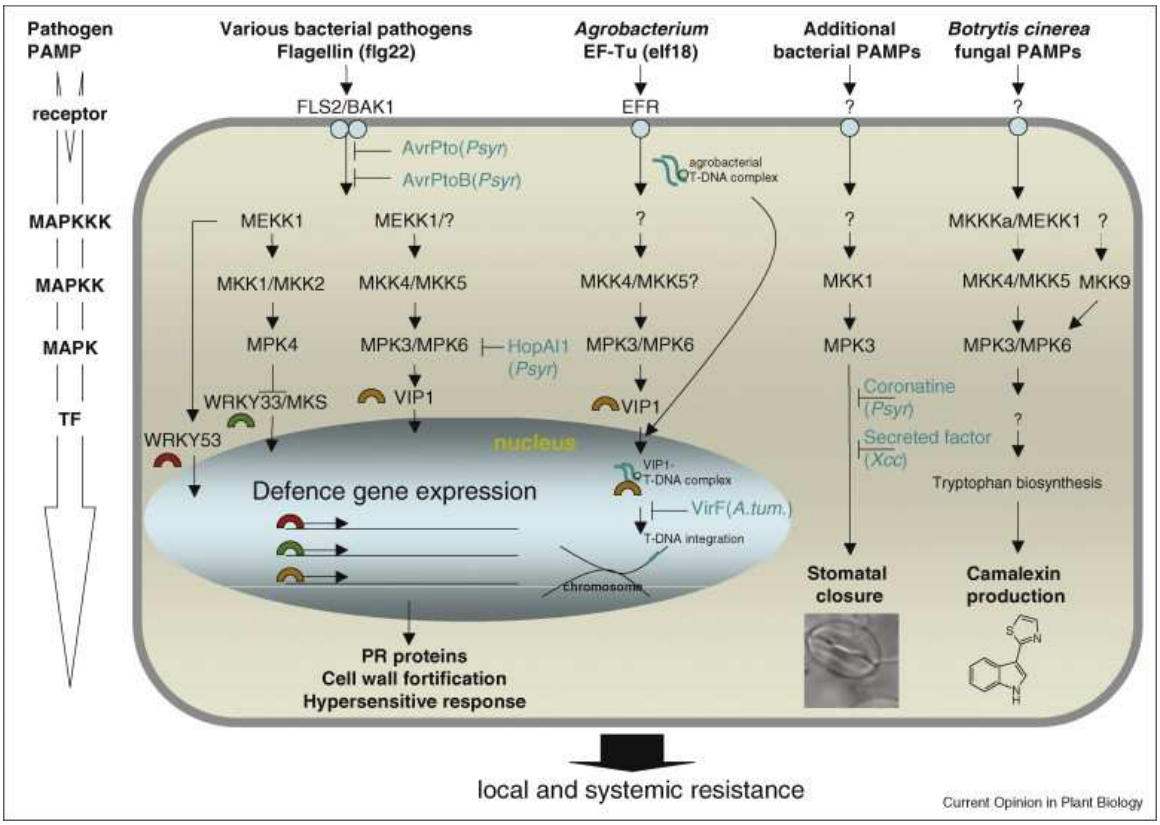

Figure 5: *PAMP-induced MAPK cascades in the plant defence to bacterial and fungal pathogens. PAMP-triggered activation of MAPK cascades initiates the synthesis of pathogen-induced synthesis of PR proteins (e.g. glucanases, chitinases), cell wall depositions, stomatal closure and phytoalexin (e.g. camalexin) synthesis. Together, these modifications result in pathogen resistance. Microbial factors interfering with the signal transduction are shown in blue. Activated transcription factors (TF) are shown as semicircles. Unknown receptors and MAPK cascade components are indicated by '?' (Pitzschke et al., 2009).*

MAPKs are also prominent targets for inactivation by effector proteins. For example, coronatine from *Pseudomonas syringae pv. tomato*, is a toxin able to re-open the stomata after they close in response to pathogen; or HopAI1 is a phosphothreonine lyase that dephosphorylates the threonine residue at which MAPKs are activated by their upstream MAPKKs (Zhang et al., 2007). It has been demonstrated that HopAI1 directly interacts with MPK3 and MPK6, thus attenuating flg22-induced MAPK activation and downstream defense responses. Strikingly, HopAI1 is also present in animal/human pathogens such as Shigella and Salmonella where it interacts with the MAPKs ERK1/2 and p38 (Li et al., 2007; Mazurkiewicz et al., 2008). Interestingly, the mechanisms of effector-mediated interruption of MAPK signalling employed by plant and animal pathogens are similar (Pitzschke et al., 2009).

Defense responses induced during pathogen invasion usually culminate in hypersensitive response cell death (Oh et al., 2006) that is a mechanism to prevent the spread of infection by microbial pathogens and is characterized by the rapid death of cells in the local region surrounding an infection. The cells involved in the HR generate an oxidative burst by producing ROS that affect cellular membrane function, in part by

inducing lipid peroxidation and by causing lipid damage. The alteration of ion components in the cell, and the breakdown of cellular components in the presence of ROS, results in the death of affected cells and the formation of local lesions. Reactive oxygen species also trigger the deposition of lignin and callose, as well as the cross-linking of pre-formed hydroxyproline-rich glycoproteins. These compounds serve to reinforce the walls of cells surrounding the infection, creating a barrier and inhibiting the spread of the infection.

The last event that occurs when a plant interacts with a pathogen is the onset of Systemic Acquired Resistance (SAR) that is an inducible defense mechanism, that is activated in the distal organs of a plant in response to a local infection of leaves with a pathogen, and confers enhanced resistance against subsequent attack by a wide array of pathogens (Vlot et al., 2008; Shah 2009). For example, root-inhabiting plant growth-promoting rhizobacteria enhance resistance against foliar pathogens (Bakker et al., 2007). Interestingly, this phenomenon has also been found in plants colonised by beneficial microbes. The biochemical and molecular mechanisms of SAR have not yet been completely clarified, but obviously a good communication is the key of this type of response. In plants, the pest-colonised organs utilise hormones, in particular SA, and secondary metabolites, some of which are systemically translocated, to communicate their stress status to the non-colonised organs and possibly adjacent plants, thus alerting them to the impending threat. Other factors, for example, ethylene, abscisic acid, Jasmonates, sugars and small peptides, also influence plant defense against pests and potentially could contribute to systemic signaling in plant defense (Shah 2009).

Clearly, the understanding of the basis of this process may lead to the development of compounds able to stimulate the SAR, reducing the use of pesticides and chemical compounds.

Fungal elicitors

The phytopathogenic fungi produce a plethora of different types of molecules that are involved in various aspects of parasitism and in the development of disease, such as the dissemination by vectors, attachment to the surface of plant organs, expression of symptoms, and elicitation of defence responses. Among these, there are essentially three classes of substances:

- cell wall components such as hepta-glucan, chitin and beta-glucan
- protein with enzymatic activity as xylanase, invertase, transglutaminase, pectinase and cellulase
- non-catalytic proteins

The third group includes various non-catalytic secreted proteins that were defined by Templeton et al. (1994) as cysteine-rich secreted proteins. The large group of cysteine-rich proteins is highly diverse and the classification proposed by Templeton groups is based on various parameters, including the number of cysteines, their molecular weight, and role in the pathogenic process, which, in many cases are not fully understood yet. Currently, referring on their primary sequence, seven non-catalytic fungal protein families have been identified, including the cerato-platanin (CP) family (CPF) (PF07249), the class I hydrophobins (PF01185), the class II hydrophobins (PF06766), the elicitins (PF00964), the PcF family (PF09461), the NIP-1 family (PF08995) and CFEM family (PF05730) (Pazzagli et al., 2014). Among these, hydrophobins have already received considerable attention in research: they are low molecular weight proteins, composed by 75-120 amino acids, and characterized by the presence of eight conserved cysteine reidues to give four intramolecular disulfide bridges.

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. 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 and render them hydrophobic (Ren et al 2013). Hydrophobins have been reported to be involved in several aspects of fungal development and in pathogen–host interactions (Sunde et al 2008).

Another family of particular interest is the CPF that was discovered only fifteen years ago and has been the main object of this thesis.

Cerato platanin family

CPF includes about one hundred proteins secreted by both ascomycete and basidiomycete. The core member of the family is cerato platanin from *Ceratocystis platani*, a necrotrophic fungus that infects plane trees (*Platanus* sp.) (Pazzagli et al 1999).

The CP proteins constitute a well conserved family with a 70% of homology, an unusually high value if compared to the other secreted fungal protein families. Most of the members have been shown to act either as virulence factors or as elicitors. Briefly, CP, and Pop1 from *Ceratocystis populicola*, induce phytoalexins production in *Platanus acerifolia* (Pazzagli et al., 1999, Comparini et al., 2009); the Snodprot1 from *Phaeosphaeria nodorum* is required for virulence (Jeong et al. 2007); the Sp1 from *Leptosphaeria maculans* induces auto-fluorescence in *Brassica napus* leaves (Wilson et al. 2002) and the MpCP1 from *Moniliophthora perniciosa* has necrosis-inducing ability in tobacco and cacao leaves (Zaparoli et al. 2009). The Sm1 and Epl1 proteins from *Trichoderma virens* and *Trichoderma atroviride* induce systemic resistance (Seidl et al. 2006, Djonovic et al. 2007, Vargas et al. 2008) and the MgSM1 CP-like protein from *Magnaporthe oryzae* is able to induce enhanced disease resistance and HR in leaves (Yang et al. 2009). HR is also elicited by the recently identified BcSpl1, one of the most abundant proteins in the *Botrytis cinerea* secretome (Frias et al. 2011, 2013). In addition, members of the 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 the Aspf13 antigen from *Aspergillus fumigatus*, both causative agents of human lung diseases (Pan & Cole 1995; Rementeria et al 2005), and of the immune-modulatory protein Aca1 from the medicinal fungus *Antrodia camphorate* (Sheu et al 2009; Pazzagli et al., 2014) (Figure 6)

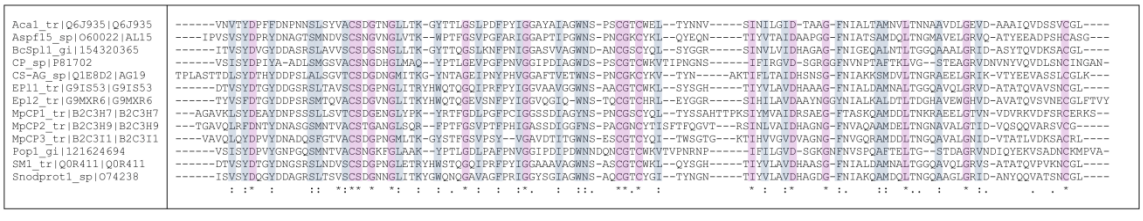

Figure 6: *Aligment of some CP proteins. The reported sequences are from proteins that have been characterized at protein level and that are cited in the paper. Aligment was performed with ClustalW by MEGA 5.2.1 (Higgins et al 1994). Invariable residues are shaded and marked with asterisks. The N-terminal secretion signal sequences of all proteins were removed to optimize the alignment. CPs proteins were from: Trichoderma atroviride Epl1 and Epl2 accession G9IS53 and G9MXR6, respectively); Botrytis cinerea (BcSpl1, 154320365); Trichoderma virens (SM1, Q0R411); Phaeosphaeria nodorum (Snodprot1, O74238); Coccidioides immitis (CS-AG; Q1E8D2); Taiwanofungus camphoratus (Aca1; Q6J935); Moniliophthora perniciosa (MpCP1, MpCP3, MpCP2, respectively B2C3H7 B2C3I1 B2C3H9), Neosartorya fumigate (Aspf15, O60022); Ceratocystis platani (CP, P71802), Ceratocystis populicola (Pop1, 121624694).*

On the basis of sequence similarity the PFAM has defined the 1–119 sequence of the mature CP as the "cerato-platanin domain" which is widespread in filamentous fungi (Chen et al 2013). All members of the family have in common the presence of this domain which is characterized by the presence of four cysteines to give two intra-molecular disulfide bridges. In addition all the 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 quite hydrophobic (Pazzagli et al 2009). Moreover, a signature sequence has been recently highlighted: 63% of the cerato-platanins proteins contain the CSD signature and 19% contained CSN (Chen et al 2013). Most of the CPs lack post-translational modifications, but some proteins such as the Sm1 from *Trichoderma virens* present the motif DNGRS, which is glycosylated.

CP is secreted into the culture filtrate, and is also found in the fungal cell wall of ascospores, conidia and hyphae (Boddi et al 2004). In 2011 de Oliveira and colleagues, determined the three-dimensional structure of the CP by NMR techniques (Figure 7A). Afterwards, other structures were determined using crystallography methods: Sm1 from *T. virens* (PDB 3m3g) and MpCP1, MpCP2, MpCP3 and MpCP5 from *M. perniciosa* (Barsottini et al 2013). The structures till now known show a high level of homology even at the 3D-level and particularly the comparison of CP, Sm1 and MpCP3 highlights the conservation of the main structural features that will be discussed below. All of them comprise a single domain containing the double-ψβ-barrel fold remarkably similar to those found in plant and bacterial expansins, lytic transglycosylases (LTs), endoglucanases, 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 through a non-enzymatic pathway.

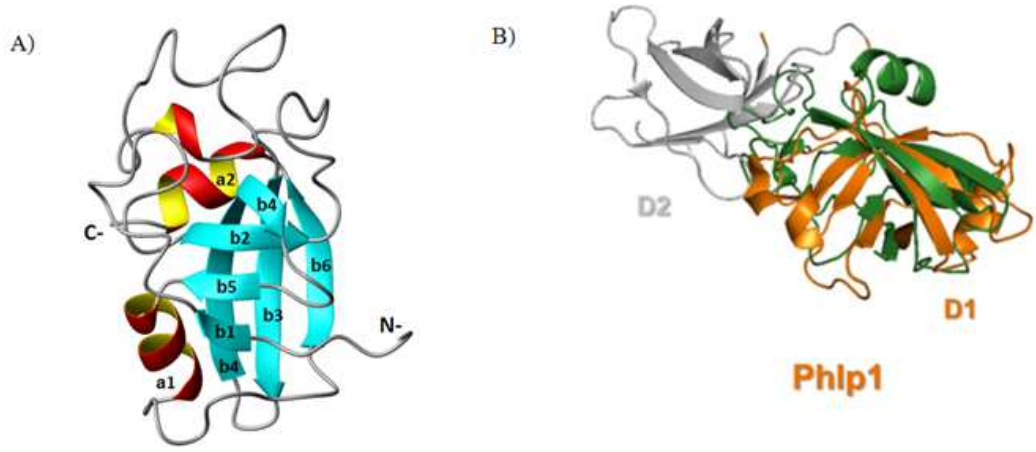

Figure 7: *The three-dimensional structure of CP:* ***A)*** *Ribbon representation of the structure;* ***B)*** *Comparison of the CP structure with expansin Phlp1 (orange)*

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 (Kerff et al. 2008). 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 them to slide against each other (Sampedro & Cosgrove 2005; Lee et al 2010). As generally observed for expansins, the CP and MpCP2 (the only tested proteins so far) did not display any detectable enzymatic activity (cellulase, endo-1,3-β-glucanase, polygalacturonase or chitinase activity), even when used at a high protein concentration (de Oliveira et al 2011; Barsottini et al 2013), and the lack of glycoside hydrolase activity can be explained by the absence of residues involved in hydrolytic catalysis in endoglucanases and LTs (de Oliveira et al 2011).
CP is also very similar to barwin proteins that contain only a single domain like cerato platanins (Gorjanovic 2009). Moreover like barwin, CP is able to bind oligosaccharides (dimers, trimers, tetramers, pentamers, and hexamers) of N-acetylglucosamine (GlcNAc, the monomer of chitin) 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 (Figure 8).

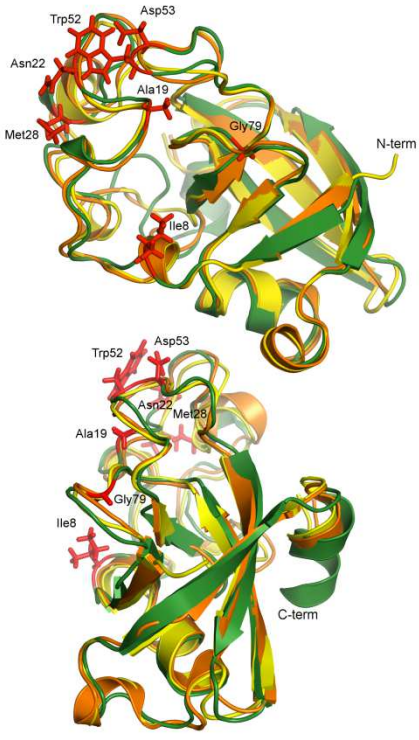

Figure 8: *3D structure alignment of cerato-platanin (CP, PDB id 2KQA, green), cerato-platanin 3 (MP3, PDB id 3SUL, yellow), and Sm1 (PDB id 3M3G,orange) observed from 2 different points of view. The side-chains of residues important for binding of GlcNAcs in CP are shown in red and labelled. Alignment was done with MultiProt [Shatsky et al 2004], structure manipulation was with PyMol (The PyMOL Molecular Graphics System, Version 1.4.1, Schrödinger, LLC.) and final assembly was with Gimp v 2.6.12.*

The high level of homology between cerato-platanins from different species and the presence of some of them in the cell wall suggest that these proteins can have an important role in the lifestyle of the fungi, but till 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 chlamydospores formation was found suggesting an important role of CP in fungal growth (Baccelli et al 2012).
Finally, all the residues involved in GlcNAc-binding are among the most conserved ones in the CP family. This data strongly support the hypothesis that one of the primary biological functions of proteins belonging to the CP family may be related to chitin and chitin-like materials and in particular in cell wall development (de Oliveira et al 2011). In fact, the similarity of structure and location between cerato platanins and expansins leads us to hypothesize a similar function, but has not yet been demonstrated.
In addition, based on the presence of the CP from *C. platani* on the 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 (Talbot, 1999; Whiteford and Spanu, 2002). As stated before, the expression of CP from *C. platani* is up-regulated during faster hyphal proliferation and spore formation (Baccelli et al 2012), MpCP2 and MpCP3 are also expressed during fast growth of *M. perniciosa* (Rincones et al 2008; Barsottini et al., 2013).
Clearly the primary biological role of the CP protein is related to fungal development although growing evidence indicates that the CP proteins do not always share the same biochemical and biological properties.

Biological activity of CP and Pop1

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.
It is apparent that some of the CP proteins enhance the virulence of necrotrophic fungi, as many of the proteins are expressed during the plant-pathogen interactions and induce the 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 (Frías et al., 2011). *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 (Jeong et al 2007). Necrosis-inducing ability was also observed for MpCP1 from *M. perniciosa*, a pathogen from cocoa (Zaparoli et al., 2009), although this toxic effect could not be reproduced (Barsottini et al., 2013)

Cerato-platanins seem to be one of a few known examples of isolated fungal molecules which are able to induce the systemic defence response by themselves, as it has been proven previously for bacterial flagellin and lipopolysaccharides (Mishina and Zeier, 2007; Newman et al 2013).

CP and Pop1 are the two certo platanins most studied in our laboratory. They are secreted by *Ceratocystis platani* and *Ceratocystis populicola* correspondingly, two fungal pathogens causal agents of canker stain of plane tree and the black cancer of poplar respectively. *C. platani* causes disease in *Platanus occidentalis*, *Platanus orientalis*, and their hybrid, *Platanus acerifolia* (Panconesi 1999; Engelbrecht et al. 2004; Ocasio-Morales et al. 2007). In the Mediterranean countries of Europe, where naturally resistant host clones are not known to exist, *C. platani* has infected and killed many centuries-old *P. acerifolia* and *P. orientalis* trees.

CP is a moderately hydrophobic and acidic protein of 120 amino acids, with an isoelectric point of 4.33. The molecular weight identified by mass spectrometry MALDI-TOF results of 12399.8 Da. The *cp* gene has two exons and an intron and recently it has been cloned into the vector pPIC9 of yeast *Pichia pastoris* to obtain large quantities of the recombinant protein (Carresi et al., 2006; Pazzagli et al., 2006).

Pop1, instead, is 122 amino acids long, its molecular weight is 12941,45 Da and has an isoelectric point of 4.58, features very similar to CP. As the *cp* gene, the *pop1* gene was cloned into the vector pPIC9 of yeast *Pichia pastoris* to obtain large quantities of the recombinant protein (Comparini et al., 2009).

CP and Pop1 are orthologous proteins with sequence identity 62% and an overall homology of 73%. Circular dichroism spectroscopy indicates some differences in their secondary structure (Figure 9), and Pop1 GRAVY index turns out to be quite different from the CP one (Comparini et al. 2009).

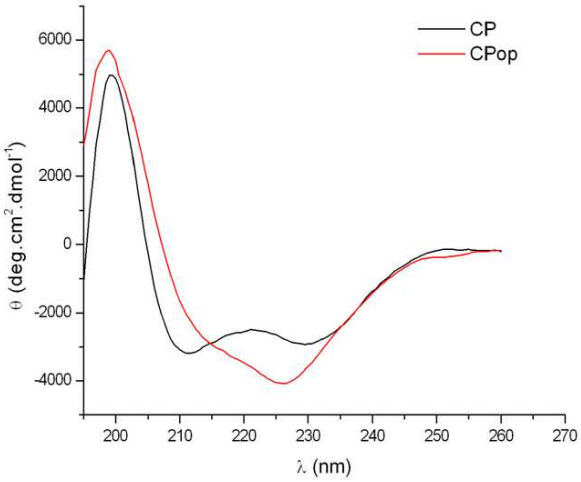

Figure 9: *Comparison of CD spectra of CP and Pop1.*

CP and Pop1 behave as elicitor because they induce phytoalexin synthesis, defense-related gene transcription, and eventually cell death (Fontana et al., 2008; Bernardi et al.,

2011). In fact, CP induce necrosis when infiltrated in tobacco leaves (Figure 10 A-B) and both the two proteins are able to induce the synthesis of phytoalexins, when applied on the lower surface of host as well as non-host leaves (Figure 10 C-D) (Pazzagli et al 1999; Comparini et al 2009).

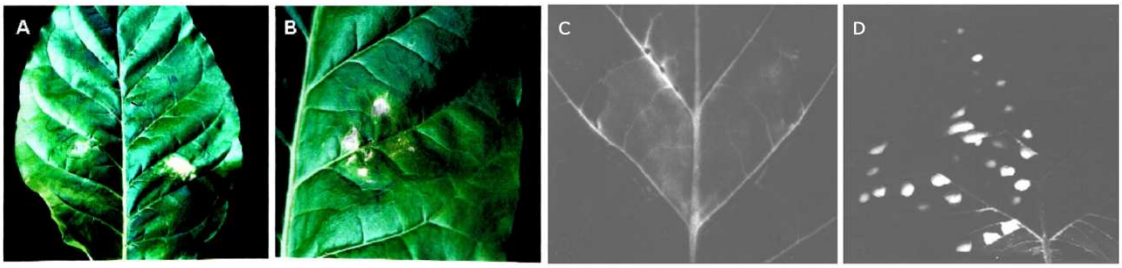

Figure 10: *Biological activity of CP:* ***A-B)*** *Leaf cell necrosis induced by pure ceratoplatanin;* ***C)*** *Fluorescence induced by 8 nmol of pure ceratoplatanin following infiltration in tobacco leaves;* ***D)****Fluorescence due to the application of CP on the lower surface of P. acerifolia.*

In addition if 24h after the application of the two proteins on the lower surface of host-leaves, about 10^3 conidia of *Ceratocystis* were added to each of the droplets containing the respective proteins, the fungal growth was inhibited (Fontana et al., 2008; Comparini et al 2009, Yang et al., 2009; Frías et al., 2013) (Figure 11).

