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"MOLECULAR STUDIES ON A LARGE MULTI-GENE FAMILY OF POLYGALACTURONASES IN TWO DIFFERENT *PHYTOPHTHORA* SPECIES."

SCIENTIFIC-DISCIPLINARY SECTOR BIO/04

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Στην Ελπίδα που ήρθε,

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Abstract

MOLECULAR STUDIES ON A LARGE MULTI-GENE FAMILY OF POLYGALACTURONASES IN TWO DIFFERENT *PHYTOPHTHORA* SPECIES.

The plant cell wall is a structural barrier to pathogens, composed of a network of polysaccharides such as cellulose, hemicellulose and pectin. The majority of pathogenic microorganisms produce cell wall degrading enzymes (CWDEs) that are essential for the invasion process. Among the different CWDEs, polygalacturonases (PGs) play a critical role since their action on pectin makes other cell wall components more accessible to other CWDEs and causes tissue maceration. PGIPs (polygalacturonase-inhibiting proteins) are plant cell wall proteins that specifically modulate the activity of the PGs, and hamper the invasion process by limiting the host tissue colonization. The PG–PGIP interaction retards pectin hydrolysis and favors oligogalacturonide (OGs) accumulation and leading to plant defense activation. This work wants to contribute to study the role of the PGs in *P. nicotianae* and *P. capsici*, among the most dangerous pathogens for many plant species: Specific points of this thesis are: 1) Identification of the whole set of the PGs from well-known oomycetes, which present different lifestyles. 2) Comparison of large PG families found in the oomycetes species using phylogenetic analysis for tracking evolutionary relationships. 3) Analysis of amino acid sequences on identified PGs to detect domains and/or amino acids involved in PG-PGIP interaction. 4) Characterization of PGs from *P. nicotianae* and *P. capsici*. 5) Construction of *P. capsici* mutants for investigate the role of PGs in the pathogenesis, using different approach of reverse genetics.

The results from this thesis enhances the hypothesis that the multiplicity of PGs may give flexibility to the pathogen, with each enzyme having its own unique properties to contribute to the performance of all the enzymes to successfully colonize plants.

MOLECULAR STUDIES ON A LARGE MULTI-GENE FAMILY OF POLYGALACTURONASES IN TWO DIFFERENT *PHYTOPHTHORA* SPECIES.

Nei primi stadi dell'infezione, i microrganismi fitopatogeni producono un arsenale di enzimi che depolimerizzano in maniera ordinata e sequenziale i componenti della parete cellulare vegetale (CWDEs - Cell Wall Degrading Enzymes). Le poligalatturonasi (PG) sono tra i primi enzimi pectici ad essere prodotti e favoriscono la macerazione del tessuto vegetale rendendo accessibili a cellulasi ed emicellulasi gli altri componenti della parete. I carboidrati rilasciati dal processo degradativo della parete cellulare vengono utilizzati dal patogeno per il sostentamento e per la crescita, conferendo al processo di degradazione un ulteriore significato biologico oltre quello di distruzione fisica della parete. Le PGIP (PolyGalacturonase Inhibiting Proteins) presenti nella parete cellulare vegetale, sono delle glicoproteine in grado di inibire e/o modulare in maniera specifica l'attività delle PG. La formazione del complesso PG-PGIP rallenta la capacità delle PG di degradare l'omogalatturonano della parete cellulare, favorendo l'accumulo di oligogalatturonidi in grado di attivare le risposte di difesa della pianta. Questo lavoro vuole contribuire allo studio del ruolo delle PG in *Phytophthora nicotianae* e *Phytophthora capsici*, che sono ritenuti tra gli agenti patogeni più pericolosi per molte specie vegetali. Argomenti specifici che vengono affrontati in questa tesi riguardano: 1) l'identificazione di PG da alcune specie di oomiceti considerate tra le più pericolose per le piante (generi *Phytophthora*, *Pythium* ed *Aphanomycetes*); 2) l'analisi filogenetica delle famiglie geniche PG; 3) l'analisi di sequenze proteiche delle PG identificate allo scopo di rilevare domini e/o amminoacidi responsabili dell'interazione PG-PGIP; 4) la caratterizzazione delle PG da *P. nicotianae* e *P. capsici*; 5) la costruzione di mutanti di *P. capsici* per indagare il ruolo di PG nella patogenesi, utilizzando diversi approcci di genetica "reverse" I risultati ottenuti in questa tesi, potenziano l'ipotesi che la molteplicità delle PG può dare flessibilità al patogeno e ogni PG, con le proprie caratteristiche uniche, contribuisce alla performance del patogeno per colonizzare le piante con successo