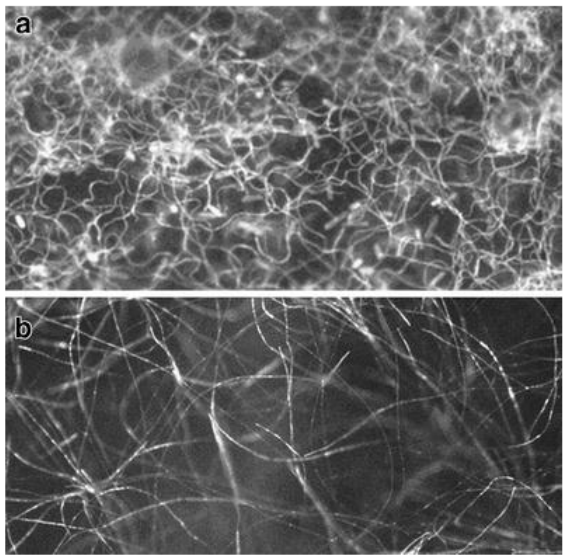

Figure 11: *Ceratocystis populicola grown for 2 days on poplar leaves that had been treated 24 h earlier wit:* ***a)*** *distilled water;* ***b)*** *10^{-4} M ceratopopulin; initial C. populicola conidial amount approximately 10^3*

In these case the induction of defense responses by CP proteins is unfavorable to the pathogens themselves. Probably these proteins are able to activate the host defenses that therefore restrict the subsequent growth of the fungus.

Other experimental data indicate that CP is also able to induce transcription of defense-related genes and cell death in various host and non-host plants (Pazzagli et al 1999, 2006, Fontana et al. 2008, Comparini et al 2009, Bernardi et al 2011). Until now, no PRR has been identified to explain the elicitation mechanism of CP and Pop1 in the host.

Recently, the unusual feature of CP and Pop1 to self-aggregate in solution has been investigated; both proteins form 'ordered aggregates', which are constituted by the unfolded chains that are able to enhance the defense responses, i.e. the production of phytoalexins and localized cell death (Figure 12) (Pazzagli et al. 2009, Martellini et al. 2012).

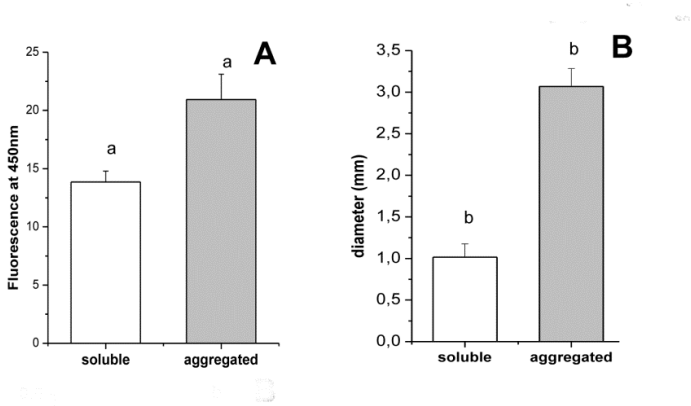

Figure 12: *Biologic activity of 1.5 9 10^{-4} M of soluble (white) and aggregated (gray) Pop1.* ***A)*** *Phytoalexins production by poplar leaves. Values shown are arbitrary fluorescence units after subtraction of the fluorescence induced by 10µl of water (controls for soluble protein) and by 1.2 µl of 10 % acetic acid vacuum dried and re-suspended in 10 µl of water (controls for aggregated protein). Values are the means of six independent experiments performed in triplicate.* ***B)*** *Cell death detection after 48 h of incubation reported as mm of cell death area.*

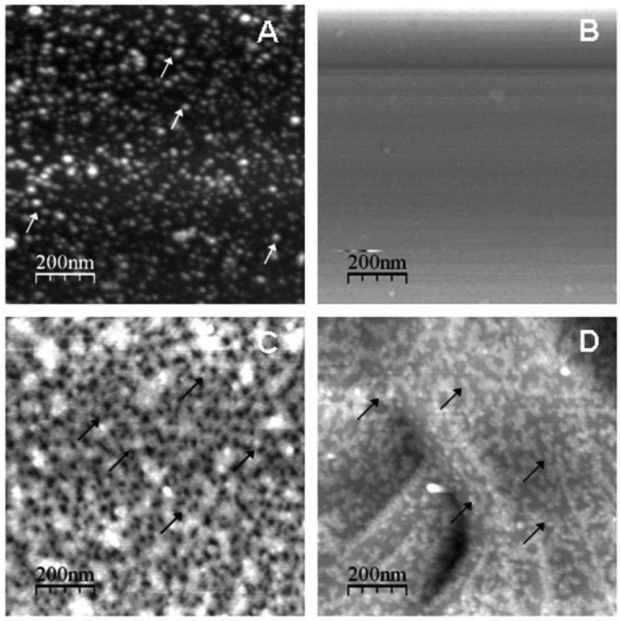

Figure 13: *AFM topography of Pop1.* ***A)*** *Pre-aggregated Pop1 adsorbed onto hydrophilic mica. 1.3 mM Pop1 was aggregated for 3 days at 37 °C in acetic acid, diluted 1:50 and adsorbed onto mica. B–D 10 µl of 26 µM soluble Pop1 adsorbed overnight onto* ***B*** *hydrophilic mica,* ***C*** *hydrophobic mica treated with APTES,* ***D*** *gold sheets. After adsorption, samples were washed with water and dried under N_2 before AFM analysis. Aggregates are imaged as light-gray on a dark background. The arrows indicate representative aggregates. Major and minor axes are measured on the aggregate profile obtained by the WSxM program.*

Interestingly, CP and Pop1 are able to interact with hydrophobic components (Figure 13) (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 (Figure 14) (Martellini et al. 2012).

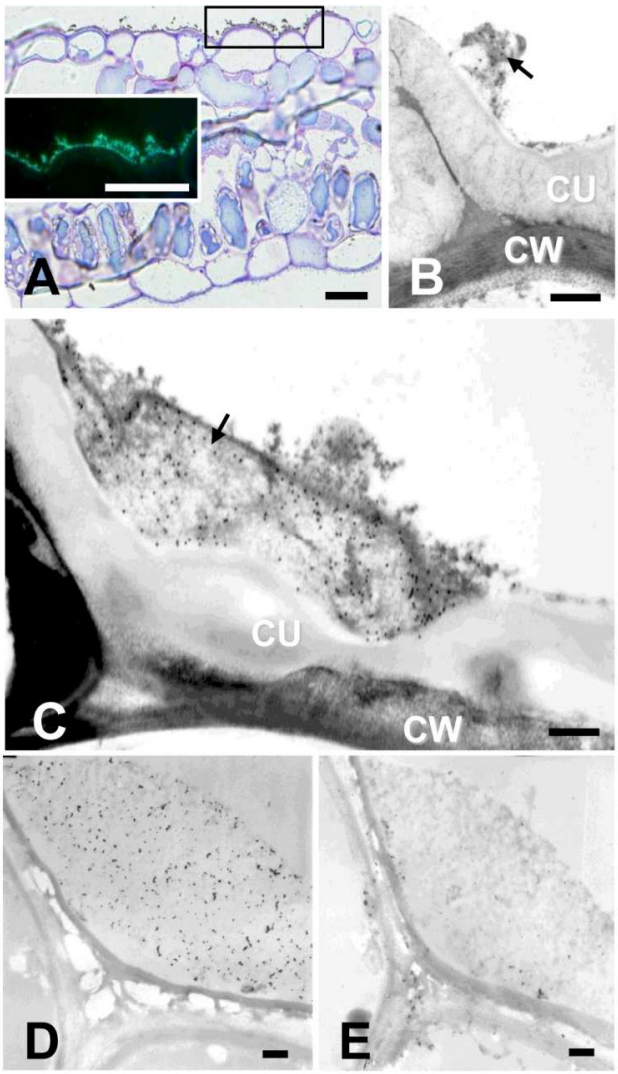

Figure 14: *Immunogold detection of CP in treated leaf tissues.* ***A)*** *Toluidine blue-stained semi-thin cross section of a leaf (upside down) after CP application: gold labeling after silver enhancement is visible only over the cuticle as black precipitate or bright fluorescent spots when observed with UV and interference contrast (inset enlargement of the framed area).* ***B)*** *Transmission electron microscopy (TEM) of a thin cross section through the epidermal cell wall (CW) and cuticle (CU) at 6 h after CP application: note that some labels very likely formed by the wax layer over the cuticle and CP, start to appear (arrow).* ***C)*** *Thin sections of the same sample as in b at 24 h after CP application: labels are larger and in some places appear as a fibrillar network (arrow) probably formed by unfolded protein and waxes.* ***D)*** *Thin section of the same sample as in b at 48 h after PC application: a more consistent labeling can be observed.* ***E)*** *Serial section of D, in which labeling was carried out with a pre-immune serum as a control: only a very small number of gold particles are present. All bars 500 nm, except for a where bar 10 µm.*

The data obtained until now, indicate that the CP proteins are certainly involved in the interaction with the host, even though many aspects remain to be clarified, in particular about the mode of action of these proteins and the primary role that they play in the fungal cell wall.

Aim of the work

The saprophytic and pathogens fungi present in their wall, and secrete into the culture medium, proteins without enzymatic activity that have recently proven to be responsible for the induction of defense responses in hosts. Among these proteins are of particular interest those belonging to the Cerato platanin family, many of which have proven to be able to act as elicitors.

The main objective of this thesis is to characterize both the biological function and the role in the interaction with plants of the core member of the family, Cerato platanin (CP) from the ascomycete *Ceratocystis platani*, and the ortholog protein Cerato populin (Pop1) from *Ceratocystis populicola*.

In particular we have developed two different lines of research:

1) **Biological function:** CP and Pop1 are presents in fungal cell wall and their three-dimensional structure is very similar to that of expansins (de Oliveira et al., 2011). In addition it has been shown that the expression of the cerato-platanin gene is related to hyphal growth and chlamydospores formation (Baccelli et al., 2012). On the basis of this data, we investigated the expansin-like activity of the two proteins.

2) **Plant-pathogen interaction:** studies carried out in previous years have shown that CP and Pop1 are capable of eliciting defense responses in host and non-host plants (Pazzagli et al., 1999; Comparini et al., 2009). The second line of research has as its objective the investigation of the eliciting ability of the two proteins on different plant tissues including the host *Platanus acerifolia* and the plant model organism *A. thaliana*.

Materials and Methods

Production and purification of CP and Pop1

CP and Pop1 used for all the experiments were produced by heterologous expression in the yeast *Pichia pastoris*. Reagents and kits for production of recombinant proteins were purchased from Invitrogen (San Diego, CA) and from Qiagen (Valencia, CA). Recombinant CP was purified from the culture medium of *P. pastoris* cells transformed with the pPIC9-cp plasmid according to the original method by Carresi et al. (2006). To obtain a higher yield of secreted CP the method was modified by inserting EA repeats after the KEX2 site in the pPIC9 plasmid (Pazzagli et al. 2009). The pPIC9-pop1 plasmid was constructed with the same methods with minor modifications (Martellini et al. 2012). The recombinant proteins were purified by reverse phase C-18 HPLC and quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) and by amino acid analysis performed on an amino acid analyzer equipped with a Beckman 4×120mm Na^+-highperformance column (Benson and Hare 1975). Purity of either CP and Pop1 was checked by SDS-PAGE and mass spectrometry and amino acid composition analysis. Secondary structure and biological activity of the recombinant proteins were tested by circular dichroism and the phytoalexin assay before use, according to Carresi et al. (2006) and Martellini et al. (2012).

Effect on cellulosic materials

Whatman no. 1 filter paper (Whatman-GE Healthcare, Milan, Italy) was used as first substrate to assay the effect of CP and Pop1. Filter paper was cut into 1-cm-diameter discs (6 mg each), and a single disc was incubated in 2 ml of 50 mM sodium acetate buffer (pH5.0) containing CP or Pop1 at three different concentrations (3×10^{-7}, 3×10^{-6} and 3×10^{-5}M). Buffer only or buffer containing the protein bovine serum albumin (BSA) at the highest concentration (3×10^{-5}M) were used as negative controls. The experiments were performed in Pyrex 20ml glass culture tubes (Sigma-Aldrich, St. Louis, MO, USA) sealed with parafilm and incubated oblique at 38°C, for 48 h, onto an orbital shaker (IKA, Staufen, Germany) at 320 rpm. At the end of the incubation period, pictures were taken, discs were removed and the absorbance at 500 nm on 1 ml of suspension was measured in order to quantify the paper fragments produced in each trial. Each measurement was taken immediately after shaking the suspension in the measuring cuvette, and three different measurements were taken for every suspension. The remaining 1 ml was used to verify the production of reducing sugars.

In order to test whether the action on cellulose was the result of a Cu-dependent polysaccharide monooxygenaselike activity, as recently found for the family-61 glycoside hydrolase (GH61) (Zifcáková and Baldrian 2012), the experiment with filter paper was repeated by assaying the incubation conditions reported for these enzymes. The paper disc was firstly incubated for 1 h in 50 mM sodium acetate buffer (pH5.0) only, or in the presence of 15 mM Na_2EDTA, used as a metal chelator in order to prevent the action of CP (incubations at 38°C, 320 rpm); CP was then added to a final concentration of 3×10^{-5}M to buffer/EDTA and buffer only, and the latter was also amended by adding $CuSO_4$ to a final concentration of 30 µM and ascorbic acid (chemical reductant) to 1 mM in order to increase the action of CP (Harris et al. 2010; Quinlan et al. 2011). Incubations were performed at 38°C, 320 rpm, for 48 h.

The ability of CP to act on cellulose was also assayed with different substrates such as microcrystalline cellulose (Avicel PH-101, catalogue no. 11365 Fluka, Sigma-Aldrich) and cotton.

CP 3×10^{-5}M was incubated with 1 mg of Avicel in 0.5 ml of 50 mM acetate buffer (pH5.0). Buffer or buffer containing BSA 3×10^{-5}M were used as a control. The experiments were performed in Pyrex 18-ml glass tubes sealed with parafilm and incubated at 38°C, for 24 h, with shaking at 320 rpm. The physical structure of Avicel after treatment was observed using a light microscope (Optika Srl, Bergamo, Italy).

Cotton fibres from pharmaceutical-grade cotton wool were firstly mercerized according to Saloheimo et al. (2002). In summary, 1 mg of cotton fibres was treated with 25 % NaOH for 15 min at 4°C, washed several times with distilled water and subsequently incubated in Pyrex 18-ml glass tubes with 0.5 ml of 50 mM acetate buffer (pH5.0) containing CP 3×10^{-5}M, buffer only, or buffer containing BSA 3×10^{-5}M. After 24 h at 38°C, with shaking at 320 rpm, the fibres were removed, suspended in distilled water with glass beads, and sonicated for 1 min at 25 µm, using a Soniprep 150 sonicator (MSE Ltd, London, UK). The physical structure of the fibres after sonication was observed by light microscopy.

Effect on chitin and colloidal chitin

CP and Pop1 3×10^{-5}M were incubated with 1 mg of chitin from crab shells (catalogue no. C9752, Sigma-Aldrich) or 1 mg of lyophilized colloidal chitin prepared as described by Roberts and Selitrennikoff (1988) from shrimp shells chitin (catalogue no. C9213, Sigma-Aldrich). Incubations were performed in 0.5 ml of 50 mM acetate buffer pH5.0, at 38°C, 320 rpm, for 72 h. At the end of the incubation period, chitin and colloidal chitin were analyzed by light microscope to observe any effect caused by CP and Pop1. Buffer and buffer containing BSA 3×10^{-5}M were used as controls.

Hydrolytic activity assay

The presence of reducing sugars after activity on filter paper was verified by dinitrosalicylic acid (DNS) method (Miller 1959) at the end of the 48h incubation period with CP and Pop1 3×10^{-5}M. One millilitre of suspension was firstly centrifuged and 300 µl of supernatant were added to 300 µl of 1 % DNS solution. The samples were then incubated at 90°C for 15 min, added with 100 µl of 40 % potassium sodium tartrate, cooled at room temperature, and the absorbance at 575 nm was then measured. The absorbance values were compared to a glucose calibration curve made in 50 mM acetate buffer (pH5.0) to determine the concentration of reducing sugars.

Analysis of optimal pH and temperature

Optimum pH and temperature for the activity of CP on cellulose were determined by measuring the activity on filter paper. Filter paper discs were prepared as previously described; CP was used at the concentration of 3×10^{-5}M. Optimal pH was established at 38°C incubating CP in 2 ml of 50 mM citrate–phosphate buffer pH3.0, 5.0 or 7.0, and Tris–HCl pH9.0, with shaking at 320 rpm. The temperature optimum was determined both in 50 mM citrate– phosphate buffer pH5.0 and distilled water (pH5.7), performing the incubation at 25, 38 and 50°C with shaking at 320 rpm. For each experiment, a corresponding control with buffer only was incubated.

The absorbance at 500 nm of the paper fragments produced in CP-treated samples and control samples was measured on 1 ml after 48 h of incubation in the various conditions. Each control value was subtracted from the corresponding CP-treated sample value and used for the graphs.

Polysaccharides binding assay

The binding ability of CP and Pop1 was tested on cellulose (filter paper and Avicel), chitin, colloidal chitin and β-1,3- glucan. CP and Pop1 3×10^{-5}M were incubated with a disc (1-cm diameter) of Whatman no. 1 filter paper (6 mg each), 40 mg of Avicel PH-101 or 40 mg of chitin from crab shells in 50 mM acetate buffer pH5.0, 38°C, with shaking at 320 rpm. The experiments were performed in 2 ml of buffer in Pyrex 20-ml tubes for the incubations with paper (48 h), and with 1 ml of buffer in Pyrex18-ml tubes for the incubations with Avicel and chitin (72 h). At the end of the incubation period, the tubes were centrifuged and 10 µl of supernatant (unbound fraction) were vacuum dried, resuspended in 10 µl of sample buffer and loaded into a 15 % SDS–PAGE gel according to Laemmli (1970). As a control, 10 µl from a tube containing the same protein concentration used for the experiments were processed and loaded. The gels were stained with colloidal Coomassie blue.

In order to further evaluate the binding ability of CP and Pop1to polysaccharides, experiments with filter paper, chitin and β-1,3-glucan (catalogue nos. C9752 and G6513,

Sigma-Aldrich) were performed monitoring the binding after 1, 5, 15, 30 min, 1, 3, 6 and 24 h of incubation.

The binding of CP to filter paper was also analyzed in distilled water (38°C) and with different temperatures (25 and 50 °C). A test was also performed with a concentration tenfold lower. The binding of CP to Avicel was also analyzed in distilled water (38°C, 24 h). Binding capacity (Bmax) and dissociation constant (Kd) were determined for CP with colloidal chitin, which was prepared as described in the previous paragraph. Binding was analyzed by depletion isotherm as described by Georgelis et al. (2011). In summary, variable amounts of CP were added to 50 mM acetate buffer pH5.0 containing a fixed amount of binding substrate. The mixture was incubated at 38°C, for 1 h, with shaking. The samples were then centrifuged at 14,000×g for 10 min to collect the binding substrate and the protein in the supernatant was quantified by bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Illkirch Cedex, France), using BSA for calibration. Soluble protein was subtracted from the protein initially added to obtain the protein bound to chitin. Dissociation constant and binding capacity were calculated by fitting the data to Langmuir isotherm.

Treatment of filter paper with CP and cellulase

In order to test whether the action of CP on cellulose could enhance the action of hydrolytic enzymes, discs of Whatman no. 1 filter paper (6 mg) prepared as described previously were incubated in Pyrex 18-ml tubes containing CP 3×10^{-6} or 3×10^{-5}M in 1 ml of 50 mM acetate buffer (pH5.0). Buffer only or buffer containing BSA 3×10^{-5}M were used as negative controls. After 48 h of incubation at 38 °C, 320 rpm, 1 U of cellulase from Trichoderma viride (Cellulase E.C 3.2.1.4, catalogue no. C1794, Sigma- Aldrich) was added to each tube and the tubes were further incubated for 3 h at 38°C, 320 rpm. The reducing sugars released were then measured by DNS method (Miller 1959) as previously described.

The experiments were also repeated decreasing the amount of cellulase from 1 to 0.2 U/ml, or diminishing the incubation time with CP (24 h or no incubation).

Plant material and growth conditions

P. acerifolia leaves were gently provided by the Department of Biology of agricultural plants of Pisa.

Arabidopsis thaliana plants, ecotype Col-0, were grown in soil in a growth chamber at 22°C with a 12-h photoperiod under light intensity of 100 µE m^{-2} s^{-1} for 4^{-5} weeks. The phosphorylation assay for MAP kinases was performed on 12-day-old seedlings as described below. Unless differently specified, the experiments were performed on detached leaves.

Silene paradoxa L. seeds were collected from plants living on non-contaminated soil (Colle Val D'Elsa), serpentine soil (Pieve Santo Stefano) and a copper mine deposit (Fenice Capanne) in Tuscany (Italy). Sites and populations were described in Chiarucci et al., (1995), Gonnelli et al., (2001), Pignattelli et al., (2012). Seeds were sown in peat soil and after six weeks seedlings of the three populations were transferred to hydroponic culture, in 1-L polyethylene pots (three plants per pot) containing a modified half strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.50 mM $MgSO_4$, 20 µM Fe(Na)-EDTA, 1 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CuSO_4$ and 0.1 µM $(NH_4)_6Mo_7O_{24}$ in milliQ-water (Millipore, Billerica, MA, USA) buffered with 2 mM 2-morpholinoethanesulfonic acid (MES), pH 5.5, adjusted with KOH (Hoagland and Arnon, 1950) and different copper ($CuSO_4$) or nickel ($NiSO_4$) concentrations.

Nutrient solutions were renewed weekly and plants were grown in a growth chamber for eight weeks (24/16°C day/night; light intensity 75 µE m^{-2} s^{-1}, 12 h d-1; relative humidity 60-65%). At the end of incubation in test solutions, root samples were desorbed with ice cold (4 °C) $Pb(NO_3)2$ (10 mM) for 30 min. Plants were then divided into roots and shoots and the dry weight of the organs was recorded after drying at 70° for 1 day.

Leaves treatment and phytoalexin assay

Pl. acerifolia, A. thaliana and S. paradoxa leaves were removed from plants and placed directly into boxes containing moist filter paper. Ten-microliter droplets containing 1.5 nmol of CP or Pop1 (1.5×10^{-4}M) were applied on the lower surface of the leaves and maintained in a moist chamber. The incubation time is indicated in each experiments. After incubation, the droplets were recovered for measurement of phytoalexin synthesis elicitation, according to Scala et al. (2004). Fluorescence was recorded with a Perkin Elmer spectrofluorimeter 650- 10S (Perkin Elmer, Wellesley, MA), using λex=365 nm, λem=460nm and slit 5 for *P. acerifolia* and *S. paradoxa* leaves, while using λex=320 nm, λem=386 nm, slit 5 for *A. Thaliana* leaves. Eliciting activity of proteins was expressed as fluorescence intensity in arbitrary units of droplets, where defense phenolic compounds accumulated.

MAPK signaling determination

Platanus acerifolia: Treated leaves were cut in 2×2 cm squares corresponding to 0.2–0.5 g of plant tissue and stored at -80°C. Various protocols were used to optimize the methods for tissue homogenization and protein extraction (Wang et al. 2003, Valcu and Schlink 2006).

Protein extraction was performed on 0.2 g of leaves, which were added to 0.02 g of polyvinylpolypyrrolidone and ground to a fine powder in liquid N_2 in a pre-chilled mortar and pestle. Samples were added to 2ml of a solution containing 5% sucrose, 4%

SDS, 5% 2-mercaptoethanol and 1:100 protease inhibition cocktail (P9599, Sigma-Aldrich, St. Louis, MO). The suspension was Ultra-Turrax (IKA, Germany) treated for 30sec. and proteins were extracted for 15min under continuous stirring. Samples were centrifuged at 10 000g for 20min at room temperature and the clear supernatants were heated at 100°C for 3min and then cooled at room temperature. Proteins were precipitated by eight volumes of cold acetone for at least 1 h at −20°C and then centrifuged at 10 000g for 30min at 4°C. Pellets were resuspended in 0.8 ml of a phenol solution (Tris buffered, pH 8.0; Sigma) and in 0.8 ml of 30% sucrose in water, and vigorously vortexed. The phenol phase was separated by centrifugation at 10000 g for 3 min. The upper phenol phase was pipette into microtubes with five volumes of cold methanol and 0.1M ammonium acetate, and stored at -20°C for 30min. Precipitated proteins were recovered by centrifugation at 10000 g for 15 min, and then washed twice with cold methanol/ammonium acetate and twice with cold 80% acetone. The final pellet was dried and dissolved in Laemmli buffer.

Arabidopsis thaliana: seeds were sterilized by treating them for 3 min in 70% ethanol, 3 min in 20% bleach with 0.1% Tween 20 and extensively washed with sterile distilled water. Subsequently, the seeds were incubated in the dark at 4°C for 1 day and then dispensed into a 24-well tissue culture plate (10 seeds per well) containing 1 ml of Murashige and Skoog Basal medium supplemented with 0.5% sucrose, pH 5.7. The plates were incubated at 22°C with a 12-h-light photoperiod (100 µE m^{-2} s^{-1} intensity). After 8 days, the medium was replaced and the treatments were performed after four additional days. Seedlings were treated for 5, 15, 30 and 60 min by adding to the medium either CP to a final concentration of 1.5×10^{-4} M or an equal volume of sterile distilled water as a control.
After seedlings were snap frozen in liquid nitrogen, and proteins were extracted with a buffer containing 50 mM Tris-HCl at pH 7.5, 200 mM NaCl, 1 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 10% (v/v) glycerol, 0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1X protease inhibitor cocktail P9599 (Sigma-Aldrich) (Galletti et al., 2011). The protein amount in the samples was determined by Bradford assay.

Silene paradoxa: leaves were excised from the plant and floated for 5h on water with gentle shaking. Leaves were infiltrated by means of a hypodermic syringe with $1{,}5x10^{-4}$ M CP. Control leaves were infiltrated with distilled water. Leaves were incubated for 15-60 minutes at room temperature and then frozen in liquid nitrogen. For protein extraction, leaves were grounded to a fine powder in liquid N_2 and added of 400 µl of the same extraction buffer of *A. thaliana* seedlings. The protein amount in the samples was determined by Bradford assay.

With all three different leaf tissues, equal amounts of proteins (about 15 mg) were resolved on 12% polyacrylamide gels and transferred onto a PVDF membrane (Biorad). Primary antibodies against human phospho-p44/42 MAP kinase (Cell Signaling Technologies) were used; horseradish peroxidase-conjugated anti rabbit as secondary antibody (Cell Signaling Technologies) and the ECL western detection kit (GE healthcare) were used. Membranes were stripped with 50mM Tris-HCl pH 7.0 buffer containing 2% SDS and 0.1M 2-mercaptoethanol for 30 min at 55°C and then incubated with *A. thaliana* MPK3 and MPK6 antibodies (Sigma-Aldrich), which were used as quantitative references. Signal detection was performed as above reported. In the case of *P. acerifolia* experiments membranes were recovered and incubated with GAPDH antibodies (Sigma; dilution 1:1000), which were used as quantitative references. Alternatively, membranes were stained with Coomassie R-250, and the band relative to the large subunit of the RuBisCo protein was used to quantify the blot results (Galletti et al. 2011).

NO and H_2O_2 visualization and determination

NO and H_2O_2 production in *P. acerifolia* and *A. thaliana* leaves treated with CP or Pop1 was visualized in situ according to Lombardi et al. (2010). Briefly, NO was stained using the fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2DA; Sigma), which reacts with NO, producing a fluorescent compound (Kojima et al. 1998). H_2O_2 was visualized by the specific probe 2'-7'-dichlorodihydrofluorescein diacetate (DCFH2-DA; Sigma), which is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of H_2O_2. After staining for 1 h with the specific probe, the samples were washed twice in fresh buffer to remove excess fluorophore, and mounted on slides. Fluorescence was then observed under a Nikon Eclipse Ti-S Microscope, equipped with filter block FITC (λex 488 nm, λem 502–540 nm). Images were captured by a QICAM digital CCD camera (QImaging, CA).

To find the minimum active concentration of CP, *A. thaliana* leaves were treated with 1:2 serial dilutions of CP (starting from 300 µM) and the production of H_2O_2 in situ was analysed after 15 and 60 min. In order to analyse H_2O_2 production on the epidermis around stomata, epidermal peels were used. *Arabidopsis* plants were kept for at least 3 h under the light to ensure that most stomata were open before beginning the experiments, then epidermis was peeled off and placed on glass slides with the cuticle side in contact with MES buffer. Peels were pre-incubated for 2 h in MES buffer (25mM MES-KOH pH 6.15, 10 mM KCl) under the light, soaked for 20 min in 10 µM H_2DCF-DA diluted in MES buffer, washed three times in MES buffer, and then incubated with 150 µM CP for 5, 10 or 30 min. MES buffer was used as a negative control, while 0.2 mg ml^{-1} chitin was used as a positive control. The experiment was also repeated by treating peels with closed stomata. In order to analyse H_2O_2 production at the level of single guard cells, an epidermal peel was placed on a glass slide with the cuticle side in contact

with CP and was visualized in continuum under the microscope for 10 min; single stomata were photographed at 2-minute intervals.

NO content in the drops recovered after the treatment was determined by a fluorometric assay based on DAF-2DA (Corpas et al. 2004, Carimi et al. 2005). DAF-2DA was added to the collected drops at the final concentration of 20 μM. After 30min incubation in the dark, fluorescence was measured in a VersaFluor Fluorimeter (Bio-Rad, Hercules, CA) (λex 495 nm, λem518 nm).

H_2O_2 in the recovered drops was determined by the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen), following the manufacturer's instructions. Briefly, droplets were pooled and mixed with the reaction buffer (1:1 v/v) and incubated for 30 min in the dark. Absorbance was measured at 560 nm and the H_2O_2 concentration was calculated from a standard curve made with H_2O_2 solutions ranging from 1 to 10 μM.

Controls were prepared by treating leaves with both sterile distilled water and the protein bovine serum albumin (BSA) 150 μM; leaves were also treated with 0.2 mg ml^{-1} chitin (chitin from crab shells, catalogue no. C9752, Sigma-Aldrich) as a positive control.

Gene expression analysis

P. acerifolia: *t*otal RNA was isolated from CP- and Pop1-treated *P. acerifolia* leaves using hexadecyltrimethylammonium bromide (CTAB) lysis buffer and isoamyl alcohol-chloroform extraction, with subsequent DNA digestion using Amplification Grade DNase I (Sigma-Aldrich). Expression analysis was carried out by semi-quantitative real time-polymerase chain reaction (sqRT-PCR).

Reverse transcription was performed with 150 ng of total RNA isolated from treated and control leaves at different time points of treatment (3, 6, 9, 12 and 24 h), using the iScript™ cDNA synthesis kit (Bio-Rad). One microliter of first-strand cDNA was used for each PCR amplification (15-μl reactions). The transcripts coding for PR-5 thaumatin-like protein, PR-14 lipid-transfer protein (LTP) and ascorbate peroxidase (APX) (GenBank Accession Nos. FN821454, AM286249 and FN821453) were analyzed using gene-specific primers:

PR-5 forward, 5'-CTGGGATATTCTCTTGTGGCACTGG-3',
PR-5 reverse, 5'-CGTATGCGTAGCTGTAGGCACGAG-3';
PR-14 forward, ;5'-GAAGCTGCCATAACATGTGGTACGG- 3',
PR-14 reverse, 5_-CCAAAAGAGCACACCGCAAGTGG-3';
APX forward, 5'-CAATGGTCTTGATATTGCTGTCAGGC-3',
APX reverse, 5'-GCATAATCTGCAAAGAAGGCATCCTC-3'.

Each target gene was amplified in a multiplex reaction along with the *18S* ribosomal gene, which was used as an endogenous control to normalize (treated versus control sample) the amount of cDNA template subjected to reaction at each incubation time.

The absence of variability in the expression of the *18S* gene in leaves treated (or not) with CP had been previously showed (Fontana et al. 2008, Bernardi et al. 2011). The *18S* ribosomal gene was amplified in the same reaction as the target gene using the Ambion's Competimer™ technique (QuantumRNA™, Universal 18S Internal Standard; Applied Biosystems/Ambion, Foster City, CA). Little variations of intensity do not influence the results because the level of expression of the target gene is obtained by dividing the signal of its amplicon by the signal of the 18S amplicon as described by Bernardi et al 2011.

A. thaliana: A primer library containing 93 gene-specific primers for pathogen-inducible genes (Primer Library for *Arabidopsis* Pathogen-inducible Genes, Sigma-Aldrich) was employed for a large scale analysis that we performed by qPCR after 6 h of treatment. This time was selected after a preliminary expression analysis performed after 1, 3 and 6 h with 8 random-selected library genes in order to find the treatment time where the greater number of genes could be found modulated (data not shown). Further 13 primer pairs were designed with Primer Express Software 3.0 (Applied Biosystems) for a detailed analysis of defence-signalling pathways that we performed at various time points (1, 3, 6, 12 or 24 h). (Baccelli et al., submitted)

Leaves from 4-week-old plants were detached and treated on the lower surface with 10-µl drops of CP 150 µM (treated sample) or sterile distilled water (control sample) as previously described and incubated in a moist chamber for 1, 3, 6 , 12 or 24 h. Three treated leaves and three control leaves were incubated per each time.

Total RNA was extracted from each leaf by using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). RNA was treated with Amplification Grade DNase I (Sigma-Aldrich) and reverse-transcribed into cDNA (400 ng per sample) by using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA).

Real-time reactions (20 µl) were carried out with 10 ng of cDNA, 250 nM primers, and 1× Fast SYBR Green® Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. PCRs were run in a StepOne™ real-time PCR System (Applied Biosystems) using the recommended thermal-cycling conditions (hold 95 ˚C, 20 s; 40 cycles 95 ˚C, 3 s; 60 ˚C, 30 s). Before performing the relative expression calculation the performance of each amplification was checked and some primers were excluded from the study.

The relative gene expression values ($2^{-\Delta\Delta Ct}$) were calculated by using *Actin-2* as the endogenous reference gene and water-treated leaves as the calibrator sample (control sample), following the calculation described by Livak and Schmittgen (2001). *Actin-2* was used as a reference gene after confirmation of its transcriptional stability in our experimental conditions (CP- vs. water-treated leaves) (data not shown). *Actin-2* primers were included in the primer library. Statistical analysis (treated vs. control) was

performed by unpaired *t*-test by using GraphPad InStat v. 3.05 (GraphPad Software, San Diego, CA, USA).

Induction of resistance assay

The capacity of CP and Pop1 to elicit defense-related responses was determined according to Fontana et al. (2008), with modifications according to Comparini et al. (2009). At 24 h after treatment with CP or Pop1, about 8×10^2 conidia of *C. platani* were added to the droplets. After another 48 h, the conidia and hyphae were stained by adding a droplet of 100mM phosphate buffer, pH 7.0, containing 0.1 $\mu g ml^{-1}$ 4'-6-diamidino-2-phenylindole (DAPI). After incubation for 10 min, the leaves were inspected under a Leitz Laborlux S fluorescent microscope (Leica, Wetzlar, Germany). The induced resistance was evaluated as the degree of inhibition of conidial germination and mycelium growth according to a four-point scale from 0 to 3, where 0=no conidial germination; 1=≤33% conidial germination and <33% droplet area weakly colonized by mycelium; 2=33–66% conidial germination and 33–66% droplet area moderately colonized by mycelium; 3=≥66% conidial germination and >66% droplet area massively colonized by mycelium.

Viability staining

The viability of cells in treated leaves was determined by staining with propidium iodide (PI, Sigma), which is excluded by viable cells but can penetrate cell membranes of damaged or dead cells (Jones and Senft 1985). Leaves were incubated with 10 μM PI for 5 min, washed, mounted on slides and observed under a Nikon Eclipse Ti-S Microscope, equipped with filter block TRITC (λex 505–565 nm, dichroic 550 nm, λem 580–630 nm). Images were captured by aQICAM digital CCD camera (QImaging).

In situ detection of DNA fragmentation (TUNEL assay)

Treated leaves were fixed overnight at 4°C in 4% (w/v) paraformaldehyde. After dehydration in an ethanol series, the samples were embedded in Paraplast Plus (Sigma). Sections of 12 μm were cut and stretched on poly-lysine coated slides. The sections were then dewaxed in xylene and re-hydrated before examination.

The TUNEL assay was performed using an 'In Situ Cell Death Detection Kit' (Promega, Madison, WI) according to the manufacturer's instructions. To facilitate the entry of TdT (terminal deoxynucleotidyl transferase) enzyme into the tissue sections, the slides were treated with proteinase K (20 mg/ml^{-1}) for 20 min. The labeling reaction was performed at 37°C in a humidified chamber in the dark for 1 h. A negative control was included in each experiment by omitting TdT from the reaction mixture. As a positive control, sections were incubated with DNase I ($10Uml^{-1}$) for 10 min before the TUNEL assay. The fluorescence of incorporated fluorescein-12-dUTP was observed with a Nikon Eclipse Ti-

S Microscope, equipped with filter block FITC (λex 488 nm, λem 502–540 nm). Images were captured by a QICAM digital CCD camera (QImaging). Counterstaining was performed with PI (5 μM) (λex 495 nm, λem 518 nm). Experiments were repeated three times, each time labeling five slides for each sample.

Transmission electron microscopy

Plane leaves treated with 10-μl droplets containing 1.5 nmol of CP or Pop1 were maintained in a moist chamber for 6, 24 and 48 h at room temperature as described above. Droplets were recovered and the application spots were washed twice with water to remove the unabsorbed protein. The droplets were assayed for phytoalexin production to confirm the induction of defense responses. Tissue fragments (1–2mm^2) cut from the application spot area and its surroundings were fixed and embedded as previously described (Faoro et al. 1991). Briefly, for ultrastructure studies, tissues were fixed in 1.2% glutaraldehyde and 3.3% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, at 4°C for 2 h, postfixed in 1% OsO_4 in the same buffer for 2 h, dehydrated in an ethanol series and embedded in Araldite. Ultrathin sections were stained with 2% uranyl acetate and lead citrate and examined with a JEOL 100SX TEM (Jeol Ltd., Tokyo, Japan).

Stomatal closure assay

Arabidopsis plants were kept for at least 3 h under the light to ensure that most stomata were open before beginning the experiments. Epidermal peels were prepared from three different fully expanded leaves as described in the previous paragraph. In those conditions, peels have about 70% open stomata (Melotto et al. 2006). The tissues were placed on glass slides with the cuticle side in contact with MES buffer or with 1.5×10^{-4} M CP dissolved in MES buffer. As a positive control, 0.2 mg ml^{-1} chitin was used; MES buffer was used as a negative control. At various time points (30, 60 and 120 min) pictures were taken of random regions and the width of the stomatal aperture was measured by using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA).

Camalexin detection

In order to confirm the release of camalexin from the leaves, the fluorescence spectrum (λex=320 nm, λem=330-550nm, slit 5) of the droplets collected after 24 h of incubation was compared to the spectrum obtained by using a pure camalexin standard (kindly provided by Dr. E. Glawischnig, University of Technology, Munich, Germany). The experiment was also repeated to compare the two leaf surfaces in their ability to release phytoalexins after 48 h of treatment.

Camalexin was extracted from CP-treated leaves (12 and 24 h) according to Beets and Dubery (2011). Leaves (about 50 mg) were covered with 1 ml of 80% hot methanol and

homogenised using a T10 basic UltraTurrax homogenizer (IKA Werke GmbH & Co. KG, Staufen, Germany). The homogenates were centrifuged for 5 min at 17,000xg, and the supernatants were transferred into microcentrifuge tubes. The pellets were resuspended in methanol, and the extraction and centrifugation steps repeated. Subsequently, the supernatants were combined, methanol was evaporated in a speed-vacuum centrifuge and the pellets were resuspended in 600 µl of methanol and filtered through 0.22 µm filters (Millipore, Billerica, Massachusetts, USA).

Ten µl of a standard stock solution of camalexin (15 µg ml^{-1} in methanol) and 40 µl of sample were analysed by RP-HPLC using a C18 column, 3 µm, 15x4.6 cm (Supelco, Bellefonte, Pennsylvania, USA). Elution gradient was performed at a flow rate of 0.8 ml min^{-1} with the following solvent system: 10mM trifluoroacetic acid (TFA) in acetonitrile (solvent A); 10mM TFA in water (solvent B). The gradient used was 10% A for 2 min, from 10% to 98% A for 13 min, holding at 98% A for 10 min, from 98% to 100% A for 5 min. Detection was based on UV absorbance at 318 nm. Under these conditions, the camalexin peak appeared at a retention time (Rt) of 11.2 min as determined by preliminary analyses.

Resistance induction assays

Arabidopsis leaves were detached from 5-week-old plants and treated on the lower surface with 10-µl drops of 1.5×10^{-4} M CP, sterile distilled water (control) or 0.1% chitosan (about 20 µM in 10 mM acetic acid; molecular weight 50-60 kDa, deacetylation degree 85%). The leaves were incubated for 24 h in Petri dishes containing 0.8% agar, after that the drops were recovered and the leaves were infected on the same foliar surface with pathogens.

Botrytis cinerea strain PM10 (Cettul *et al.,* 2008) was kindly provided by Prof. F. Favaron, University of Padua, Italy. The fungus was grown on potato dextrose agar (PDA, Difco, Detroit, MI) at 22°C for 8 days before collecting conidia. Inoculations were performed by placing a single 10-µl drop per leaf of a suspension $2x10^{5}$ conidia ml^{-1} in 1% Sabouraud Maltose Broth (SMB) (maltose 8 g l^{-1}, peptone 2 g l^{-1}) on a side of the middle vein. Leaves were incubated at 22°C for 3 days before measuring the lesion diameter and taking photographs. In order to test for a direct inhibiting effect of CP against the germination of *B. cinera* conidia, two drops of conidial suspension were placed on a glass slide and one of these was added with CP whereas the other with sterile distilled water for an equal volume and germination/growth monitored.

Pseudomonas syringae pv. *tomato* (*Pst*) strain DC3000 was kindly provided by Prof. R. Buonaurio, University of Perugia, Italy. *Pst* DC3000 was cultured at 28°C, 300 rpm, in Luria-Bertani (LB; tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 5 g L^{-1}) medium supplemented with 50 mg l^{-1} rifampicin (Sigma-Aldrich) until an OD_{600} of 0.8 was reached (24-26 h). Bacteria were collected by centrifugation and resuspended in sterile water

containing 0.02% Silwet L-77 (Momentive, Columbus, OH, USA) to the final concentration of 10^8 colony-forming units (CFU) ml^{-1} (OD_{600}=0.2). Inoculations were performed by placing a single 10-µl drop per leaf on a side of the middle vein. Leaves were incubated at 28°C for 3 days after that photographs were taken and the leaf bacterial titer was determined. The titer was determined by grounding the infected side of the leaf in 1 ml of 10 mM $MgCl_2$ and by overspreading serial dilutions on LB agar (10 g l^{-1} agar) plates containing 50 mg l^{-1} rifampicin. CFU were counted after 2 days of incubation at 28°C. In order to test for a direct inhibiting effect of CP on the bacterial growth, *Pst* DC3000 was grown with 150 µM CP for 3 days.

Leaves treatment and sample preparation for 2D electrophoresis

A. thaliana leaves are treated for 8h on the abaxial page with 10 µl droplets of 1.5×10^{-4} M CP (treated) or H_2O (control). Subsequently, on the treated leaves was carried out a proteins extraction as previously describe (Galletti et al., 2011). In order to eliminate part of the RuBisCo present in the extracts we treated samples with protamine sulfate 0.05% (PS Sigma P4020) that is capable of binding to this enzyme (Kim et al., 2013). After the samples were centrifuged at 12000 rpm to precipitate the Rubisco-PS complex. On the supernatant was subsequently carried out a protein precipitation by methanol-chloroform method and the final pellet was resuspended with 9M urea, 4% CHAPS and 20mM DTT.

2D-Electrophoresis and image analysis

The two-dimensional electrophoresis experiments were performed using the Amersham Pharmacia Biotec IPGPhor for the first dimension isoelectric focusing and the BioRad PROTEAN II xi Multi-Cell to perform sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension.

The first dimension separation, involving the isoelectric focusing of proteins present in the protein extracts, was performed using 18 cm (pH 3-10, nonlinear) immobilized pH gradient (IPG) strips (GE Healthcare), which were passively rehydrated overnight in Urea 8M, CHAPS 2% and Bromophenol blue. 600 µg of proteins were loaded for for each gels. The isoelectric focusing of proteins was performed using preprogrammed five-step method:

S1: 200V 1h
S2: 300V 1h
S3: 3500V 4h gradient
S4: 5000V 2h
S5: 8000V to the end

Prior to the embedding of the IPG strips on prepared SDS-PAGE gels for second dimension separation, the proteins underwent an equilibration step. Briefly, the strips

were initially saturated in 6 Murea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol, and 10mg/ml DTT, with gentle agitation at room temperature for 10 min. The strips were equilibrated again in 6 M urea, 2% SDS, 50 mM Tris-HCl, pH 8.8, 30% glycerol, and 135 mM iodoacetamide, with gentle agitation at room temperature for 10 min. Following the equilibration steps, the IPG strips were carefully embedded within a molten agarose solution (Bio-Rad) directly on top of a 9-16 % gradient SDS-PAGE gel. The SDS-PAGE was performed at 1000 V and 20 mA for gels for about 6h. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue G250.