1. INTRODUCTION

1.1 Oomycetes and fungi

Fungi and oomycetes are the causal agents of many of the world's most serious plant diseases and are unique among the microbial pathogens, are being able to breach the intact surfaces of host plants, and quickly establish infections that can have disastrous consequences for the environment and large-scale agricultural production. Fungi and oomycetes can also cause disease in animals and humans, and in certain cases mortality (Soanes, Richards, & Talbot, 2007). Through convergent evolution, oomycetes and fungi have acquired striking similarities in their mechanisms of host colonization, including physiological adaptations, mechanisms of adhesion, modulation of host defenses, and strategies of nutrient acquisition (Kale, 2011). Pathogenesis by a fungus or oomycete is a complex process and include the following steps (Meng *et al.*, 2009) (Figure 1.1):

- dispersal and arrival of an infectious particle (usually a spore) near the host,
- adhesion to the host, recognition of the host (which may occur prior to adhesion),
- penetration into the host,
- invasive growth within the host,
- lesion development in the host,
- production of additional infectious particles.

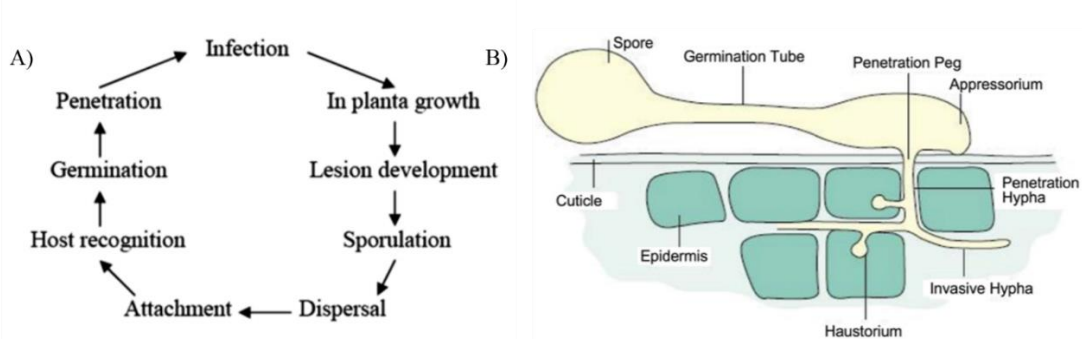


Figure 1.1: Generalized diagram displaying infection and disease cycle caused by fungi and oomycetes. Adapted by Meng *et al.*,2009.

Plant pathogens can be divided into groups based on the different strategies they employ to colonize plants. (Figure 1.2) (Latijnhouwers, Wit, & Govers, 2003).

1.1.2 Biotrophs

Biotrophy is a lifestyle generally associated with a narrow host range, requiring living host tissue, and involving the establishment of intimate interactions with living plant cells for the

exchange of nutrients and signals. They possess haustoria for retrieval of nutrients from plants. Secrete limited amounts of lytic enzymes and cause little damage to the host plant. Their sporangia ripen simultaneously, giving these pathogens a defined infection, proliferation and reproduction phase. (Oliver & Ipcho, 2004). Biotrophic pathogens evade or suppress defense responses (Vleeshouwers & Oliver, 2014).

1.1.3 Necrotrophs

Necrotrophy is a lifestyle generally associated with a wide host range (Lévesque *et al.*, 2010), in which the pathogen invades the host tissue, immediately kills host cells and lives on dead plant material. They have very destructive pathogenesis strategies resulting in extensive necrosis, tissue maceration and plants rots. Necrotrophs secrete disease agents including cell wall degrading enzymes and toxins both prior and during colonization (Laluk & Mengiste, 2010; Oliver & Ipcho, 2004). *Cochliobolus* and *Botrytis* species are examples of fungal necrotrophs.

1.1.4 Hemibiotrophs

Hemibiotrophy is a lifestyle that is generally associated with a more limited host range. Hemibiotrophic pathogens initially establish a relationship with living host cells, extracting nutrients from them, yet subsequently kill these host cells as the infection proceeds. Spores are formed while at the same time new plant tissues are being infected. During the biotrophic stage, these pathogens form invasive structures called haustoria. These specialized structures breach the plant cell wall, yet do not penetrate the host cell membrane. Haustoria are thought to serve both in nutrient uptake and delivery of factors for manipulate living host cells (Jonge, 2013; Oliver & Ipcho, 2004).