The gels were scanned using the Epson Expression 1680Pro scanner to produce images that could be compared using the accompanying Progenesis SameSpot software (Totallab).

Results

Cerato-platanin shows expansin-like activity on cellulosic materials

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APPLIED MICROBIOLOGY AND BIOTECHNOLOGY 2013

In the CP family, the molecular mechanism of action on plants and above all the biological role in fungal life are little-known aspects. On the basis of the structural data that indicate a strong similarity between CPs proteins and expansins (de Oliveira et al., 2011) and that the expression of the *cp* gene is related to hyphal growth and chlamydospores formation, an expansin-like function has recently been suggested concerning CP. Expansin-like proteins have the ability to act non-hydrolytically on cellulose. In the present work, the expansin-like activity on pure cellulose and the carbohydrate-binding properties of CP and Pop1 were investigated.

Weakening activity on filter paper by CP and Pop1

First, we investigated the activities that show the two proteins on cellulosic substrates: CP and Pop1 were able to weaken filter paper in a concentration-dependent manner (Figure 15). The action on filter paper discs was clearly visible as production of paper fragments in suspension. To clearly observe the production of paper fragments, an incubation was needed with vigorous shaking in at least 2 ml of buffer. The fragments were more abundant as the concentration increased, and CP produced a higher amount of fragments than Pop1. Unlike the incubations with CP and Pop1, when paper discs were incubated with buffer or BSA, a very poor amount of fragments was detectable. The spectrophotometric analysis of the suspensions allowed quantification of the paper fragments produced, showing that the minimum active concentration was 3×10^{-7}M for CP and 3×10^{-6}M for Pop1 (Figure 16A).

As expected on the basis of previously obtained results with CP on carboxymethylcellulose (de Oliveira et al. 2011), during the action of CP and Pop1 on filter paper, no detectable amount of reducing sugars was produced (data not shown). It has recently been shown that glycosyl hydrolases GH61 can internally cleave cellulose molecules in a direct oxidative way that does not lead to the generation of new reducing ends (Westereng et al. 2011). In order to exclude this mechanism, we tested whether CP

could take advantage, on filter paper, of the incubation conditions suitable to these enzymes. GH61 are metal-dependent enzymes and require an external electron donor. Any trace of copper from the solution or substrate can be enough to activate these enzymes, but GH61s are generally blocked by the presence of a metal chelator, such as EDTA (Harris et al. 2010; Quinlan et al. 2011). However, in its weakening activity, CP was neither blocked in the presence of EDTA nor amplified in the presence of copper ($CuSO_4$) and ascorbic acid (electrons donor) (Figure 16B).

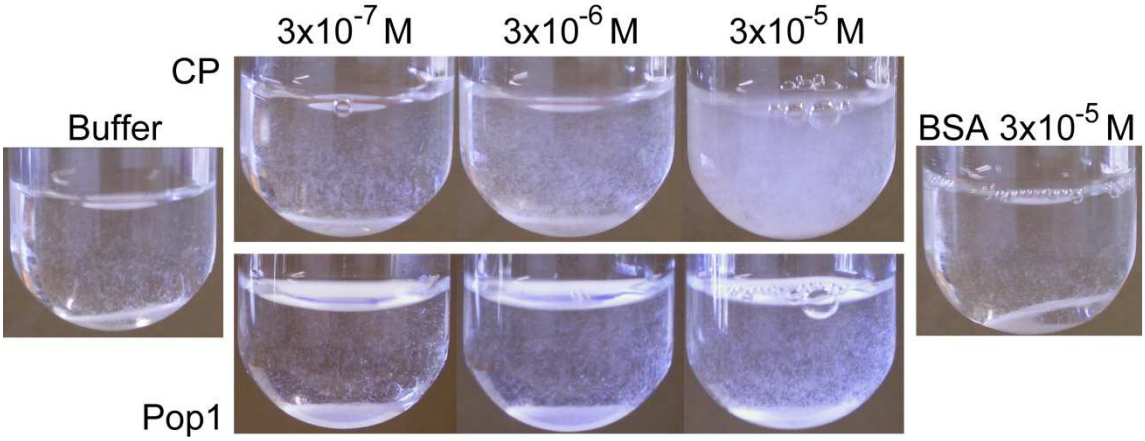

Figure 15: *Weakening activity of CP and Pop1 on filter paper. Each tube contained a filter paper disc (6 mg) in 2 ml of 50 mM sodium acetate buffer (pH5.0), and CP or Pop1 at three different concentrations (3×10-7, 3×10-6 and 3×10^{-5}M). Buffer only or buffer containing the protein bovine serum albumin (BSA) at the highest concentration (3×10^{-5}M) were used as negative controls. The action was visible as paper fragments released in suspension from the paper disc (discs visible on the bottom of the tubes) after 48 h, at 38°C, with shaking at 320 rpm.*

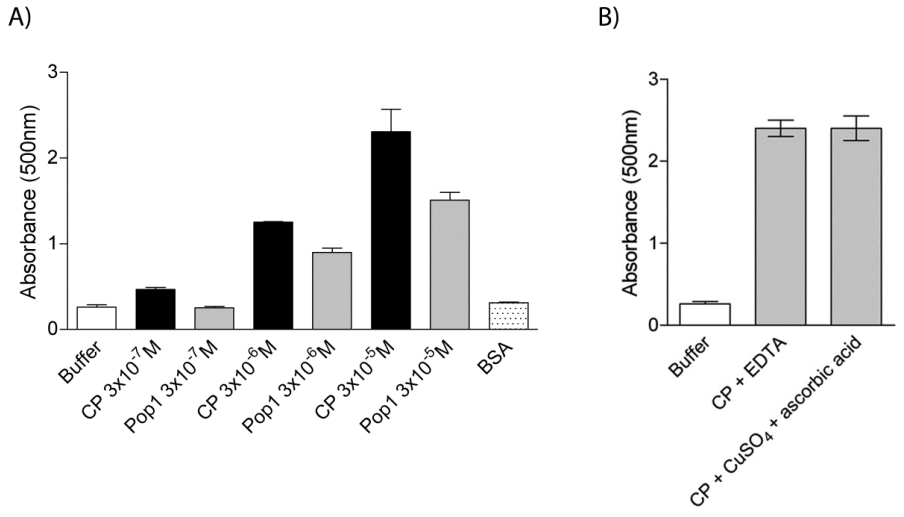

Figure 16: *CP and Pop1 activity on filter paper:* ***A)****quantification by spectrophotometer analysis of the paper fragments produced. Absorbance at 500 nm was measured on 1 ml of suspension at the end of the incubation period with paper discs (48 h, 38 °C, 320 rpm). Error bars indicate the standard deviation of three measurements;* ***B)*** *weakening activity of 3x10^{-5} M CP on filter paper in the presence of 15 mM EDTA or 30 μM $CuSO_4$ and 1 mM ascorbic acid. Each tube contained a filter paper disc (6 mg) in 2 ml of 50 mM sodium acetate buffer (pH 5.0). The incubations were performed for 48 h, at 38 °C, with shaking at 320 rpm. The activity was quantified by spectrophotometer analysis (Abs 500 nm) on 1 ml of suspension. Error bars indicate the standard deviation of three measurements.*

Effect on Avicel, cotton fibres and chitin

The effect of CP was analyzed on other cellulosic materials: Avicel (crystalline cellulose) and cotton fibres. Cotton fibres were firstly mercerized and the effect of CP was observed after sonication, in accordance to other similar works carried out with expansin-like proteins (Quiroz-Castañeda et al. 2011; Saloheimo et al. 2002). CP was able to act both on Avicel and cotton. When Avicel was incubated with CP, it showed fragmentation into smaller particles that were observable on most of the sample (Figure 17a). Some particles remained apparently intact. However when Avicel was incubated with buffer or BSA it showed principally intact particles and only some small particles. The effect was quantified in about 15±6 intact particles (length≥50 μm) per field analysed at ×100 after incubation with CP, against about 33±8 intact particles after incubation with buffer or BSA (n=3).

Cotton incubated with CP showed breakage of the fibres and defibration (Figure 17b). Conversely, fibres remained essentially undamaged by sonication when incubated with buffer only or BSA. The effect was quantified in about 95 broken/defibrated fibres per 100 fibres analysed after incubation with CP, and about 1 or 10 damaged fibres per 100 fibres analysed after incubation with buffer or BSA, respectively. No swollen areas or enlarged "bubbles", as described by Saloheimo et al. (2002) and Quiroz-Castañeda et al. (2011), were observed on CP-treated fibres.

On chitin and colloidal chitin CP and Pop1 did not cause any apparent activity (expansion or weakening) as observed by light microscope (data not shown).

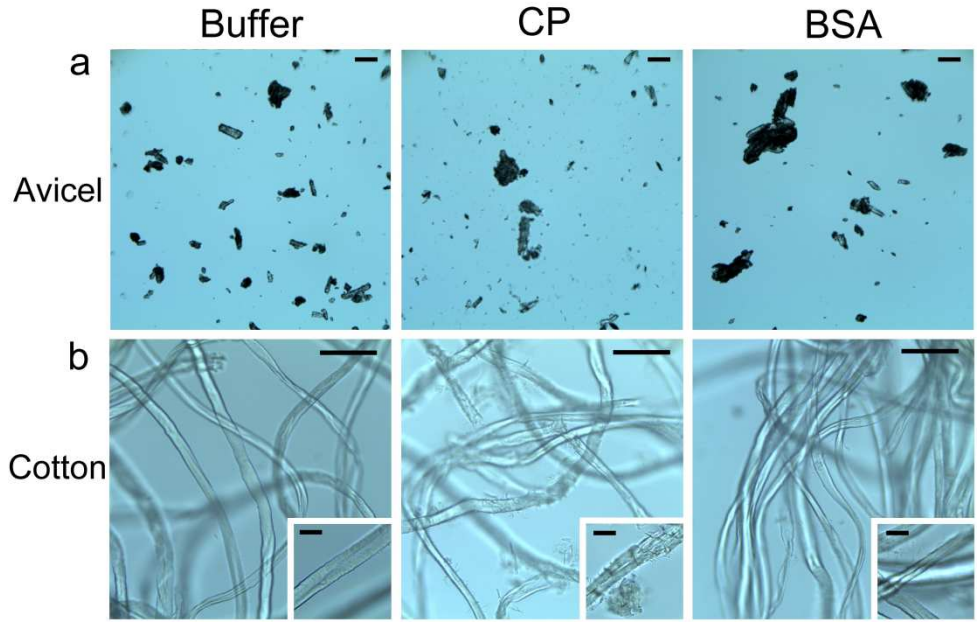

Figure 17: *Light microscopy of Avicel and cotton fibres treate with CP 3×10^{-5}M.* ***a)*** *Avicel (1 mg) incubated with CP in 0.5 ml of 50 mM acetate buffer (pH5.0) for 24 h, at 38 °C, shaking at 320 rpm. Bars=100 μm.* ***b)*** *Mercerized cotton fibres (1 mg) incubated for 24 h (38 °C, 320 rpm) with CP in 0.5 ml of 50 mM acetate buffer (pH5.0), and then sonicated for 1 min at 25 μm. Bars=100 and 25 μm (images for details). Buffer only or buffer containing BSA 3×10^{-5}M were used as controls.*

Optimum pH and temperature

Since the spectrophotometric analysis turned out to be a reliable method of quantifying the activity of CP and Pop1 on filter paper, the same system was employed to determine the optimal values of pH and temperature for the weakening activity shown by CP. The best conditions turned out to be pH5.0 and 38 °C (Figure 18). However, CP worked with all the pH values tested (Figure 18a) and with all the temperatures (Figure 18b). In addition, the incubation in distilled water increased the production of paper fragments by CP as compared to the incubation in buffer pH5.0 (Figure 18b).

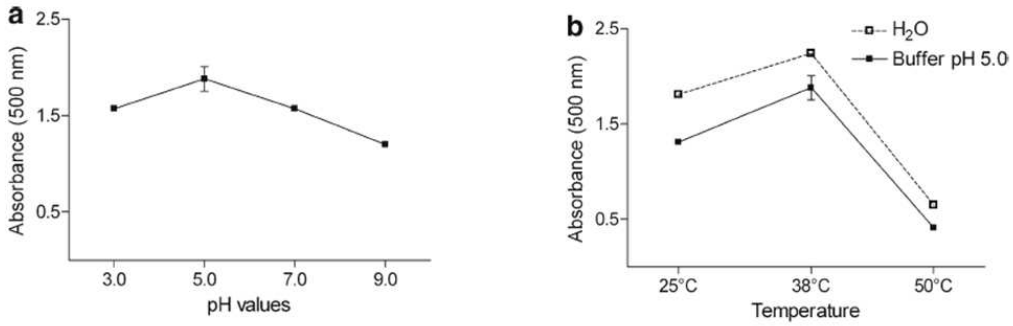

Figure 18: *Optimum pH and temperature for the activity of CP on cellulose. The values were determined by measuring the absorbance at 500 nm of the paper fragments produced after 48 h of incubation with CP 3×10^{-5}M.* ***a)*** *The optimum pH was established at 38 °C incubating CP in 2 ml of 50 mM citrate–phosphate buffer (pH3.0, 5.0 or 7.0) or Tris–HCl buffer (pH9.0), with shaking at 320 rpm.* ***b)*** *The optimum temperature was determined both in 50 mM citrate–phosphate buffer (pH5.0) and distilled water, at 25, 38 and 50 °C, with shaking at 320 rpm. Controls (buffer only) were prepared for each experiment, and their absorbance value was subtracted from the value obtained in the CP-treated samples. Error bars indicate the standard deviation of three measurements.*

Binding to polysaccharides

The binding ability of CP and Pop1 was tested on cellulose (filter paper and Avicel), chitin, colloidal chitin and β-glucan. The ability of CP and Pop1 to bind the substrates was detected by SDS-PAGE, analyzing aliquots of the supernatants in order to visualize the unbound fraction. Results showed that neither CP nor Pop1 were able to bind cellulose (Figure 19a). Further experiments performed with CP showed that even changing the incubation conditions (temperature and buffer) or the time of sampling (few minutes), or diminishing the concentration, the binding between CP and cellulose did not occur. CP and Pop1 were also unable to bind β-1,3-glucan.

On the contrary, both CP and Pop1 were able to bind chitin and colloidal chitin. CP bound to chitin fragments very quickly (1 min) and compared to Pop1 the binding occurred earlier and to a greater extent (Figure 19b).

The binding capacity of CP and Pop1 was the same both in the presence of chitin fragments and colloidal chitin (Figure 20A). Therefore, we used colloidal chitin to determine the kinetic curve: the binding parameters for CP turned out to be Bmax=2.07±0.19 μmol/g of chitin, and Kd=40.56± 9.50 μM (Figure 20B). The Kd value of

Pop1 was of at least tenfold higher (>400 μM), due to the low binding affinity, in accordance with the SDS-PAGE result (Figure 19b).

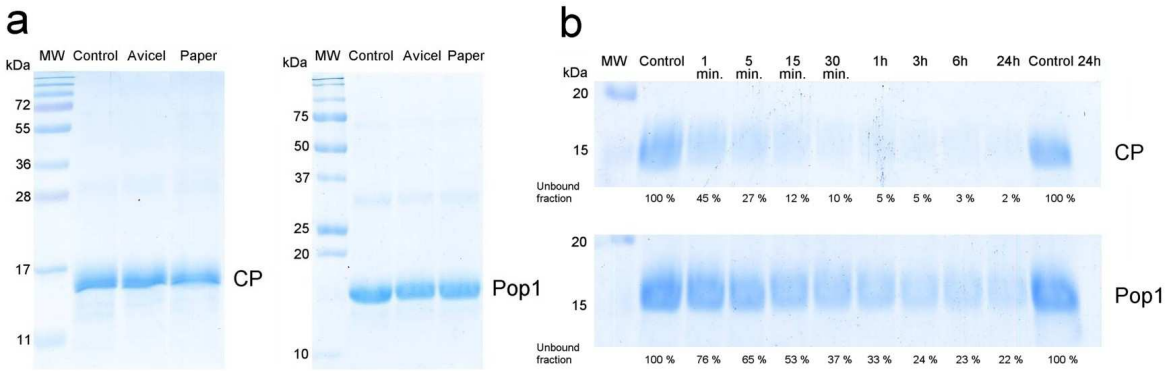

Figure 19: *Binding assay with cellulose and chitin.* ***a)*** *CP and Pop1 3×10^{-5} M incubated with filter paper (6-mg disc) for 48 h, or 40 mg of Avicel PH-101 for 72 h.* ***b)*** *Binding over time (from 1 min to 24 h) of CP and Pop1 3×10^{-5}M to chitin from crab shells. All the incubations were performed in 50 mM acetate buffer (pH5.0), at 38 °C, with shaking at 320 rpm. Aliquots of the supernatants (unbound fraction) were centrifuged and analyzed by 15 % SDS–PAGE and Coomassie blue staining. Percentage values were calculated with Kodak MI software (Carestream Health, Inc.). Control, protein not incubated. Control 24 h, protein incubated for 24 h without any substrate.*

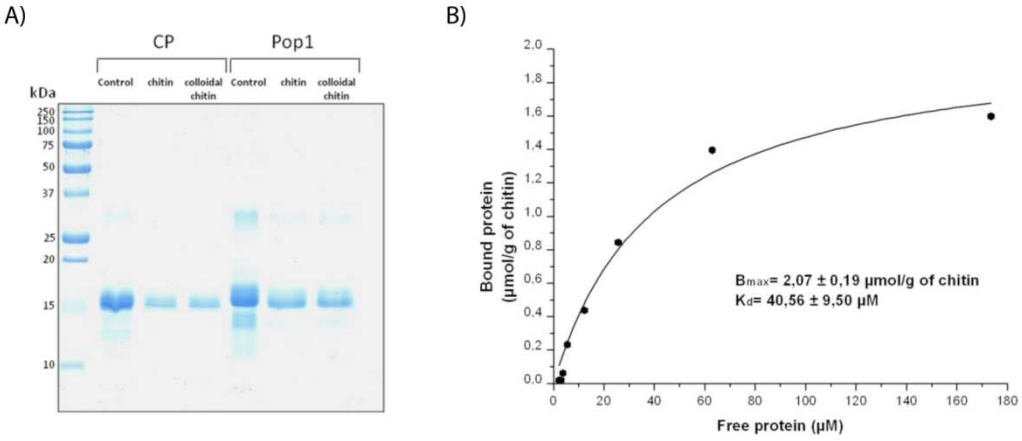

Figure 20: *Binding of CP and Pop1 to chitin and colloidal chitin.* ***A)*** *SDS–PAGE of CP and Pop1 3x10^{-5} M incubated with chitin and colloidal chitin for 72 h in 50 mM acetate buffer (pH 5.0), at 38 °C, with shaking at 320 rpm. Aliquots of the supernatants (unbound fraction) were centrifuged and analyzed. Control, protein not incubated.* ***B)*** *Binding isotherm of CP to colloidal chitin*

Combined action on filter paper by CP and cellulose

As expansin-like proteins can enhance the yield of reducing sugars produced by hydrolytic enzymes when applied together on cellulosic materials (Chen et al. 2010; Kim et al. 2009; Lee et al. 2010; Quiroz-Castañeda et al. 2011; Wang et al. 2010, 2011; Zhou

et al. 2011), accordingly the synergistic effect of CP and cellulases was tested in the present study.

CP was incubated with filter paper for 48 h at 38 ˚C using two different concentrations (3×10^{-6} and 3×10^{-5}M) and applying the conditions used for the previous experiments. Subsequently, 1 U of cellulase from T. viride was added to the tubes, and further incubation (3 h, 38 ˚C) was performed.

Results showed that the incubation with CP increased the yield of reducing sugars produced by cellulase by about 50 % more than the incubation with buffer only (Figure 21). However, the same enhancing effect was also found with BSA. We were also unable to find a significant yield increase compared to BSA even when reducing the amount of cellulase to 0.2 U/ml (the minimum active concentration), or diminishing the incubation time with CP (24 h or no incubation) before adding 1 U of cellulase (data not shown).

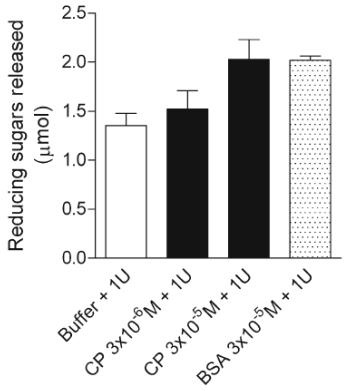

Figure 21: *Synergistic effect of CP and cellulase on filter paper. Filter paper discs were incubated with CP 3×10−6 or 3×10^{-5}M in 1 ml of 50 mM acetate buffer (pH5.0) for 48 h, at 38 °C, with shaking at 320 rpm. Subsequently, 1 U of cellulase from Trichoderma viride was added to each tube and further incubation was performed (3 h at 38 °C, 320 rpm) before measuring reducing sugars released by DNS method. Buffer only or buffer containing BSA 3×10^{-5}M were used as negative controls. Concentration of reducing sugars was determined comparing to a glucose calibration curve. Error bars indicate the standard deviation of three measurements.*

Differential timing of defense-related responses induced by cerato-platanin and cerato-populin, two non-catalytic fungal elicitors

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PHYSIOLOGIA PLANTARUM 2013

Cerato platanin is a non-catalytic protein with a six-stranded double-*ψβ*-barrel fold and is secreted by *Ceratocystis platani*, the causal agent of the canker stain of plane. Cerato populin is an ortholog showing low sequence identity with CP and is secreted by Ceratocystis populicola, a pathogen of poplar. CP and Pop1 have been suggested to act as PAMPs because they induce phytoalexin synthesis, transcription of defense-related genes, restriction of conidia growth and cell death in various plants. In the present work we treated plane leaves with CP or Pop1, and monitored defense responses to define the role of these elicitors in the plant interactions.

Resistance-inducing activity of CP and Pop1

When plane leaves were treated with 1.5×10^{-4}M CP they acquired the ability to slow the growth of *C. platani* until it was almost blocked (Table 2). Few conidia of *C. platani* germinated and the germ tubes produced were only slightly stretched. In contrast, the effect of treatment with 1.5×10^{-4}M Pop1 was not statistically different from the control. In this case nearly all conidia germinated, and an abundant mycelium grew in the droplets (2.6 ± 0.6 and 2.8 ± 0.7 relative units, respectively).

Resistance inducing activity of CP and Pop1	
	Fungal growth
Distilled water	2.8±0.7a (n=19)
CP	0.2±0.1b (n=20)
Pop1	2.6±0.6a (n=19)

Table 2: *The resistance inducing activity (^) was evaluated by growth of Ceratocystis platani on plane (Platanus acerifolia) leaves treated with CP or Pop1. Fungal growth: Growth of C. platani on plane (P. acerifolia) leaves treated for 24 hours with distilled water droplets containing 1.5×10^{-4} M CP or Pop1 or nothing (initial conidial concentration approximately 8×10^{2}). Fungal growth was evaluated according to the three-point scale (0–3 relative units) described in the section Materials and methods. Values are the means of data from two independent experiments±SD. Values marked with different letters are significantly different (at $P \leq 0.001$, according to ANOVA).*

MAPK activation

Several extracellular signal-regulated kinase (ERK)-type MAPKs involved in plant defense signaling have been identified in herbaceous and plant species (Lumbreras et al. 2010, Hamel et al. 2012), and recently it has been shown that the MAPK pathway is involved in ROS homeostasis (Pitzschke et al. 2009). Therefore, we analyzed the expression level of two protein kinases using the anti-human phospho- ERK1/2 antibodies because phosphorylation sites of plant kinases show a sufficient level of homology with the phosphorylation sites of mammalian kinases. Moreover, the ERK1/2 kinases are found to be equivalent to MAPK6 and MAPK3 from *Arabidopsis* (Wan et al. 2004, Galletti et al. 2011) and to the 47 and 44 kDa kinases from poplar (Hamel et al. 2005), all of which are involved in the defense response.

Using the extraction methods reported in the literature as references (see section Materials and methods), we set up an unusual method to extract protein from plane leaves, which are a recalcitrant tissue. Using the described method, we were able to obtain 3.1±0.10mg of total protein, starting with about 0.2 g of treated leaves. Before performing kinase activation analysis, the quality of each protein extract (treated and control) was examined by SDS-PAGE (data not shown).

In addition to the incubation times used to detect H_2O_2 and NO production we analyzed the level of MAPKs phosphorylation at 15, 30 and 60 min, as the kinase cascade activation is one of the first events in plant defense signaling. Results show that two bands of 47 and 44 kDa positively react with anti-phospho- ERK1/2 antibodies confirming the involvement of these kinases in defense. Immunoblot analysis of CP-treated leaves showed that only after 1 h of incubation the ERK1/2 kinases were phosphorylated up to 10-fold the control value. Then the signal decreased after 9–12 h and got back to control values at 24 h of incubation (Figure 22A, B). In particular, the 47 kDa band was the first to be induced, while the 44 kDa band showed its maximum after 1–3 h of incubation.

The expression of these kinases in leaves treated with Pop1 also showed an increase in the phosphorylated form but with different timing when compared to the MAPKs activated by CP: an increase in phosphorylation was visible after 9 h of incubation and the signal was maintained for up to 12 h of incubation (Figure 22B, D).

The increase in phosphorylation was higher for the 47 kDa kinase than for the 44 kDa one, in agreement with literature (Wan et al 2004, Hamel et al. 2005, Galletti et al. 2011).

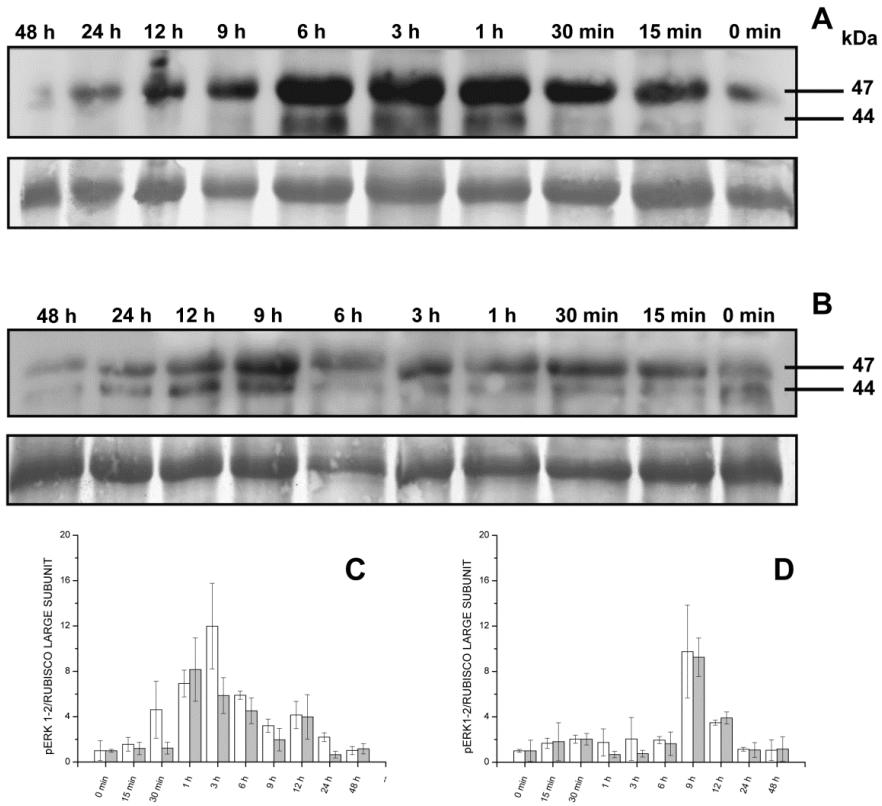

Figure 22: *MAPK activation in plane leaves treated with CP or Pop1.* ***A)*** *Treatment with 1.5 nmol/10 µl of CP. Upper panel: immunoblot analysis obtained with antibodies against human phospho-ERK1/2 (a-pTEpY). Bands corresponding to the 44 and 47 kDa phosphorylated kinases are indicated by arrowheads; lower panel: RuBisCo large subunit was used as a loading control to obtain the quantitative measurement of the immunoblot signals.* ***B)*** *Treatment with 1.5 nmol/10 µl of Pop1. Details are the same as in (A).* ***C, D)*** *pERK1/2-rubisco ratio of four separate experiments of CP and Pop1 leaves treatment, respectively.*

NO and H_2O_2 production

A very rapid ROS production in infected plant cells usually precedes the hypersensitive cell death and is widely recognized as the inducer of cell death (Rodrıguez-Serrano et al. 2012).

As a consequence of CP or Pop1 treatment, leaf cells produced and released H_2O_2, which could be identified and quantified inside the drops recovered after treatment. As shown in Figure 23A, H_2O_2 production and release into the drops was already visible after 3 h of treatment. Its level was higher with CP than with Pop1, and it slightly increased until 12 h of treatment. After this time point, the presence of CP continued to increase H_2O_2 release, while treatment with Pop1 did not induce further production of H_2O_2, whose concentration in the drops started to decrease because of chemical breakdown.

The presence of the two fungal proteins also induced the release of NO to the drops used for the treatment (Figure 23B). Unlike H_2O_2, NO release into the drops was not slow and continuous, but showed an initial burst, followed by a rapid decrease. CP induced NO production more rapidly than Pop1, as can be observed by the level of NO in the

drops after 3 h of treatment. The burst induced by the presence of Pop1 was quantitatively similar, but occurred slightly later.

H_2O_2 production as a consequence of CP or Pop1 treatment on the leaf was also visualized in situ by DCFH2-DA, which is rapidly oxidized to highly fluorescent DCF in the presence of H_2O_2. The intense green fluorescence in the cells indicated the presence of endogenous H_2O_2. Its production increased significantly at the site of treatment with either CP or Pop1, and remained constantly high throughout the duration of the treatment (48 h). The CP-induced accumulation of H_2O_2 was more rapid, as it was visible just over 3 h after the addition of CP, whereas Pop1 required at least 6 h to induce H_2O_2 production in the leaf (Figure 23C).

Treated leaves also produced NO, as demonstrated by staining with the NO-specific fluorescent probe DAF- 2DA. This probe has been widely used to identify and quantitatively measure NO production in plants or cell cultures (Lombardi et al. 2010). CP and Pop1-treated leaves produced NO at the site of treatment; however, unlike H_2O_2 whose accumulation was continuous, NO production was transient. CP induced NO synthesis as early as 3 h after treatment, with a gradual increase until 6 h of treatment. After 6 h, no more NO was produced in the treated leaf. In the presence of Pop1 little NO production was observed only after 6 h of incubation. Similarly to CP, Pop1 was able to induce NO accumulation only for a short period; however, it is worth noting that in the presence of Pop1 a second burst of NO took place after 48 h of incubation (Figure 23D).

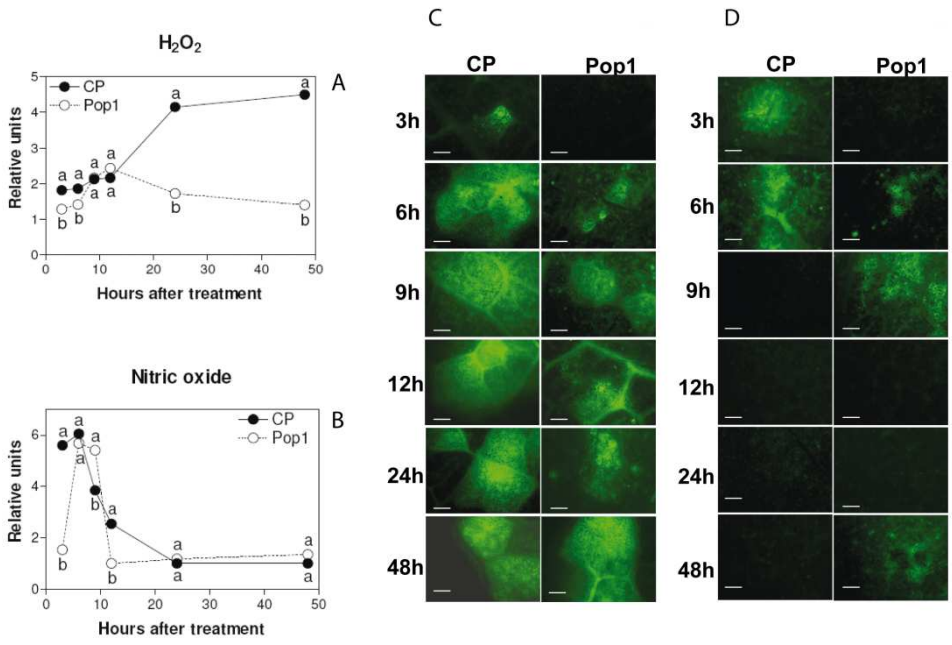

Figure 23: *Hydrogen peroxide and nitric oxide produced after CP and Pop1 treatment:* ***A-B)*** *Hydrogen peroxide and nitric oxide released by treated leaf cells was measured in the drops containing CP, Pop1 or distilled H_2O (control) and recovered after treatment. (A) Hydrogen peroxide content was estimated by the Amplex Red Assay and reported as µM. (B) Nitric oxide content in the droplets reported in arbitrary fluorescence units. Values are the means of 30 measurements from three independent experiments (where no error bars are present SE is smaller than the size of the symbol). For each time point the values marked with different letters are significantly different (at P ≤0.05).* ***C)*** *H_2O_2 was visualized in situ by the fluorescent probe DCFH2-DA.* ***D)*** *Nitric oxide was visualized in situ by the fluorescent probe DAF-2DA. Bars=1 mm.*

Expression of defense-related genes

CP and Pop1 had a different effect on the transcription of the analyzed genes (Figure 24). Generally speaking, CP affected gene transcription for shorter periods of time compared to Pop1, which induced changes at each time point, as shown by the modulation of PR-5.

Moreover, CP never induced gene expression after 6 h of treatment. Also, it is important to emphasize that the APX transcript was underexpressed after 3 h of CP-treatment, while it was overexpressed after Pop1- treatment. Note that the 6 h time point showed the strongest gene modulation for all the analyzed genes. In addition, the 6-h-treatment showed the samemodulation pattern, with no differences between the two elicitors.

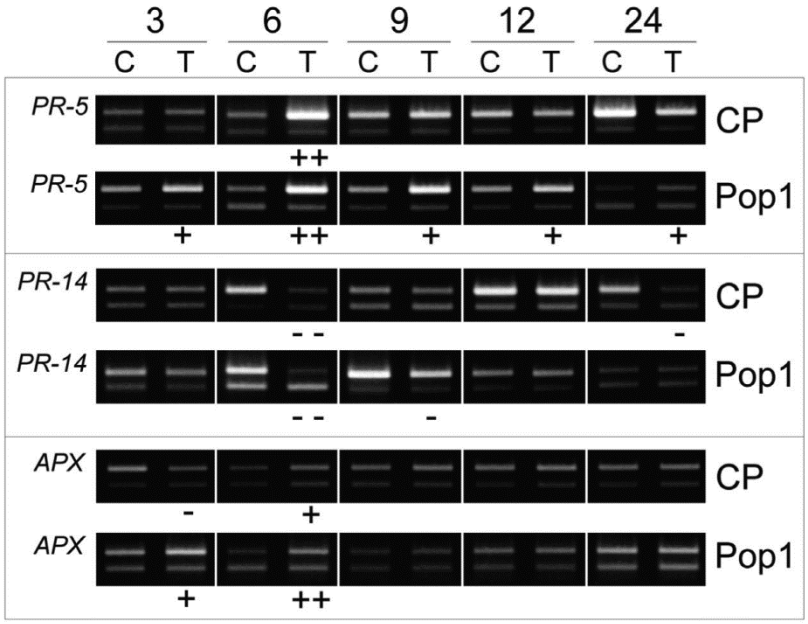

Figure 24: *sqRT-PCR of defense-related genes in Platanus acerifolia leaves treated for 3, 6, 9, 12 or 24 h with the proteins CP or Pop1. C, control leaves (water-treated); T, treated leaves. Upper PCR products correspond to the pathogenesis-related proteins family 5 (PR-5), family 14 (PR-14) or ascorbate peroxidase (APX). Lower products of each panel correspond to the endogenous control gene 18S used to normalize the values. Overexpression (+, ++) or underexpression (-, - -) of the target genes in the treated leaves compared to the control leaves are reported. Data are representative of three replicates.*

Cell death

Cell death in the treated leaves was assayed using PI staining, which can only enter damaged and dying cells (El-Maarouf-Bouteau et al. 2011). In the presence of CP, cell death was visible at the site of treatment after 24 h of incubation, while Pop1 induced cell death after 48 h (Figure 25A).

To investigate the type of cell death and, in particular, to detect specific features of the PCD mechanism a TUNEL assay was performed on sections of treated leaves. The TUNEL assay showed that DNA degradation took place in nuclei of mesophyll cells undergoing

cell death as a consequence of CP and Pop1 treatments. It is worth noting that DNA fragmentation occurred in nuclei located in the spongy mesophyll, while nuclei of the palisade, clearly visible after PI staining, were not TUNEL-positive (Figure 25B).

PCD was also confirmed by transmission electron microscopy (TEM). Early signs of PCD were detected in the epidermis and in the adjacent spongy mesophyll cells already at 6 h after CP treatment, and consisted of slight plasmolysis of plasma membranes, swelling of mitochondria and chloroplasts, and initial chromatin condensation (Figure 26A) (Pavet et al. 2006). A middlestage PCD was observed after 24 h, with disruption of nuclear and tonoplast membranes and organelles, and chromatin condensation (Figure 26B), followed by cell collapse (Figure 26C) (Faoro and Iriti 2009). Similar responses occurred in Pop1-treated leaves, however, they were delayed by about 24h (Figure 26D). The early detection of PCD by TEM compared with other microscopy techniques is in agreement with previous studies (Bakeeva et al. 2005, Dzyubinskaya et al. 2006).

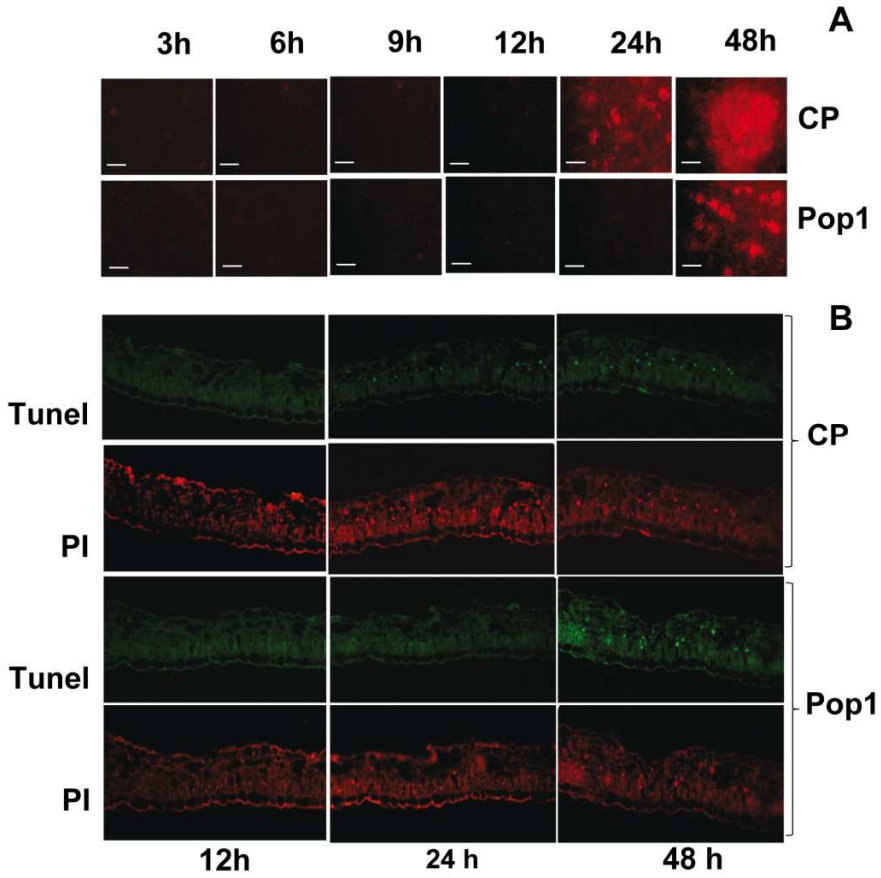

Figure 25: *Viability staining of the leaf after treatment with CP or Pop1.* ***A)*** *Cell death was visualized by PI staining in the area of the leaf treated with CP or Pop1. Bars=1 mm.* ***B)*** *Nuclear DNA fragmentation in the leaf cells was visualized by the TUNEL assay on tissue sections. Green stained nuclei indicate DNA fragmentation. Nuclei were counterstained with PI (red staining).*

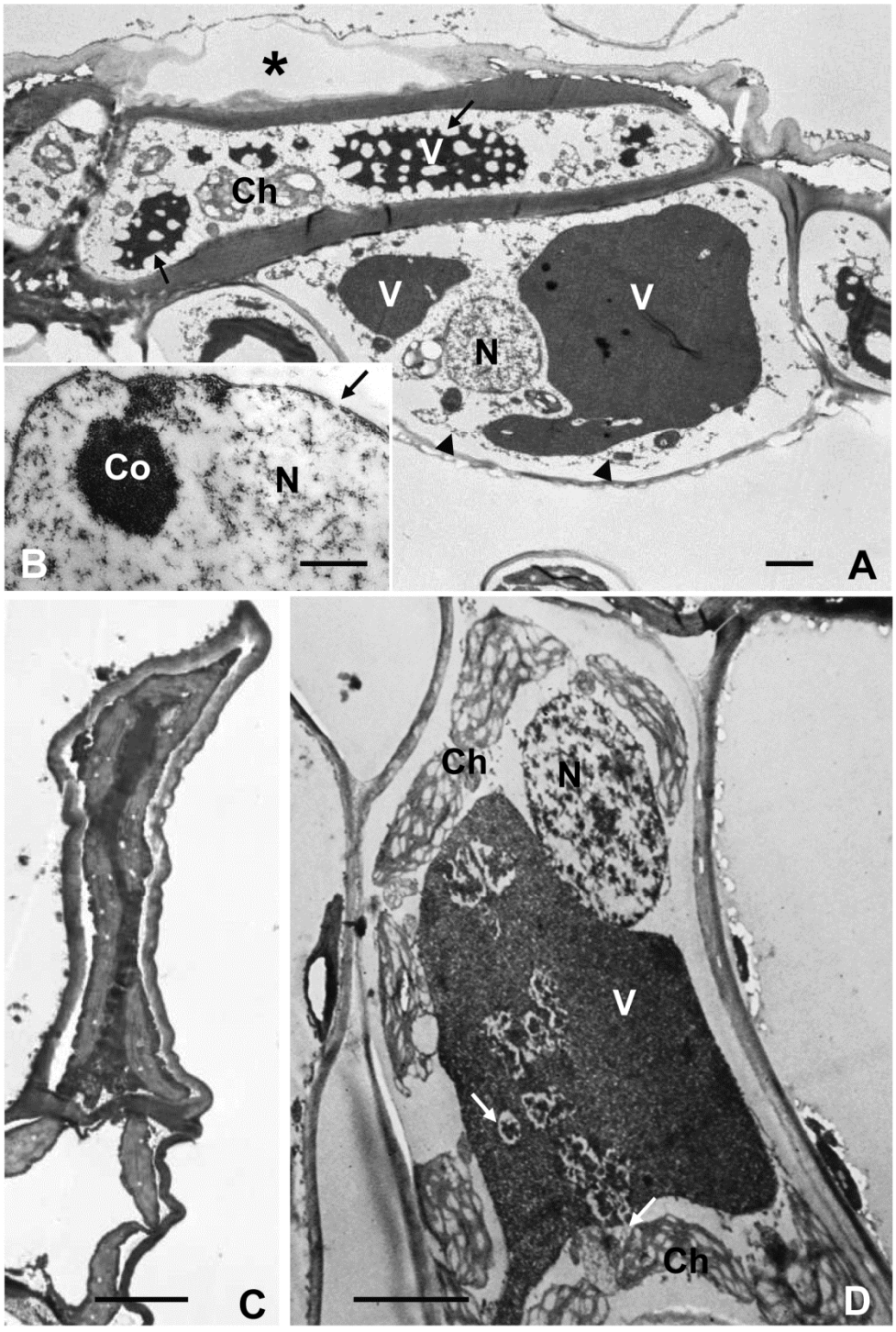

Figure 26: *Ultrathin cross sections of plane leaves treated with CP (A, B, C) and Pop1 (D).* ***A)*** *Epidermal cells in an early stage of PCD at 6 h after treatment, showing incipient plasmolysis with plasma membrane (arrowheads) detached from the cell wall, swollen chloroplasts (Ch), vacuoles (V) containing tannins in the course of being released (arrows), possibly because of tonoplast damages, and nuclei (N) showing early stages of chromatin condensation; note the cuticle (asterisk) alteration where the CP drop was placed.* ***B)*** *A nucleus (N) with ruptures in the envelope (arrows) in the intermediate stage of chromatin (Co) condensation at 6 h after treatment.* ***C)*** *A mesophyll cell adjacent to the epidermis completely collapsed 24 h after treatment.* ***D)*** *A mesophyll cell adjacent to the epidermis beneath the point of treatment 24 h after Pop1 drop deposition: the cell is just plasmolyzing, with initial chloroplast swelling and chromatin condensation in the nucleus (N); tannins start to be released from the vacuole (V, arrows). Bars in (A), (C), (D)=5 μm; in B=1 μm.*

Stomata sense cerato-platanin and trigger resistance to pathogens in *Arabidopsis* leaves

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SUBMITED AT MOLECULAR PLANT PATHOGY

Microbe-associated molecular patterns (MAMPs) lead to the activation of the first line of plant defence. Few fungal molecules are universally designed as MAMPs and this designation is appropriate for the fungal proteins of the "cerato-platanin family".
In the present study we show that stomata have a key role in the *Arabidopsis* immunity induced by CP. The guard cells were the first cells to perceive the presence of CP by initiating the production of H_2O_2, and this response was influenced both by the density and aperture of the stomata. Stomata represent a natural entry site for potentially harmful microbes and plants close their stomata upon perception of MAMPs. In this paper we show that CP caused this early defense response in *Arabidopsis* leaves. CP induced very rapidly MAPK phosphorylation and then up-regulation of salicylic acid (SA)- and ethylene (ET)-signalling genes, while jasmonic acid (JA)-dependent genes were down-regulated. Finally, the phytoalexin camalexin was produced within 12 hours following treatment with CP and the leaves showed reduced susceptibility to *Botrytis cinerea* and *Pseudomonas syringae* pv. *tomato*.