Plant-pathogenic oomycetes include species with diverse life styles. Downy mildews (*Peronosporales*) are biotrophic pathogens. *Albugo spp*. that causes white rust on *Arabidopsis* are obligate biotrophs (Links e*t al.*, 2011). The biotrophic downy mildew pathogen *Hyaloperonospora arabidopsidis* also infects *Arabidopsis* and, this plant–oomycete interaction is well characterized (Coates & Beynon, 2010).

Phytophthora spp. include mostly hemibiotrophic pathogens and cause devastating diseases on a variety of staple crops, fruit, ornamentals, and trees (Erwin & Ribiero 1996*).*

Pythium spp. inhabit water and soil habitats and display necrotrophic growth. On a broad range of plants, *Pythium spp*. can cause root rot and damping off on seedlings (Laluk & Mengiste, 2010).

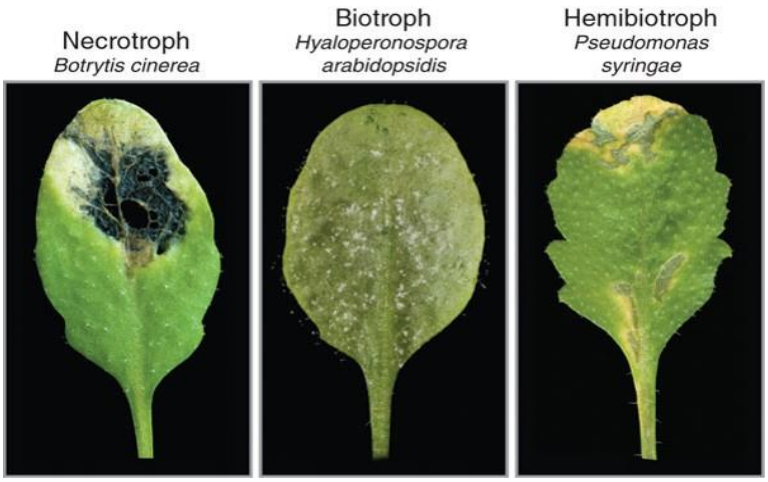


Figure 1.2: Disease symptoms on Arabidopsis leaves caused by the necrotrophic fungus *Botrytis cinerea*, the biotrophic oomycete *H. arabidopsidis* and the hemibiotrophic bacterium *Pseudomonas syringae*. Adapted from Corné M J P. *et al*., 2009

1.2 Oomycetes evolutionary relationship

Currently, at least 800 oomycete species are known, but depending on the definition of a species, this number might actually reach 1500. Nevertheless, the species richness seems low when compared to the number of fungal species known to date: 30000 basidiomycete species have been described and ascomycetes reach a similar number It is, however, likely that there are many oomycetes out there yet to be discovered (Bouwmeester, Poppel, & Govers, 2009). Due to their shared morphology (filamentous, branched somatic structures that bear spores), oomycetes and fungi were traditionally classified in the same kingdom, the Fungi (Erwin & Ribeiro, 1996). For this reason, oomycetes were for a long time considered a class within the kingdom fungi. Oomycetes, also known as water molds, resemble fungi in many ways (Sleigh, M.A. 1989). Like fungi, oomycetes have a global distribution and prosper in quite diverse environments. Both, show filamentous growth in their vegetative stage, produce mycelia and form spores for asexual and sexual reproduction. In recent years, barcode sequences as rRNA internal transcribed spacer (ITS) or cytochrome oxidase (COX) regions have been used for the identification of the species of the oomycetes. This new insights based on molecular phylogeny and comparative genomics reshaped again the tree of life (Cooke, *et al.*, 2000). In this classification, the ascomycete and basidiomycete fungi are grouped together with animals in the supergroup Unikonts (Burki, 2014). Modern molecular and biochemical analyses suggest that oomycetes have taxonomic affinity with to brown algae (heterokonts) in the Stramenopiles, one of several major eukaryotic kingdoms (Figure 1.3) (Sophien Kamoun, 2003).