The production of H_2O_2 after treatment with CP spreads from stomata to the neighbouring cells

As the oxidative burst is one of the first responses occurring in plants after MAMP recognition (Boller and Felix, 2009), we assessed the ability of CP to elicit *Arabidopsis* leaves by analysing the production of hydrogen peroxide. H_2O_2 was first analysed in situ by treating the lower (abaxial) leaf surface with 10-µl drops of 150 µM CP, according to our previous works performed on *P. acerifolia* (Baccelli et al., 2013b; Lombardi et al., 2013). H_2O_2 was visualised by the fluorescent probe 2,7-dichlorofluorescin diacetate (H2DCF-DA). Fluorescence was already visible after 15 min of treatment. Some stomata were more fluorescent than the background. After 1 h the fluorescence was already very intense, differently from what occurred when we treated the upper (adaxial) surface of the leaf, which developed a weak fluorescence at that treatment time (data not shown). Leaves remained fluorescent for 24 h.
In order to find the minimum active concentration of CP, the production of H_2O_2 in situ was analysed by treating leaves with serial dilutions of CP, for 15 and 60 min. The minimum active concentration turned out to be 75 µM; neither of these analyses showed H_2O_2 production with lower concentrations (data not shown).

Since it was known from *P. acerifolia* that CP cannot overcome the cuticle (Martellini *et al.,* 2012), we hypothesized a role for stomata in the perception mechanism of CP. Accordingly, we investigated stomata as a signalling source of H_2O_2 and we used epidermal peels to analyse the evolution of H_2O_2 production on the epidermis.

Epidermal peels were first loaded with the fluorescent probe and then treated on the cuticle side with 1.5×10^{-4} M CP. H_2O_2 production by single stomata over time and on the same peel is shown in Figure 27A. Guard cells turned out to be the first cells on the epidermis to become fluorescent, starting to synthesise H_2O_2 after 4-6 min of treatment. Before that time, the fluorescence was only due to the oxidizing environment of the chloroplast (Allan and Fluhr, 1997). After 8-10 min the epidermal cells surrounding the stomata also became fluorescent. Because of the impossibility of analysing a single peel under the microscope for a longer period, we decided to repeat the experiment with different epidermal peels. Peels were treated for 5, 10 and 30 min. As shown in Figure 27B, once again the first fluorescence signals occurred in the stomata; after 30 min the fluorescence had spread to most epidermal cells. In the same experiment, closed stomata did not produce H_2O_2. In addition, no fluorescence was observed in a 2h period when we treated epidermal peels obtained from plants with stomata still closed (data not shown).

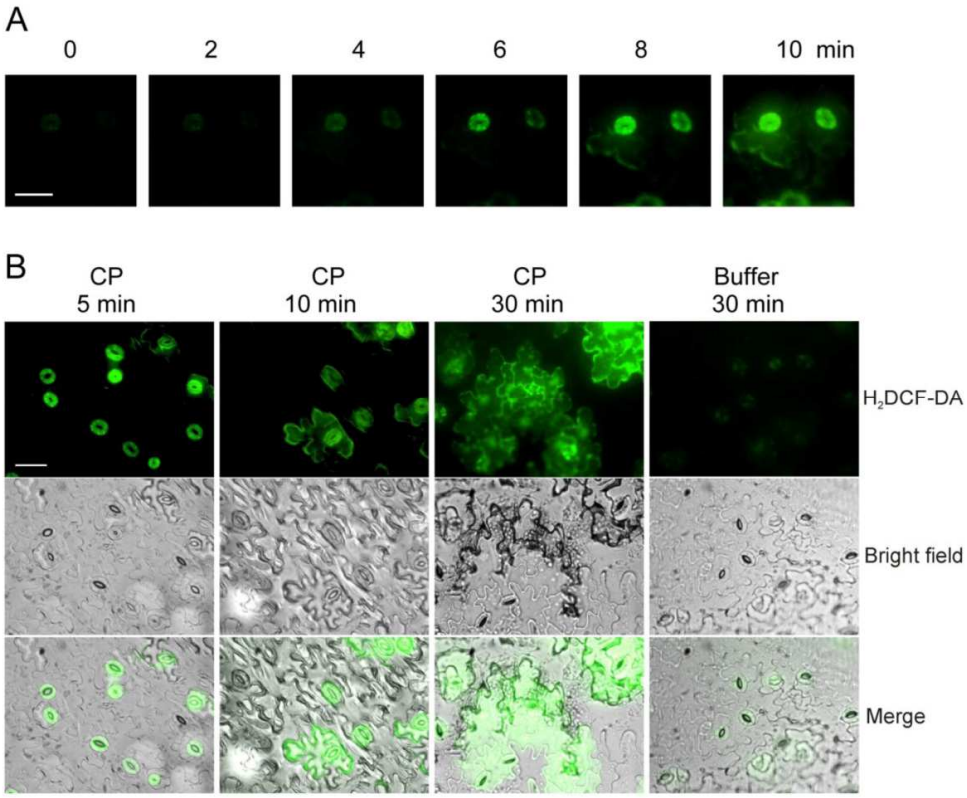

Figure 27: *H2O2 production at the level of stomata. Epidermal peels from Arabidopsis leaves were loaded with the fluorescent probe H2DCF-DA and treated on the cuticle side with 1.5×10^{-4} M CP to analyse the origin of ROS signalling.* ***A)*** *Single guard cells photographed at 2-minute intervals after treatment with CP (same peel). Bar=30 µm.* ***B)*** *Photographs of representative regions of three different peels treated with CP for 5, 10 or 30 min respectively, or 30 min with MES buffer (control). Fluorescence microscopy (H2DCF-DA), light microscopy (bright field) and merged pictures (merge) are shown in order to facilitate the localization of stomata. The bar is 40 µm and applies to all photographs.*

CP induces stomatal closure

Since epidermal peels allow better microscopic recording, they were used to investigate the ability of CP to influence the stomatal movement, according to previous studies performed with different elicitors (Lee *et al.,* 1999; Melotto *et al.,* 2006). Epidermal peels were taken in the morning, kept floating on buffer under continuous light for 2 h, and then treated on the cuticle side with CP for 30 min, 1 h and 2 h.

As shown in Figure 28, CP induced a progressive reduction in the average width of the stomatal aperture. The reduction was already significant after 30 min, and after 2 h the stomata were almost completely closed. The treatment with MES buffer, which was used as a control, did not influence the stomatal movement.

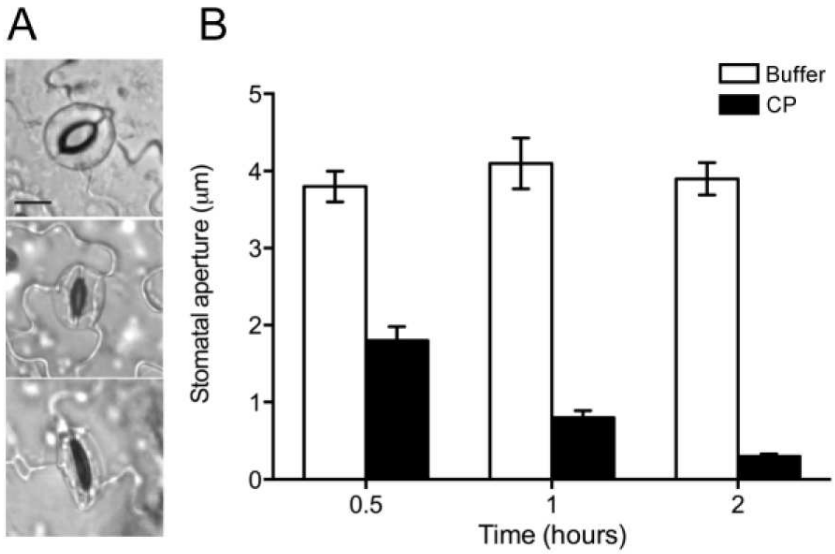

Figure 28: *Stomatal closure induced by CP.* ***A)*** *Open and closed stomata in Arabidopsis epidermal peels. Bar=10 µm.* ***B)*** *Measurement of stomatal aperture after 30 min, 1 and 2 h of treatment with 1.5×10^{-4} M CP or MES buffer (control). Results are the mean of 100 measurements ± SEM.*

Phosphorylation of MAP kinases

The activation of the MAPK cascade following treatment with CP was studied in *Arabidopsis* seedlings by analysing the phosphorylation of MAPK3 and MAPK6, according to the recent evidence that indicates a key role of kinase induction in *Arabidopsis* defense responses (Galletti *et al.,* 2011; Ren *et al.,* 2008; Wan *et al.,* 2004). For example, MPK3 and MPK6 play roles in camalexin induction, the major phytoalexin in *Arabidopsis*, and posses a critical role in priming *Arabidopsis* plants for full induction of defense responses during induced resistance (Beckers *et al.,* 2009; Mao *et al.,* 2011).

The analysis was carried out with specific phospho-p44/42 (pERK1/2) antibodies on protein extracts obtained after 0, 5, 15, 30 and 60 min of treatment with 150 µM CP. As shown by immunoblot analysis, both MPK3 and MPK6 turned out to be rapidly phosphorylated (Figure 29A). After only 5 min of treatment the ERK1/2-type kinases

were phosphorylated up to 20-fold the control value (Figure 29B). As expected, when leaves were treated with sterile distilled water MAPKs were not phosphorylated (data not shown).

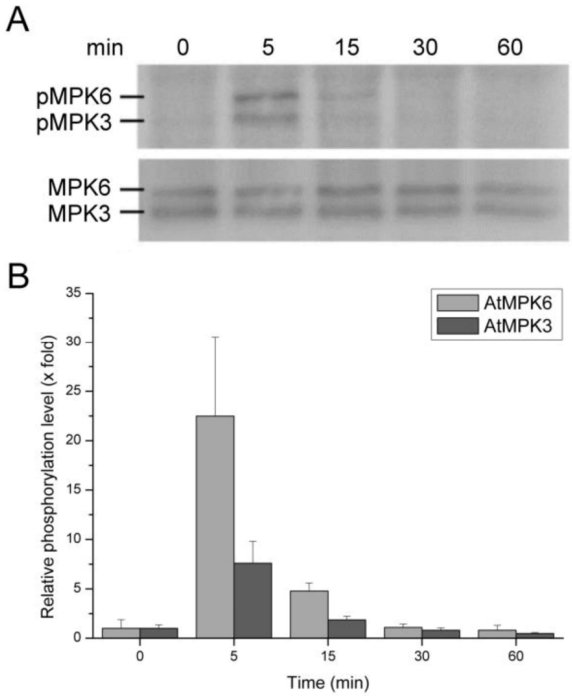

Figure 29: *Phosphorylation of Arabidopsis MPK6 and MPK3 after treatment with 1.5×10^{-4} M CP.* ***A)*** *Analysis carried out with human phospho-p44/42 antibodies (pERK1/2) on protein extracts obtained from Arabidopsis seedlings treated for 0, 5, 15, 30 and 60 min. pMPK6 and pMPK3 indicate phosphorylation.* ***B)*** *Image analysis showing the phosphorylation level normalized on the respective protein amount. Error bars indicate SD of three replicates.*

CP up-regulates SA- and ET-signalling genes, but not JA-signalling genes

The ability of CP to act at the level of gene transcription on *Arabidopsis* leaves was evaluated by real-time PCR (qPCR). As a first approach, we used a primer library purchased from Sigma-Aldrich containing gene-specific primers for pathogen-inducible genes. The study was performed after 6 h of treatment, which was the time in which the greatest number of genes was expected to be modulated (preliminary data not shown). By this analysis, we identified 56 up-regulated genes out of 67 genes analysed. It is worth mentioning genes involved at different levels with the oxidative stress, such as glutathione *S*-transferases (*GSTF3, GSTF7*), peroxidase 50, late embryogenesis abundant-like protein 5 (*LEA5*), blue copper protein (*BCB*) and respiratory burst oxidase homologue D (*RBOHD*) (Ezaki *et al.,* 2000; Mowla *et al.,* 2006; Torres and Dangl, 2005; Wagner *et al.,* 2002); or genes codifying for various receptor kinases, suchs as cysteine-rich receptor-like protein kinases (*CRK5*, *CRK7*, *CRK10*, *CRK36*), lectin-receptor kinase, receptor-like protein kinase 1 (*RLK1*), wall-associated receptor kinase 1 (*WAK1*) and somatic embryogenesis receptor kinase 4 (*SERK4*) (Schwessinger and Ronald, 2012).

Many other up-regulated genes could instead be related to defense-signalling pathways, mainly SA and ET. For this reason, we focused our attention on the expression of SA-,

ET- and JA-dependent genes. In order to perform a detailed study, we designed 13 more primer pairs to include, in the analysis, some of the most studied genes that we found in the literature. The study was performed at various treatment times (1, 3, 6, 12 and 24 h) on a total of 20 genes (7 were from the Sigma-Aldrich primer library). Among these, we analysed the expression of *MAPK4* and the transcription factor *WRKY33*, which act upstream of the synthesis of camalexin (Qiu *et al.,* 2008), and *RBOHD* (respiratory burst oxidase homologue D), which is implicated in the production of reactive oxygen species (Torres and Dangl, 2005). The expression pattern of the analysed genes is shown in Figure 30. CP induced the expression of SA- and ET-dependent genes starting from 6 h after the treatment, and this was also the treatment time in which most genes were found modulated following application of CP. This did not occur for JA-dependent genes, which were instead downregulated by CP. *COI1* (coronatine insensitive 1) and *MYC2* (jasmonate insensitive 1, *JIN1*), two key regulators of JA signalling (Bari and Jones, 2009), were down-regulated after only 1 h of treatment, while *PDF1.2a* (plant defensin) was strongly down-regulated after 12 and 24 h.

The expression of pathogenesis-related (PR) genes was analysed taking into account their differential expression in SA- and JA-/ET-signalling pathways (Thomma *et al.,* 2001). As shown in Figure 30, PR1, PR2 (β-1,3-glucanase), PR3 (basic chitinase) and PR4 (hevein-like protein) turned out to be all up-regulated by CP, starting from 6 h after the treatment. However, their up-regulated status was differently maintained. In particular PR1 was up-regulated until 24 h. PR5 (thaumatinlike) was instead down-regulated by CP.

MAPK4 and *WRKY33* were both up-regulated by CP: *WRKY33* turned out to be the earliest upregulated gene (1 h) and its status was maintained until 6 h; *MAPK4* was up-regulated after 6 h and its status was maintained until 12 h. Finally, *RBOHD* was up-regulated after 6 h of treatment.

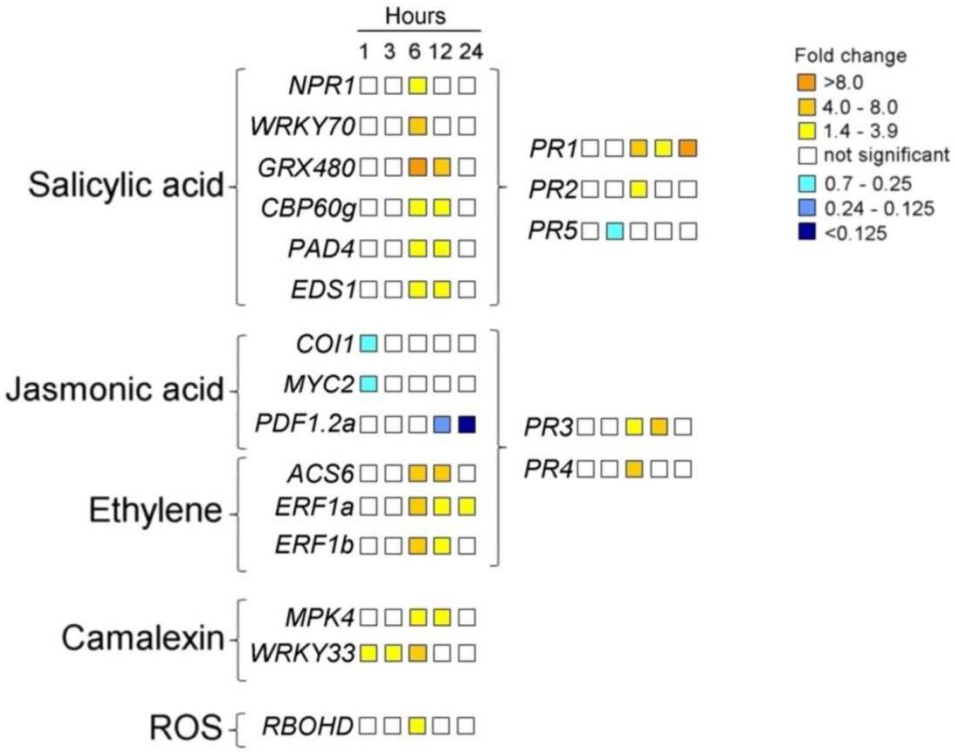

Figure 30: *Expression analysis of key genes related to defence-signalling pathways, camalexin synthesis and ROS production in Arabidopsis leaves treated with CP. Significant variations (P≤0.05) in the level of expression are reported in the figure with a colour scale (up-regulated genes = yellow; down-regulated genes = blue). Arabidopsis leaves were treated on the lower surface with 10-µl drops of 1.5×10^{-4} M CP (treated sample) or sterile distilled water (calibrator sample) for 1, 3, 6 , 12 or 24 h. Actin-2 was used as the endogenous reference gene. Relative gene expression values (2-ΔΔCt or fold change values) from three biological replicates are shown.*

CP induces the biosynthesis of camalexin

The production of the *Arabidopsis* phytoalexin camalexin was evaluated on leaves treated with 10 µl drops of 150 µM CP. Phytoalexins are phenolic compounds whose production after treatment with CP is known in different plants as a release of fluorescence substances (Comparini *et al.,* 2009; Pazzagli *et al.,* 1999; Scala *et al.,* 2004). Accordingly, the synthesis of phytoalexins was first determined in *Arabidopsis* leaves by measuring the fluorescence of the droplets recovered from the leaves. Drops were collected after 15 and 30 min, 1, 3, 6, 9, 12 and 24 h of treatment with CP from the lower leaf surface where they had been applied. As shown in Figure 31A, the fluorescence in the droplets significantly increased starting from 12 h following treatment and became about six times higher after 24 h. In order to determine whether the fluorescence was due to camalexin biosynthesis, the emission peak of a camalexin standard was compared to the peak obtained from the recovered droplets. The two spectra almost overlapped, thus suggesting that the fluorescence emitted after treatment with CP was due to camalexin production (data not shown). To confirm this observation and to investigate the presence of camalexin in the tissues, treated leaves were extracted with methanol and analysed by reverse-phase chromatography (Figure

31B). The analysis revealed the presence of a main peak (with a retention time of 11.2 min) that matches the retention time of a camalexin standard analysed with the same method. To further confirm the result, the eluted peak at 11.2 min was recovered and its fluorescence emission compared to the standard (Figure 31C). The overall results indicate that camalexin was produced by *Arabidopsis* leaves following treatment with CP. As these results were obtained by treating the lower leaf surface, we compared the two leaf surfaces in their ability to release phytoalexins after treatment with CP. After 48 h of treatment, the fluorescence emitted by the droplets recovered from the lower surface was about double that emitted by the droplets recovered from the upper surface (data not shown).

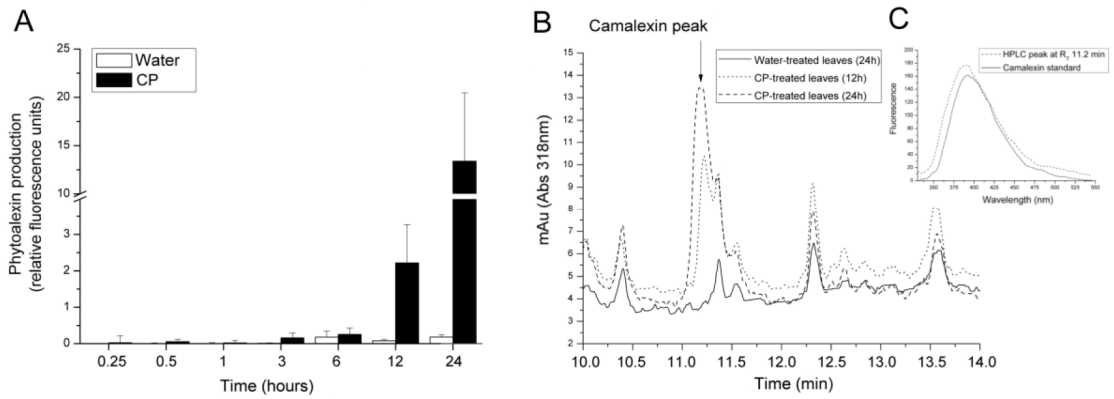

Figure 31: *Phytoalexin production by Arabidopsis leaves treated with CP.* ***A)*** *Leaves were treated on the lower surface with 10 µl drops of 1.5×10^{-4} M CP or sterile distilled water (control). The drops were collected after 15 and 30 min, 1, 3, 6, 9, 12 or 24 h of treatment and phytoalexin release measured by fluorescence analysis (λex=320 nm, λem=386 nm). The fluorescence value was normalized to the number of droplets analysed and expressed as relative fluorescence units. Error bars indicate SD of three measurements.* ***B)*** *Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) of the phytoalexin camalexin extracted from the leaves treated for 12 and 24 h. The retention time (Rt) of the peak indicated by the arrow is the same of a camalexin standard, as experimentally determined.* ***C)*** *Fluorescence emission spectrum of the peaks eluted at 11.2 min compared to a camalexin standard.*

CP protects *Arabidopsis* leaves from Botrytis cinerea and Pseudomonas syringae pv. tomato

In order to assess the effectiveness of CP in protecting the plant from pathogens, *Arabidopsis* leaves were treated on the lower surface with 10 µl drops of 150 µM CP, sterile distilled water (control) or 0.1% chitosan (50-60 kDa, deacetylation degree 85%). After 24 h of treatment, the drops were recovered and the leaves were infected on the same foliar surface with virulent pathogens. *B. cinerea* strain PM10 was inoculated by placing a single 10 µl drop of a suspension $2x10^5$ conidia ml^{-1} on a side of the middle vein. After 3 days of incubation at 22°C, CP-treated leaves showed a consistent reduction of the disease symptoms (Figure 32A). The lesion size in CP-treated leaves was statistically similar to what occurred after the treatment with chitosan. The lesions remained basically limited to the point of application of the conidial suspension,

although after the treatment with chitosan some leaves showed a complete absence of lesions.