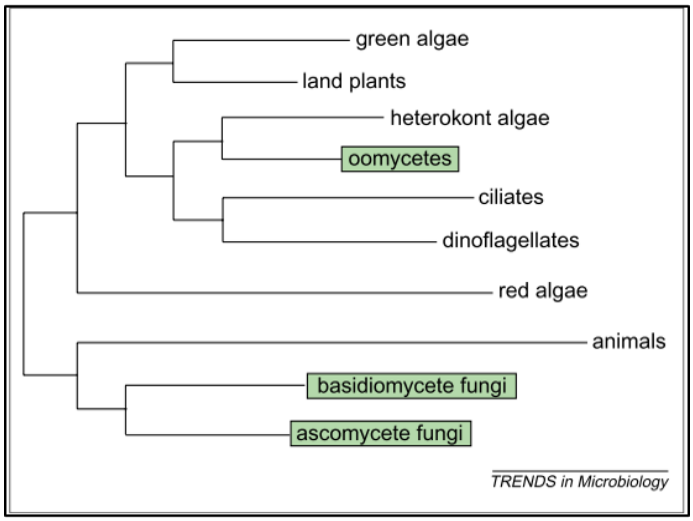


Figure 1.3: Phylogenetic tree showing the evolutionary relationships between the major eukaryotic groups. The Oomycetes and the *Ascomycetous* and *Basidiomy* fungi are highlighted in green. To be note the evolutionary distance between the Oomycetes and the fungi. Adapted from Latijnhouwers, Wit, & Govers, 2003

1.3 Morphological and physiological differences between oomycetes and fungi.

There are important differences, which can justify the phylogenetic distance observed among fungi and oomycetes, including both, morphological and physiological characteristics.

1. Fungi are haploid or dikaryotic during the major part of their lifecycle, whereas oomycetes are diploid and homologous recombination has not been found to occur. Oomycetes are far less tractable to genetic manipulation than many fungi.

2. Fungal hyphae are septate, whereas Oomycete hyphae are non-septate also called coenocytic mycelium.

3. Many Oomycetes are (partial) sterol auxotrophs. Their membranes contain lipids with unusual structures and long-chain fatty acids that presumably replace sterols in mycelial membranes. Stacked Golgi cisternae (versus unstacked in fungi) and, tubular mitochondrial cisternae (versus disclike in fungi).

4. Fungi and Oomycetes synthesize lysine by different pathways. The Oomycetes use the a, ε-diaminopimelic acid pathway, whereas fungi synthesize this amino acid by the α-aminoadipic acid pathway.

5. Oomycetes cell walls lack chitin but are composed of a mix of cellulosic compounds and glycans. In detail the cell wall consists mainly of 1,3-β-glucans, some 1,6-β-glucans and

1,4-β glucans (cellulose). Chitin, which is a major constituent of fungal cell walls, is detected in small amounts only in a few oomycetes (Bouwmeester *et al.,* 2009; Latijnhouwers *et al.,* 2003).

6. Most characteristic for oomycetes are the zoospores, the free-swimming asexual spores that are propelled by two unequal flagellae and explain why a moist environment is most favorable for these water molds. One of the flagella has lateral hairlike structures called mastigonemes that contain the β-1,3-glucan mycolaminarin, an energy storage molecule that is also found in brown algae and diatoms (Feofilova, 2001). Literally, oomycetes means 'egg fungi', a name based on the egg-shaped resting spores, named oospores. Oomycetes can be either homothallic or heterothallic. Sexual reproduction is initiated upon release of hormones that trigger the formation of gametangia (♀ oogonium and ♂ antheridium) in which meiosis takes place. The diploid oospores are produced as a result of oogamous fertilization when a haploid oosphere fuses to a haploid gamete. Thick-walled oospores are most durable propagules that can survive harsh environmental conditions and are important for the generation and maintenance of genetic variation in a population (Bouwmeester *et al.,* 2009). The role of the zoospores is the transmission of the pathogen from host to host. Zoospores are chemotactically and electrotactically attracted to the surface of potential host plants (Jung, 2007). Spores can also be moved to adjacent places by the wind, explaining their fast dissemination. They are also essential for targeting the site of infection (Figure 1.4-1.5) (Walker & West, 2007).

1.4 Economically relevant Oomycete species

Oomycete diseases occur on nearly every agricultural crop across the globe. Oomycetes include both saprophytes and pathogens of plants, insects, crustaceans, fish, vertebrate animals, and various microorganisms. Some of the most damaging oomycete genera are *Aphanomyces* (Diéguez-Uribeondo *et al.,* 2009), *Peronospora* (Cooke *et al.,* 2000), *Phytophthora* (Bouwmeester *et al.,* 2009), *Plasmopara* (Göker M *et al.,* 2006), *Pseudoperonospora* (Runge *et al.,* 2011*)*, and *Pythium* (André LéVesque & De Cock, 2004).

In particular, severe damages are provoked from the obligate biotrophs *P, viticola* (the agent of downy mildew of grapevine), *Albugo*, *Bremia*, and *Peronospora* species, which cause white rust and downy mildew on several crops. Other important pathogens include more than a hundred species of the genus *Pythium*, which are abundantly present in water and soil habitats and cause a diversity of plant diseases, mainly in root tissue.

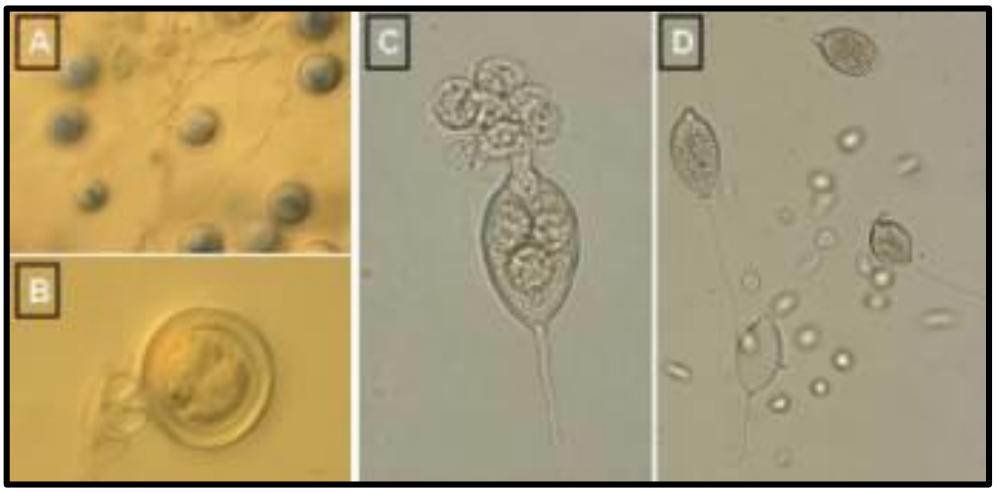


Figure 1.4: A) oospores; B) an oospore; C) a sporangium releasing zoospores; D) sporangia and zoospores.

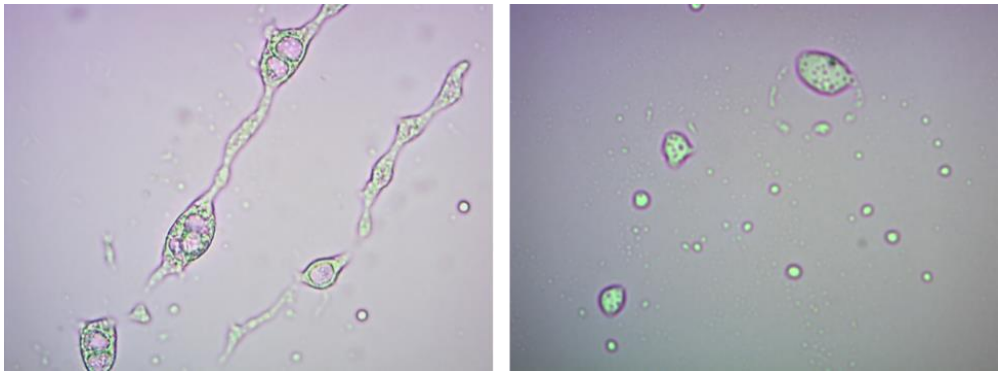


Figure 1.5: Mycelia disks (5mm diameter) of *P. nicotianae* were cut from the periphery of 4 to 6 days old cultures on V8 agar were transferred individually to the center of new plates. After different periods of incubation the plates were flooded with distilled water to induce the formation of sporangia (under microscope Primo Star Zeiss 100X). M. Maistrou observation

Pythium infections are usually limited to the meristematic tips, epidermis, cortex of roots, and fruits, but occasionally, severe *Pythium* infections occur when the pathogen moves deeper into plant tissue and reaches the vascular system. Some *Pythium* species, such as *Pythium oligandrum*, are essentially beneficial and reduce infections caused by more-severe pathogenic microbes. This can occur directly, through antagonistic effects or mycoparasitism, or indirectly, by induction of defense responses in plants (Sophien Kamoun, 2000, 2003). Most dangerous *Phytophthora* spp will be analyzed next paragraph.