P. syringae pv. *tomato* (*Pst*) strain DC3000 was similarly inoculated by placing on the leaf surface a single 10 µl drop of a suspension 10^8 colony-forming units (CFU) ml^{-1}. After 3 days of incubation at 28°C, the leaves were photographed and the bacterial titer was determined. As shown in Figure 32B, the treatments with CP and chitosan reduced both symptom development and bacterial growth compared to water-treated leaves. Chitosan appeared more effective than CP in limiting the growth of *Pst* DC3000, but the difference compared to CP was again not significant.

No direct inhibiting effect of CP against the germination of *B. cinera* conidia or the growth of *Pst* DC3000 was found when the pathogens were grown in vitro in the presence of 150 µM CP (data not shown).

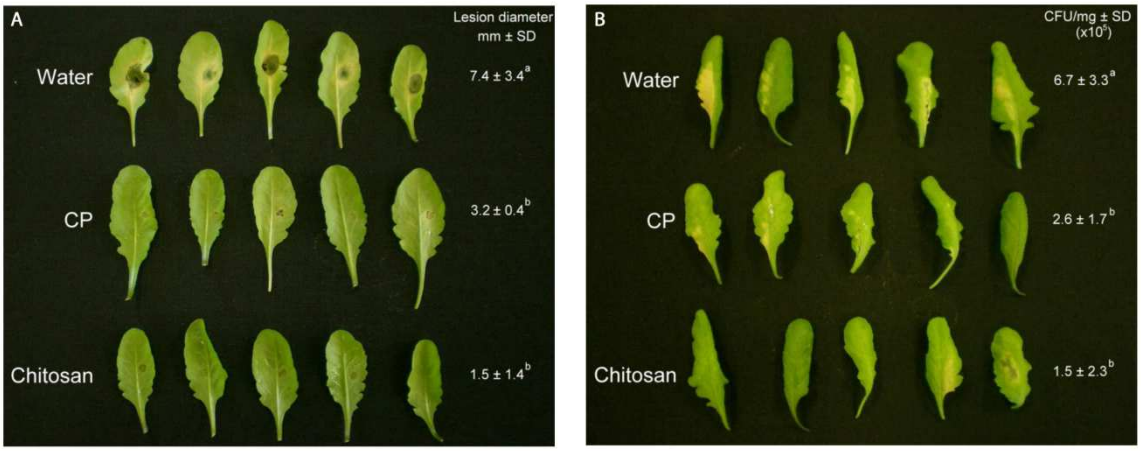

Figure 32: *Resistance induction assay against Botrytis cinerea strain PM10: (A) and Pseudomonas syringae pv. tomato (Pst) strain DC3000 (B). Arabidopsis detached leaves were treated on the lower surface with 10 µl drops of 1.5×10^{-4} M CP, sterile distilled water (control) or 0.1% chitosan for 24 h before pathogen inoculation.* ***A)*** *B. cinerea was inoculated by placing a single 10 µl drop of a suspension $2x10^5$ conidia ml^{-1} in 1% Sabouraud Maltose Broth on a side of the middle vein. Infected leaves were incubated for 3 days at 22°C before taking photos and measuring the lesion size. Lesion diameter (mm) ± SD refers to 8 replicates per treatment.* ***B)*** *Pst DC3000 was inoculated by placing a single 10 µl drop of a suspension 10^8 colony-forming units (CFU) ml^{-1} (OD600=0.2) in sterile distilled water containing 0.02% Silwet L-77 on a side of the middle vein. Infected leaves were incubated for 3 days at 28°C before taking photos and determining bacterial titer. CFU mg-1 ± SD refers to 8 replicates per treatment. All the values are $x10^5$. Statistically significant differences among treatments are indicated at $P\leq0.05$.*

Can adaptation to metalliferous environments affect plant response to biotic stress?Insight from *Silene paradoxa* L. and phytoalexins

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ENVIRONMENTAL AND EXPERIMENTAL BOTANY 2013

Plants are exposed to varying environmental conditions that generally include the combination of biotic and abiotic factors. In this context, an interesting and appropriate model system to investigate such intriguing topic can be represented by the study of responses of heavy metal adapted plants to pathogen attack. In this work we investigate the ability of CP to induce MAPK activation and phytoalexins production in three different populations of *Silene paradoxa* from a non-contaminated soil, a serpentine soil and a copper mine soil respectively cultivated in the presence/absence of nickel or copper. This work confirmed the role of general elicitor of CP that can be used as PAMPs to activate the plant defense response.

MAPK activation and phytoalexins production

In this case CP protein was used to induce the phytoalexins production in *Silene paradoxa* leaves when the plants are previously treated with nickel, copper or water. Leaves were treated for 24h of CP and assayed for phytoalexins production by fluorescence spectroscopy. When leaves were not exposed to CP, in the sensitive and the copper mine populations the levels of fluorescence were not significantly different between control and metal treated plants, whereas in the serpentine population such levels were significantly higher in metal treated plants as compared to control plants.

After the exposition to CP, all the samples showed significantly higher values of fluorescence, from a two-fold to a six-fold increase in the different cases. Comparing such data in the case of metal exposed plants, the copper mine population displayed a higher value of fluorescence when grown in the presence of copper and a lower one when grown in the presence of nickel, whereas in the other two populations the fluorescence values obtained where not dependant on the growth conditions (data not shown).

Similarly we investigate the ability of CP to induce the MAPK cascade activation. For this analysis we used the anti-human phospho-ERK1/2 antibodies because plant kinases show a sufficient level of homology with the phosphorylation sites of mammalian kinases. ERK1/2 are equivalent to MAPK6 and MAPK3 from *Arabidopsis* which are involved in the defense response (Galletti et al., 2011).

The level of MAPK phosphorylation was measured at 15 and 60 min, as the kinase cascade activation is one of the first events in plant defence signalling (Pitzschke et al., 2009). Immunoblot analysis of CP-treated leaves showed that either in copper mine and

sensitive plants treated with water the activation of kinases was not detectable (Figure 33A,C). On the contrary, the treatment with CP induced an increase in kinases phosphorylation and such increase was more evident in both sensitive and copper mine plants grown in copper containing medium when compared to the same plants grown in control medium (Figure 33B,D). Results also showed that the kinase activation was one of the first signal in defence responses: in all the assayed samples the phosphorylation signal started after 15 min and it was off after 60 min of incubation. Finally, in the copper mine population grown in 5µM copper a basal level of kinase activation was observed also without CP treatment (figure 33D).

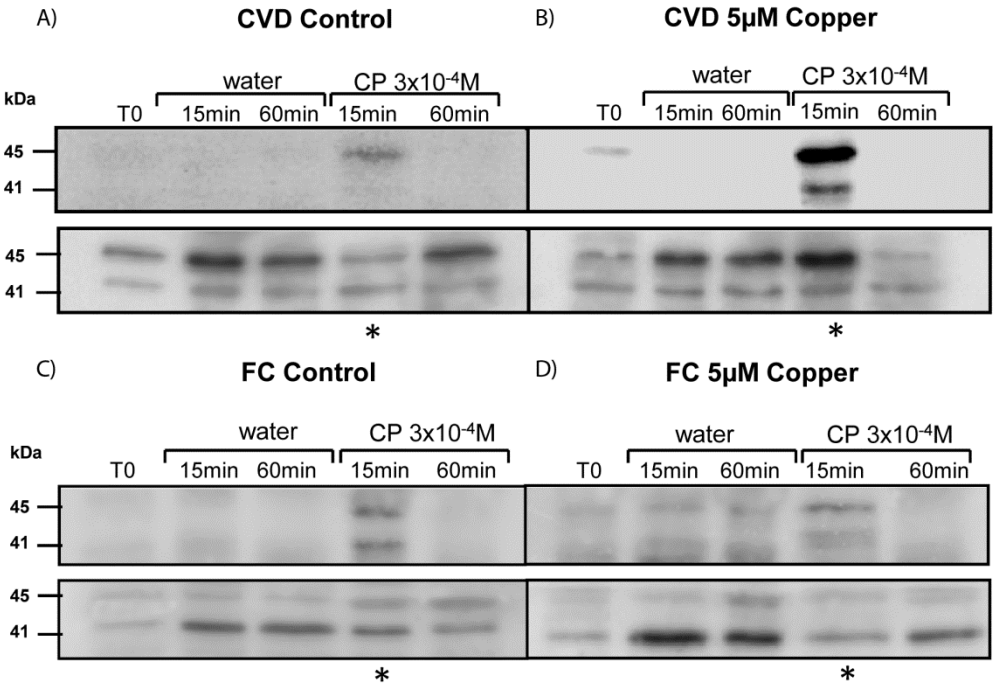

Figure 33: *MAPKs activation in Silene paradoxa leaves treated with 3 nmol of cerato-platanin (CP). Phosphorylation of MAPK in sensitive* ***(A)*** *and copper mine* ***(C)*** *leaves grown in water (left panel) and respectively* ***(B and D)*** *in copper medium (right panel) visualized with anti ERK1/2 antibodies. In each figure the lower panel represents the results obtained with anti MAPK3 and MAPK6 antibodies; the band intensities were used to normalize the results. The figure and the normalization results are representative of three independent experiments performed in duplicate.*

A. thaliana-CP interaction: a proteomic approach

With the aim to further clarify the mode of action of CP, we are now studying how changes the expression profile of *A. thaliana* leaves treated for 8h with CP. To this end, we are conducting a proteomics study between control and treated leaves by two-dimensional electrophoresis.

Samples preparation and 2DE

We used PS to precipitate part of the RuBisCo present in the leaves extracts and this method was efficient in decreasing the percentage of enzyme (data not shown). Subsequently, we performed proteomic analysis by 2DE of the control and treated samples according to the procedure described in materials and methods. Extracted proteins were subjected to 2D gel electrophoresis and representative 2D gels are shown in Figure 34.

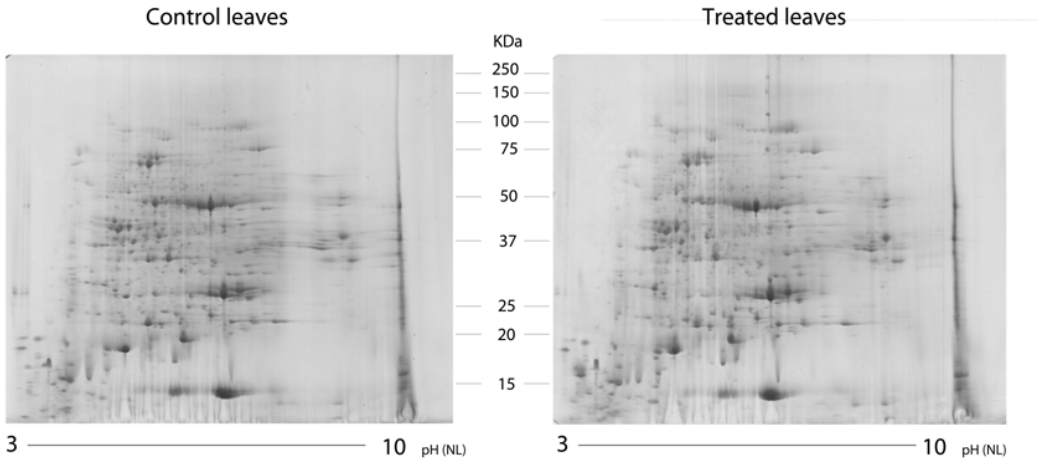

Figure 34: *Images from 2-DE analysis: equal amounts (600µg) of control and treatment samples were loaded on the gels and electrophoresis was performed as detailed in the section: materials and methods. The MW markers and pI are indicated.*

To detect proteins variably expressed in the treatment, 2-DE patterns from control and the corresponding CP treatment were compared. Using the software SameSpots for the image analysis, around 1493 spots were resolved and detected by Coomassie staining in the pH range of 3–10.

The comparative image analysis revealed 33 spots, which showed at least 1,5-fold different protein expression by comparing samples (Figure 35). Twenty of these spots were increased and thirteen protein spots showed decreased accumulation in the treated leaves when compared with the controls (Table 3).

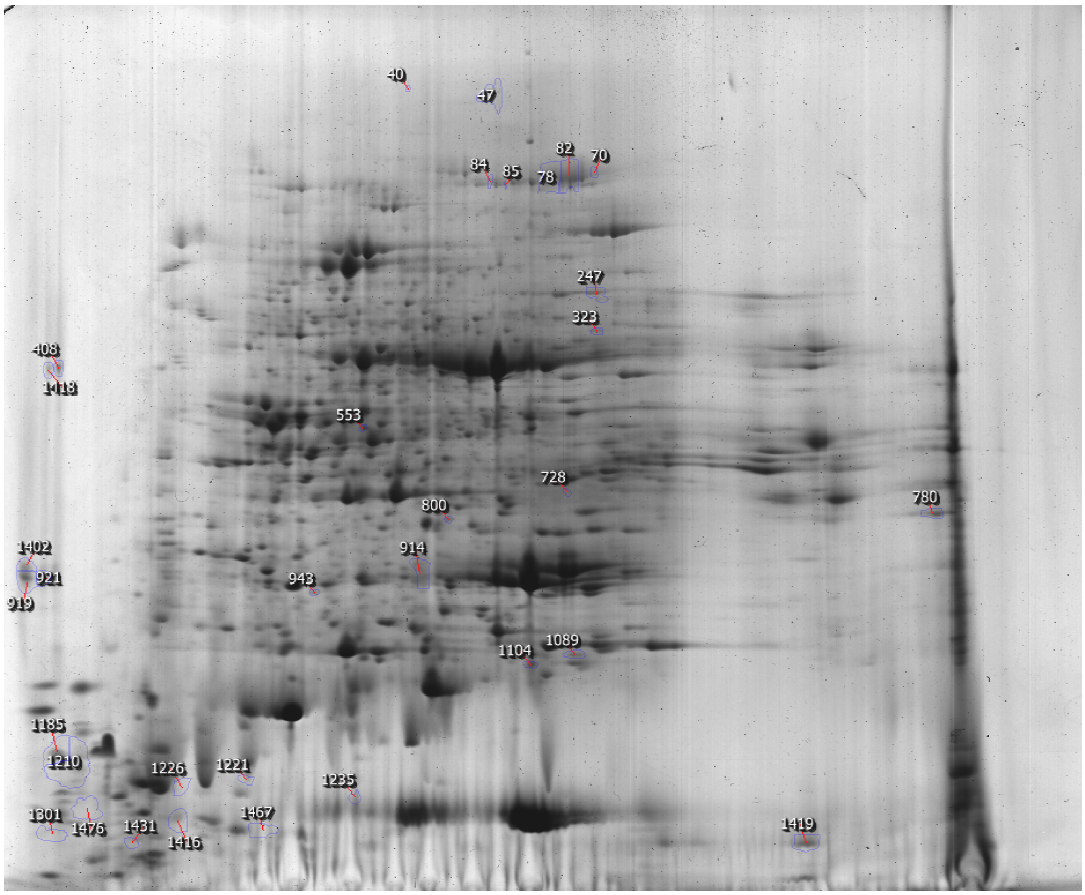

Figure 35: *Reference gel chosen for image analysis: the differentially expressed spots are circled in blue and are indicated by the ID numbers.*

SameSpots software is also able to calculate the presumed molecular weight and isoelectric point which are reported in Table 3.

Is of particular interest that many of the differentially expressed spots are located in the region of acidic low molecular weight and is generally known that the PR proteins are acidic proteins of low molecular weight (Figure 36) (Leubner-Metzger et al., 1999).

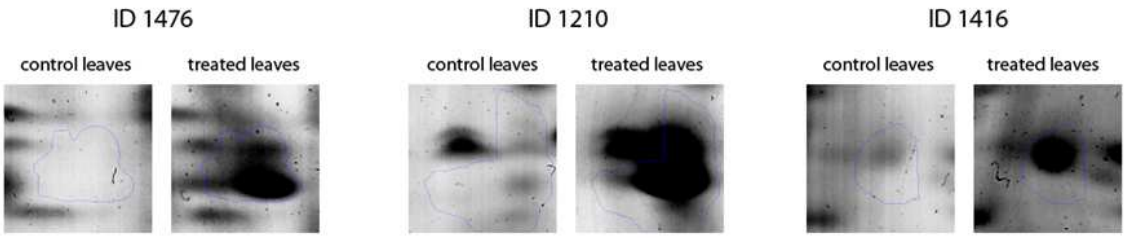

Figure 36: *Comparison of three spots that are differentially expressed in the acidic region and at low molecular weight. The ID number corresponds to that of Figure X and Table X. In this case all three spots are overexpressed in the treated leaves compared to the controlleaves.*

ID spots	Anova (p)	Fold	pI	MW	
40	0,015	1,7	6,01	191.667	↑
47	0,025	1,9	6,63	179.167	↑
70	0,008	1,5	7,43	99.138	↑
78	0,015	1,6	7,16	97.414	↑
82	0,04	1,5	7,23	96.552	↑
84	0,016	1,5	6,65	95.936	↑
85	0,037	1,6	6,74	95.936	↑
247	0,038	1,7	7,38	63.491	↑
323	0,004	1,9	7,43	54.954	↑
408	0,009	3,9	3,4	48.327	↓
553	0,035	4,1	5,69	40.818	↓
728	0,041	1,6	7,19	34.714	↑
780	0,018	3	9,98	32.755	↓
800	0,031	1,8	6,32	32.293	↓
914	0,025	1,9	6,13	27.776	↓
919	0,018	3,5	3,15	27.694	↓
921	0,021	3,7	3,28	27.667	↓
943	0,018	2,1	5,31	26.551	↓
1089	0,004	1,8	7,25	21.837	↑
1104	0,03	2,1	6,93	21.148	↓
1185	0,031	2	3,44	16.911	↑
1210	7,02E-04	3,6	3,47	15.444	↑
1221	0,046	2,1	4,8	14.627	↑
1226	4,42E-04	3	4,29	14.176	↑
1235	0,003	1,7	5,63	13.843	↓
1301	7,07E-04	3,1	3,36	11.275	↑
1402	0,02	4,1	3,1	28.347	↓
1416	0,047	1,8	4,31	11.667	↑
1418	0,007	4,6	3,35	48.756	↓
1419	0,027	2,5	9,04	10.863	↓
1431	0,048	3	3,91	11.020	↑
1467	0,019	1,8	5,04	11.706	↑
1476	1,87E-04	6,9	3,68	12.667	↑

Table 3: *Relative changes in the abundance of 33 protein spots with at least 1,5-fold alteration after CP treatment. MW and pI were predicted by the SameSpots software on the basis of the markers present in the gels; ↑ overexpressed spots in treated leaves; ↓ downexpressed spots in treated leaves.*

However, the analysis is only at a preliminary stage; in the future we aim to identify the differentially expressed spots extracting them from the gels and identifying them by mass spectrometry.

Discussion

The Cerato-platanins are non-catalytic low molecular weight proteins characterized by the presence of four cysteine residues to give two intra-molecular disulfide bridges (Pazzagli et al., 1999). They are secreted and present in the cell wall of hyphae and conidia of phytopathogenic fungi ascomycete and basidiomycete (Scala et al., 2004; Frias et al., 2013). The members of this family are capable to induce defense-related responses in both animals and plants (Pazzagli et al., 1999; Sheu et al., 2009) and currently have been identified about one hundred of proteins belonging to the family, including the proteins CP and Pop1 that have been the main object of this thesis.

In the CPF, the molecular mechanism of action on plants and above all the biological role in fungal life are little-known aspects. Therefore, the understanding of the role of CP and Pop1 in the physiology of the fungus and in the disease can help us to set up a model valid for all the family members.

The first issue that we discussed was the role that CP and Pop1 play in the fungal life. Previous studies have shown that expression of the *cp* gene is correlated with the fungal growth: when the growth of the fungus is reduced the *cp* gene is down-regulated; instead, the *cp* gene is up-regulated when the fungus grows (Baccelli et al., 2012). In addition, de Oliveira and colleagues (2011) indicate that CP structure is very similar to those of proteins capable of interacting with sugars like expansins, endoglucanases and the plant-defence protein barwin. In this regard, we have investigated the ability of the two proteins to interact with wall polysaccharides and in particular their expansin-like activity. Expansins are proteins mainly found in plants, where they have cell wall-loosening activity and various roles in growth and developmental processes (Sampedro and Cosgrove 2005). They are believed to disrupt non-covalent bonds of wall polysaccharides without any hydrolytic activity. However, expansin-like proteins have also been found in fungi and bacteria (often called swollenins or loosenins) (Bouzarelou et al. 2008; Brotman et al. 2008; Chen et al. 2010).

We have demonstrated that CP, like the orthologous protein Pop1, weakened filter paper in a concentration-dependent manner. No detectable amount of reducing sugars was released by CP and Pop1 during the action on filter paper, thus confirming a lack of β–1,4–glucanase activity that had already been reported for CP on carboxymethylcellulose (de Oliveira et al. 2011).

In order to further understand the nature of the weakening activity shown by CP and Pop1, other substrates such as Avicel, cotton, chitin, colloidal chitin and β-1,3-glucan were used. Avicel and cotton turned out to be susceptible to the action of CP, suggesting

an interaction with these cellulosic materials. The interaction, however, did not involve a stable bond with the substrates, since both CP and Pop1 were recovered from the supernatants of the incubation mixtures, as revealed by SDS-PAGE. The proteins did not bind even β-1,3-glucan. However, both CP and Pop1 bound quickly to chitin and colloidal chitin. CP bound to colloidal chitin with the following binding parameters: Bmax=2.07±0.19 μmol/g of chitin, Kd=40.56±9.50 μM, whereas Pop1 had an affinity of about tenfold lower.

The effect caused on filter paper by CP and Pop1 was very similar to that reported by Chen et al. (2010) for the expansin-like protein Swo1 from Aspergillus fumigatus, while on Avicel the effect was very similar to that reported both by Chen et al. (2010) and Wang et al. (2011). Conversely, on cotton CP did not cause the formation of swollen areas or enlarged "bubbles" as reported for two expansin-like proteins (Quiroz-Castañeda et al. 2011; Saloheimo et al. 2002), but breakage and defibration. Pop1 acted to a lesser extent than CP and the minimum active concentration was 3×10^{-7}M for CP and 3×10^{-6}M for Pop1.

The optimum of pH and temperature for the activity of CP were also determined: the optimum values were pH 5.0 and 38 °C, substantially in accordance with expansins and expansin-like proteins (Chen et al. 2010; Kim et al. 2009; Quiroz-Castañeda et al. 2011; Sampedro and Cosgrove 2005; Wang et al. 2011).

Canonical expansins are small proteins of about 26 kDa consisting of two compact domains: the N-terminal domain (D1) constitutes the double ψβ-barrel fold; the C-terminal domain (D2) forms an Ig-like β-sandwich fold (Kerff et al. 2008; Yennawar et al. 2006) that have the essential role of binding to cellulose and to matrix polysaccharides (Georgelis et al. 2011). Since CP lacks D2, this could account for its inability to bind cellulose. However when D1 from EXLX1 was separated from D2 it did not weaken paper (Georgelis et al. 2011), but it should be noted that LOOS1, the only expansin-like protein found in nature without D2, showed instead both binding ability and disrupting activity on cellulose (Quiroz-Castañeda et al. 2011). Thus, the behaviour of CP differed from that of both EXLX1 and LOOS1. Georgelis et al. (2011) reports that several polar residues in D1 could contribute to the weakening activity of EXLX1 and in particular Asp-82, an amino acid considered essential for this activity and also well conserved among expansins. Cerato-platanins have the residue Asp-77 well conserved among members of the family (de Oliveira et al. 2011) and its role will be the object of further investigation.

The results we obtained also indicate that CP and Pop1 not show synergistic effect with cellulases. The synergism between cellulase and expansins is a known phenomenon for all espansin proteins since CP is the only protein with an expansin-like domain that does not show a synergistic effect with cellulases and the only protein active on cellulose without binding to it; the lack of synergistic effect could somehow be related to its inability to bind.

Finally, CP and Pop1 are able to binding chitin and colloidal chitin but these two matrices did not appear damaged and/or altered by the incubations, as observed by optical microscope. The binding result was partially expected because de Oliveira et al. (2011) showed that the residues involved in binding to β-(1,4)-linked N-acetyl-Dglucosamine oligomers are the most conserved in the CP family and also, the ability to bind chitin had been already reported for expansin-like proteins from fungi, although it is not clear whether these proteins caused changes in the chitin structure (Chen et al. 2010; Quiroz-Castañeda et al. 2011).

In addition Barsottini and colleagues (2013) have seen that even MpCP2, another CP proteins, presents expansin-like activity very similar to CP and Pop1. CP, Pop1 and MpCP2 are enough representative of the CP family, 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.

The ability to bind chitin and the localization in the cell wall suggests for CPs 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 (Baccelli et al 2013a). However, it is now clear the similarity between CPs and expansins and this opens, also, new perspectives in the understanding of the role of CPs in plants interaction, as we will discuss later.

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. In this regard, the second part of this thesis concerned the characterization of the interaction of Pop1, and in particular of CP, with different types of plant. For all the experiments we used both the two proteins CP and Pop1 at 1.5×10^{-4}M, being this concentration the most used in characterizing the biological activity of these proteins (Scala et al. 2004). However, the used concentration is in the same range of the ones used in the study of other fungal elicitors (Wan et al., 2004; Buensanteai et al., 2010; Frias et al., 2011).

First of all, we evaluated the action of the two proteins applied on the lower surface of leaves of *P. acerifolia*. To unravel the upstream events in priming defense we decide to analyze one of the first events following PAMPs recognition that is the activation of the MAPK cascade (Tena et al. 2011, Hamel et al. 2012). MAPKs are activated by dual phosphorylation of specific threonine and tyrosine residues leading to various cellular responses, i.e. through the activation of other protein kinases or through phosphorylation of transcription factors or components of the cytoskeleton (Mishra et al. 2006). Results obtained by antihuman ERK1/2 antibodies on plane leaf extracts showed that both CP and Pop1 activated the MAPK cascade, as confirmed by the increased levels of phosphorylated MAPKs after 60min of treatment. Being these levels already decreasing after 12 h this confirms once again that MAPKs are one of the first events in plant defense. The molecular weight of the positive phospho-ERK kinases is of

about 44 and 47 kDa and it matches those of MAPK3 and MAPK6, respectively, which are activated in *Arabidopsis* and in poplar leaves (Hamel et al. 2005, Han et al. 2010). Therefore, we can state that either CP and Pop1 are able to activate the MAPK cascade by phosphorylation of two kinases of 44 and 47 kDa, probably homologous to the MAPK3 and MPK6 from *Arabidopsis*.

As a consequence of MAPKs activation, a plethora of downstream signals are activated. In this study we showed that CP and Pop1 induce NO and H_2O_2 production in plane leaves: both fluorescence microscopy and quantitative assays in droplets indicated that after 3 h of treatment H_2O_2 generation began and reached steady state shortly after on. Contrarily, NO peaked around 6 h and was switched off after 12 h of treatment.

Among the other downstream signals activated by CP and Pop1 we looked at the expression of three defense-related genes during the first hours of elicitation. Our results showed that CP and Pop1 are able to induce the modulation of these genes: *PR5* transcription levels enhanced after 6 h of incubation with CP and from 3 to 24 h of incubation with Pop1; *LTP* was not activated from both the two proteins; the *APX* gene expression was up-regulated only at the first hours of elicitation and after no over- or under-expression were detected. This may explain the time course of H_2O_2 production in plane leaves upon treatment with CP: after 6 h of incubation *APX* was not over-expressed anymore and H_2O_2 reached high values that could be correlated with the appearance of HR visible symptoms. The plant HR is a form of PCD, sharing some cytological features with animal PCD (Mur et al. 2008). We showed that both CP and Pop1 induce such cytological features, i.e. organelle swelling, tonoplast rupture and chromatin condensation, cell collapse and DNA fragmentation typical of PCD.

Among the results obtained, it is of particular interest the faster activity of CP compared to Pop1. A clear difference in the response timing and sometimes in the magnitude of the signal can be observed for MAPK activation, H_2O_2 and NO production and for cell death induction. In addition it must be noted that the fungal growth observed in droplets containing CP was strongly reduced, while Pop1 was unable to stop the *Ceratocystis* growth (Table 2).

These differences could be explained by the structural data obtained so far on CP and Pop1 (Comparini et al. 2009, de Oliveira et al. 2011). We suggest that the difference in the induction of defense responses may be due to the different hydropathy profile and GRAVY (Grand average of hydropathicity) index of CP and Pop1 (0.002 and −0.304, respectively). Because CP is more hydrophobic than Pop1, the interaction with cuticular waxes can be faster and stronger with CP than with Pop1. We also considered that many non-catalytic fungal proteins such as cryptogein (Lamotte et al. 2004) and other hydrophobins interact with substrates by a helix preformed in the molecule, or induced by the substrate itself (De Vocht et al. 1998). A difference in helix content between CP and Pop1 is suggested by the circular dichroism spectra of these two proteins (Figure 9) and by the 3D structure of CP (de Oliveira et al 2011, Martellini et al 2012). Probably, as

indicated by secondary structure prediction methods, Pop1 lacks the α2-helix that could be involved in substrate recognition.

We are currently determining the three-dimensional structure of Pop1 (Baroni et al., 2013) and we hope that the information about the structural differences between the two proteins will help us to understand the differences in the action on plants.

Once established that CP and Pop1 act as elicitors of plane leaves, we went to investigate the ability of CP to act on *A. thaliana* leaves. In this case we used only the CP, at the same concentration of the experiments on plane leaves, since this protein has been shown to be more effective in eliciting host defenses. We have also decided to carry out experiments on *A. thaliana* because it is the plant model organism for the study of elicitors. It is a small plant that completes its life cycle in one month and whose genome has been completely sequenced.

Initially we focused on the ability of CP to induce the production of H_2O_2 through a series of experiments similar to those carried out on plane. ROS are central players in the complex signalling network of cells. An initial burst of ROS production can trigger a cascade of cell-to-cell communication events that propagate the signal over long distances like a wave (Mittler *et al.,* 2011). Therefore we analysed the production of H_2O_2 with the objective to investigate the origin of the ROS signalling.

Hypothesizing a role for stomata in the perception mechanism of CP, we assumed that H_2O_2 had to be produced first by the stomata. Accordingly, we designed the experiment to monitor the H_2O_2 evolution on the epidermis and we started the analysis almost instantaneously (2 min).

Other cerato-platanins had been reported to induce H_2O_2 production in *Arabidopsis* leaves, namely MgSM1 and BcSpl1 (Frias *et al.,* 2011; Yang *et al.,* 2009), but MgSM1 had been assessed 24h after the ectopic expression in transgenic plants, whereas BcSpl1 had been assessed 4h after the infiltration. Our results suggest that CP elicited defense responses by entering through the stomata, and perhaps its perception occurred at the level of the inner surface of the guard cells. This action model was suggested by some clear observations: H_2O_2 production after the treatment with CP was initiated by guard cells (4-6 min) and only subsequently spread from the stomata to the neighbouring epidermal cells (not *vice versa*); the treatment of the lower surface, which normally presents a higher stomatal density (Kumar *et al.,* 2013; Lake *et al.,* 2002), resulted in a higher production of H_2O_2 and phytoalexins; the treatment performed with closed stomata did not result in H_2O_2 production; CP is unable to penetrate the leaf cuticle (Martellini *et al.,* 2012) and thus different entrance routes seem unlikely.

Accordingly, we can deduce that (**a**) stomata have a key role in the *Arabidopsis* immunity triggered by CP; (**b**) the guard cells probably possess a receptor for this MAMP. This assumption is in accordance with the role in MAMP sensing attributed to guard cells by Melotto *et al.* (2006), who also hypothesized the presence in these cells of several receptors for multiple MAMPs, and with the actual presence in these cells of the flagellin

receptor (Robatzek *et al.,* 2006). At the same time our results do not conflict with the presence of a CP receptor also in the plasma membrane of epidermal or mesophyll cells, as suggested by Frias *et al.* (2013b). However, this receptor could only sense cerato-platanins produced by fungi able to break through the leaf cuticle and cannot explain the elicitation process without cuticle penetration, as it is the case of the surface application.

Stomata represent a natural entry site for potentially harmful microbes. It is known that the stomata close upon perception of MAMPs as an innate immune response active at the pre-invasive level (Melotto *et al.,* 2006; Sawinski *et al.,* 2013). It is also known that the generation of H_2O_2 is crucial to initiate this process and that PAMPs rely on RBOHD as the primary NADPH oxidase for ROS production (Sawinski *et al.,* 2013; Wang and Song, 2008). CP induced a progressive reduction of the stomatal aperture similarly to well-known PAMPs, such as flagellin-22 (flg22) and lipopolysaccharides (Melotto *et al.,* 2006). As found by qPCR, *RBOHD* was effectively up-regulated by CP but the timing (6h) could not explain the early oxidative burst observed. Therefore we can assume that the rapid generation of H_2O_2 was caused by this enzyme in the form already present in the cells.

Also, on *A. thaliana* leaves we verified the ability of CP to induce the MAPK cascade activation. In this case, the phosphorylation occurs at very early times: both kinases, MPK3 and MPK6, are activated within 5 minutes of incubation and the signal persists until 15 minutes. This result confirms that the activation of the kinases cascade is one of the first events in plant defense signaling.

Subsequently, we performed an extensive gene expression analysis never performed before for cerato-platanins. The results obtained indicate that CP is able to modulate the phytohormone homeostasis, in particular are up-regulated metabolic pathways related to SA and ET while it is down regulated JA signaling. *NPR1* (nonexpressor of PR genes 1), *EDS1* (enhanced disease susceptibility 1), *PAD4* (phytoalexin-deficient 4), *GRX480* (glutaredoxin 480), *WRKY70* and *CBP60g0* (calmodulin binding protein 60g) were up regulated by CP within 6 h of treatment and have been reported to contribute to PAMP-induced SA accumulation or suppression in JA pathways (Wang *et al.,* 2009; Pieterse *et al.,* 2009), while *COI1, MYC2,* both involved in JA perception and regulation respectively, were both rapidly down-regulated (1h) (Katsir *et al.,* 2008; Lorenzo *et al.,* 2004). Finally *ACS6* (1-amino-cyclopropane-1-carboxylic acid synthase 6), *ERF1a/ERF1b* (ethylene response factor 1), three ET-dependent genes, were up-regulated by CP.

This result, confirming the well-known antagonism between SA and JA signaling (Thaler *et al.,* 2012), leads us to predict a role for SA and ET, rather than JA, in CP-induced defense signaling in

Arabidopsis. This role for SA is further supported by the experimental evidence that the cerato-platanin BcSpl1 induces SA production and SAR in tobacco (Frias et al., 2013a).

We also investigate the production of phytoalexins, and in particular of camalexin that is the major phytoalexin in *Arabidopsis* (Ahuja et al., 2012; Qiu et al., 2008). Phytoalexins are secondary metabolites that show biological activity against a variety of pathogens and are considered molecular markers of disease resistance (Ahuja *et al.,* 2012). By fluorescence spectroscopy and HPLC analysis we demonstrated that CP induces the biosynthesis of camalexin after 12h of incubation. To confirm this, we have seen that CP up-regulates the expression of *MAPK4* and *WRKY33*, two genes involved in camalexin biosynthesis. Interestingly, the resistance induced by elicitors in *Arabidopsis* to *B. cinerea* is independent of SA, ET, or JA signaling, whereas the biosynthesis of camalexin and other secondary metabolites seems to have a prominent role (Ferrari *et al.,* 2007; Kliebenstein *et al.,* 2005).

The final experiment on *A. thaliana* leaves concerned the induction of resistance against pathogens. We investigated if CP was able to protects the plant tissues against two foliar pathogens, the necrotrophic fungus *B. cinerea* and the hemibiotrophic bacterium *P. syringae* pv. *tomato*. To date the effectiveness of CP against pathogens had only been demonstrated against *C. platani* (Fontana *et al.,* 2008). In that report, plane tree leaves treated with CP hampered *C. platani* germination and growth when inoculated with conidia. But *C. platani* is not a foliar pathogen. The data we had obtained clearly demonstrated that CP was able to induce disease resistance towards the two pathogens and the level of protection was similar to what obtained with chitosan, a well-known MAMP that we used for comparison (Iriti and Faoro, 2009). Frias and colleagues (2013) reported that the CP family protein BcSpl1 induce systemic resistance to both *B. cinerea* and *P. syringae* pv. *tabaci* in tobacco and, in addition, *Arabidopsis* transgenic plants expressing MgSM1, a cerato-platanin protein from *Magnaporthe grisea*, showed enhanced resistance to *B. cinerea* and *P. syringae* pv. *tomato* (Yang *et al.,* 2009). All these data lead us to think that all CPs have a protective effect against these two pathogens and that phytoalexins and SA play an important role in induction of resistance.

At the same time, we confirmed the role of CP as PAMPs through experiments on *S. paradoxa* leaves. Even in this case, CP is able to induce the synthesis of phytoalexins and activate the MAPK cascade in all the differnt varieties tested.

Until now, the mechanism of CPs on plants is still unclear and recently have been hypothesized three different modes of action (Figure 37) (Pazzagli et al., 2014):

A) CPs weakening activity on the plant cellulose 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.
B) CPs penetrate in the host through stomata and are recognized by membrane receptors from guard cells or epidermal/mesophyll cells. Until now, no PRRs (pattern

recognition receptors) have been identified although, the involvement of the BAK1 kinase in the cascade signaling of BcSpl1 protein in *Arabidopsis* has been proposed (Frias et al 2011).

C) Another mechanism by which CPs can act in plant cells may be derived from the ability of CPs to bind chitin and N-acetylglucosamine oligomers via amino acid residues that are conserved in all the 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 the over-expression of plant cell wall degrading enzymes, which in turn would produce chitin oligomers (Kishimoto et al 2010). CPs contribute to pathogen virulence by sequestering chitin oligosaccharides released by the fungus during infection, thereby blocking the activation of host chitin receptors (Miya et al 2007). 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(de Jonge et al 2009; 2010; Mentlak et al. 2012). 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 bring to sequester the elicitor chitin and, as a consequence, to induce phytotoxic effect. On the contrary a low affinity for chitin could enable the induction of defense.

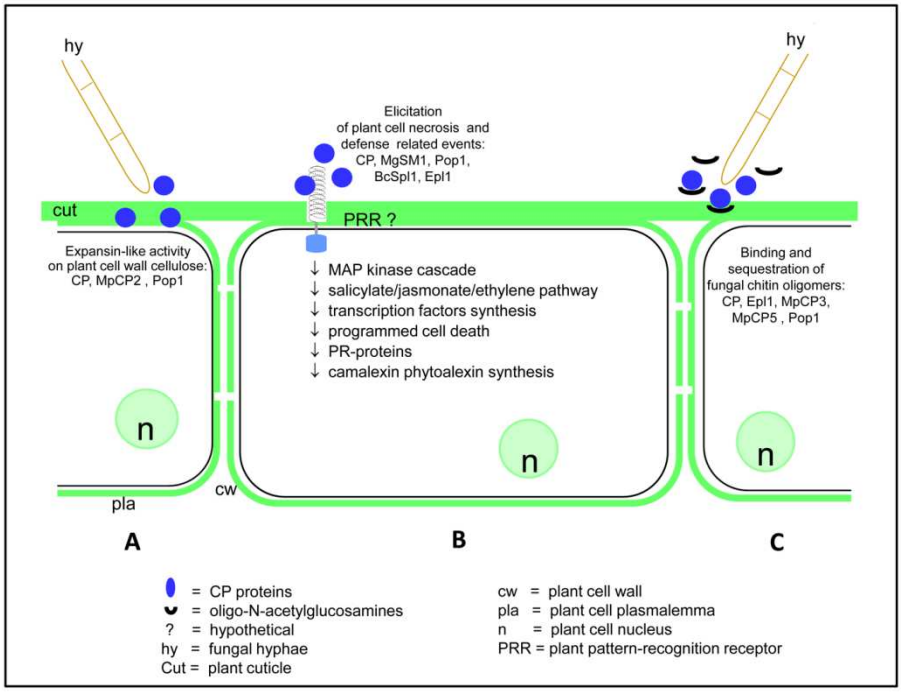

Figure 37: *Schematic rapresentation of the possible interaction between CP proteins and plant. From left to right: A) expansins-like activity on cell wall; B) PAMP activity of CP proteins upon interaction with a putative receptor; C) phytotoxic activity as a consequence of chitin oligomers sequestration*

We do not know yet which of the three mechanisms proposed is fair, probably the real mode of action of CPs is a combination of all three models. We hope that the proteomic approach that we are developing will help us to clarify this issue and to better understand the proteins involved in the induction of resistance. The ultimate goal is arrive to use this type of elicitors for crop protection.

To this aim, engineered proteins as well as peptides derived from them could be designed in order to obtain "the best" elicitor to be used for plant defense. Recently some peptides ranging from 13 to 79 kDa secreted from *S. mycoparasitica* have been patented and preliminary tested against *Fusarium*, but more information and research are needed to obtain a small, easily to produce, and stable peptide to test as inductor of defense.

In this regards, our laboratory in the near future plans to test four different peptides derived from the amino acid sequence of CP. These peptides were designed on the basis of similarities with known PAMPs as fl22 or elf18 or on the basis of protein fragments that are formed during the aggregation process. Recent works have shown that both CP that Pop1 undergo self-assembling in solution, forming ordered aggregates which can enhance the fluorescence of dyes commonly used in studying protein aggregation (Pazzagli et al., 2009; Martellini et al., 2012). Self-assembling of CP and Pop1 takes place after the unfolding of the polypeptide chain, leading to the formation of ordered, amyloid-like structures. During this aggregation process both proteins lose a peptide of 6 amino acids at the N-terminus and it has been shown that the aggregated form of the proteins have greater eliciting activity (Pazzagli et al., 2009; Martellini et al., 2012).

Based on this knowledge, we decided that we will test the following peptides (Figure 38):

1. N-terminal peptide→ VSISYD
2. peptide 1-19→ VSISYDPIYAADLSMGSVA
3. peptide fl22-like→ ADLSMGSVA{Cys(CAM)}SNGDHGLMAQY
4. peptide elf18-like→ WKVTIPNGNSIFIRGVDSGRG

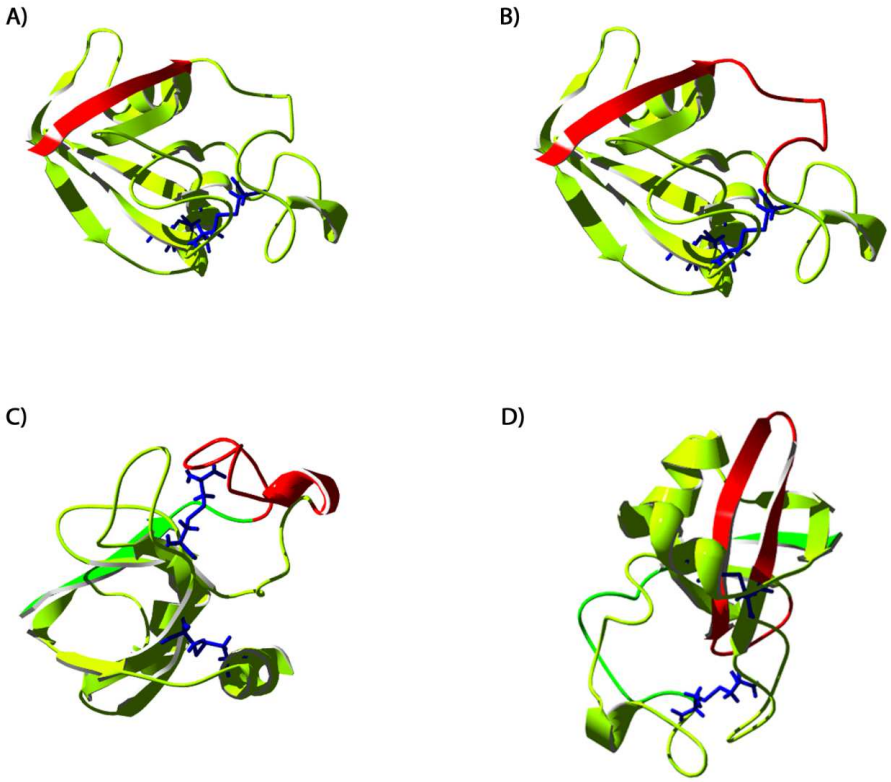

Figure 38: *Localization of peptide 1-4 within the CP structure. Peptides are shown in red and in blue the disulfide bridges.* ***A)*** *N-terminal peptide;* ***B)*** *peptide 1-19;* ***C)*** *peptide fl22-like;* ***D)*** *peptide elf18-like*.

This peptides will be tested for the ability to induce synthesis of secondary metabolites that help the plant to survive under stress challenge; e.g. some oxygenated forms of fatty acids (oxylipins) that can inhibit mycelial growth and spore germination of several eukaryotic microbes (Zaparoli et al., 2009) and for the ability to induce synthesis of salicylic acid, which along with its signaling components is key for SAR induction (Zhou et al., 2011). In fact, induced resistance offers the hope to devise a durable and broad-spectrum disease control that exploits the plant's own defense mechanisms (Pazzagli et al., 2014). 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.

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