Animal-pathogenic oomycetes, such as species in the genus *Saprolegnia*, can cause severe losses in aquaculture and fisheries (Bruno, D. W, & B. P. Wood. 1999). At least one

species, *Pythium insidiosum*, is known to infect various mammals, including humans, horses, and dogs. *P. insidiosum* colonizes cutaneous and subcutaneous tissues and can invade blood vessels and bones, resulting in fatal lesions (Ravishankar, J. P *et al.,* 2001). One genus, *Aphanomyces*, includes both plant and animal pathogenic species. A facultatively parasitic oomycete, *Lagenidium giganteum*, infects the larval stage of many mosquito species, and spore formulations of this oomycete have been used for biocontrol of mosquitoes (Woodring *et al.,* 1995).

1.5 Genus Phytophthora

There are more than 60 species of the genus of *Phytophthora that* are arguably the most devastating pathogens of dicotyledonous plants and cause enormous economic damage to important crop species such as potatoes, tomatoes, peppers, soybeans, and alfalfa, as well as environmental damage in natural ecosystems. Virtually every dicot plant is affected by one or more species of *Phytophthora*, and several monocot species are infected as well. (Erwin DC & Ribeiro OK,1996; Adhikari *et al.,* 2013).

The most notable pathogenic oomycete is *P. infestans*, well known for causing the disease that triggered the Irish potato famine in the mid-nineteenth century resulted in the potato blight famine (Figure 1.6) with death and displacement of millions of people (Sophien Kamoun, 2003). Today, *P. infestans* remains a devastating pathogen, causing losses as high as $5 billion in potato production worldwide through losses in potato and increased fungicide costs. The appearance of highly aggressive and fungicide- insensitive strains in North America and Europe in the 1990s resulted in a new wave of severe and destructive potato and tomato late-blight epidemics (Sophien Kamoun & Smart, 2005).

Other economically important *Phytophthora* diseases include root rot of soybean, caused by *P. sojae*; black pod of cocoa, a recurring threat to worldwide chocolate production, caused by *P. palmivora* and *P. megakarya*; dieback and related root rot diseases in crops and native plant communities, caused by *P. cinnamomi*; and sudden oak death, caused by *P. ramorum* (Sophien Kamoun, 2003).

1.5.1 Life cycle and reproduction of Phytophthora species

Phytophthora species are able to survive unsuitable environmental conditions over several years as dormant resting spores (oospores or chlamydospores) in the soil or in infected tissue. When environmental conditions become suitable (high soil moisture, soil

temperature > 10 °C) the resting spores germinate by forming sporangia which release motile, biflagellate (Jung, 2007; Hardham, 2007) In *Phytophthora*, infection generally starts when motile zoospores released from sporangia reach a leaf or root surface, encyst, and germinate (Sophien Kamoun, 2003).

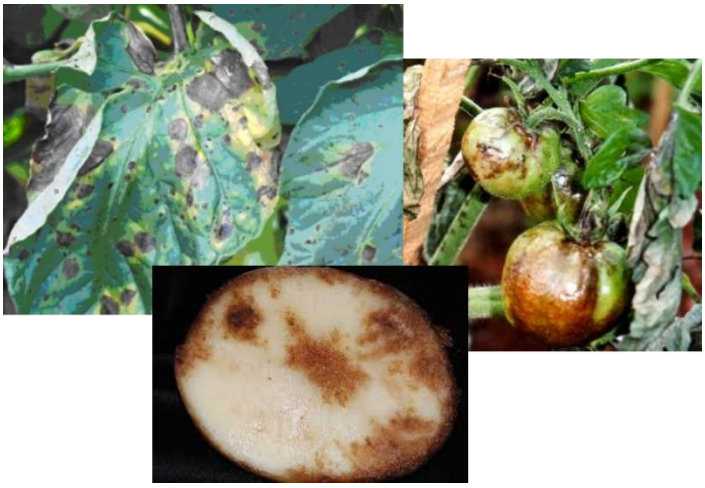
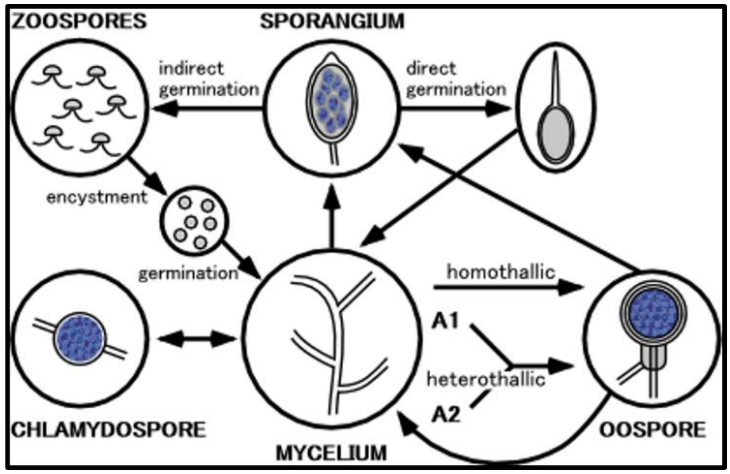


Figure 1.6: Late blight infection on tomato and potato plants. Lesion can be seen on the stem, on the foliage as well as the fruits.

The life cycle of *Phytophthora* includes sexual and asexual reproduction, with flagellated free-swimming spores. The mycelium of *Phytophthora* produces branched sporangiophores containing asexual sporangia at their tips. When the sporangium bursts, releases motile zoospores with two flagella, which is typical for heterokonts. Upon the contact with the host, zoospores become immotile and encyst, which leads to the formation of a cell wall. A germ tube protrudes from the cyst forming an appressorium that resembles a swollen tip of the germ tube. The appressorium penetrates the host tissue directly or enters via stomata in the leaf (Figure 1.7_B), and forms an infection vesicle. The mycelium then grows from the infection vesicle in between the plant cells, sometimes with haustoria, which is a mycelium that has penetrated the host cell wall and invaginated the plasma membrane (Grenville-Briggs *et al.*, 2008). Older infected plant cells die while the mycelium continues to spread into the host tissue (Agrios, 2005; Hardham, 2007). The sexual reproduction of Oomycetes results in thick walled resistant oospores (Figure 1.4_A). Oospores are formed when the male reproductive organ, the antheridium, fertilizes the female reproductive organ, the oogonium. For oospore formation in *P. infestans* to take place, strains of two mating types are needed, A1 and A2. It was not until recently that the A2 mating type emerged from

Mexico and spread in the rest of the world, thereby making possible the sexual reproduction of *Phytophthora*. This has lead to the emergence of more virulent strains due to genetic recombinations of pathogenic characteristics of the mating strains (Figure 1.7_A,) (Agrios, 2005).

A)



B)

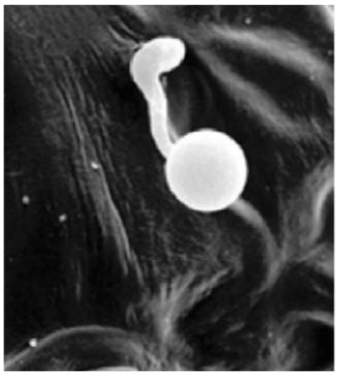


Figure 1.7: **A)** Life cycle of *P. infestans* with sexual and asexual sporulation. **B)** Germinated cyst of *P. infestans* with its appressorium on a potato leaf and (from Grenville-Briggs et al., 2008)

1.6 Oomycetes-plant communication

Like many other species that survive via dependent interaction with other organisms, oomycetes secrete a suite of proteins with functions including basic metabolic processes, nutrient acquisition, cell wall manufacture/adhesion/digestion, and virulence (Misner *et al.*, 2014). The definition of "pathogen effectors" is: molecules that manipulate host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defence responses (avirulence factors or elicitors) (Kamoun 2006). Over recent years, a number of oomycete genomes have been sequenced (Table 1.1), providing many information for studies of oomycete effectors, with important roles in pathogenicity mechanisms.

Phytophthora sojae	(Tyler *et al.* 2006)
Phytophthora ramorum	(Tyler *et al.* 2006)
Phytophthora infestans	(Haas *et al.* 2009)
Phytophthora capsici	(Lamour *et al.* 2012)
Pythium ultimum	(Lévesque *et al*. 2010)
Albugo laibachii	(Kemen *et al*. 2011)
Hyaloperonospora arabidopsidis	(Baxter *et al*. 2010),
Saprolegnia parasitica	(Jiang *et al.* 2013)

Table 1.1: oomycete sequenced genomes.

The secretome is defined as the sum of all proteins secreted by an organism often reflecting the niche that these microbes reside in, rather than there phylogenetic affinities (Soanes *et al.,* 2008). Analyses of the genomes identified a large repertoire of secreted proteins, known as effectors, during plant infection (Haas *et al.,* 2009; Lamour *et al.,* 2012; Lévesque *et al.,* 2010) such as pectinases, cutinases, protease inhibitors, *Nep*1-Like Proteins (NLPs), Crinklers (CRNs), elicitins, RXLR-effectors and many more (Haas *et al.,* 2009; Tyler *et al.,* 2006) associated with plant pathogenicity.

Effectors are secreted by the invading pathogen but their site of action in the host differentiates them into two main categories. Extracellular effectors remain in the apoplastic space where they interact with extracellular host molecules. Cytoplasmic effectors, on the other hand, move across the host cell's plasma membrane and function inside the plant cell (Figure 1.8) (Hardham & Cahill 2010).

Effectors are often under diversifying selection, as they are in a continuous co-evolution with factors from the host (Stahl & Bishop, 2000). Many oomycete effectors have been found in families with extensive gene duplication and gene loss, speeding up the process of diversifying selection. In this category, we can include: cell wall degrading enzymes, trypsin-like serine proteases, berberine-bridge enzymes, carbonic anhydrases, small cysteine-rich proteins, and repeat-containing proteins (Seidl, *et al.*,2012).

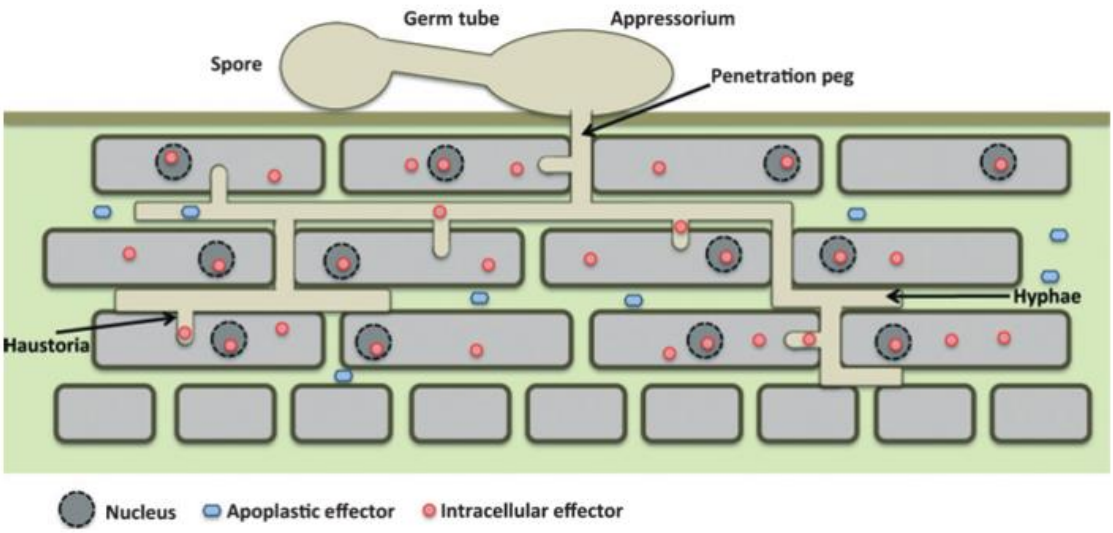


Figure 1.8: Role of effectors in fungal and oomycete colonization. Oomycete secrete effectors to facilitate host colonization. A subset of effectors localize and function in the apoplast. Other effectors are able to translocate into host cells and localize in diverse host compartments. These effectors have a variety of cellular targets and many contribute to colonization by modulating host defense machinery. Some effectors are delivered through the haustorium, a site of intimate interaction between pathogen and host formed by certain pathogens, while other effectors enter directly from the apoplast. Adapted from Kale, 2011 .

Despite considerable progress in the control strategies of plant disease, our global food supply is still threatened by a multitude of pathogens and pests. Plant diseases can dramatically reduce crop yield and the impact of disease outbreaks is particularly acute in developing nations. Pesticides provide effective protection but their applicability can be compromised by adverse environmental effects and by the emergence of resistant pathogen strains. For these reasons, much effort has been invested to understand innate resistance mechanisms in plants. Plants can activate a very effective arsenal of inducible defense responses, comprised of genetically programmed suicide of infected cells (the hypersensitive response, HR), as well as tissue reinforcement and toxin production at the site of infection (McDowell & Woffenden, 2003). Plants do not have the benefit of a circulating antibody system so plant cells autonomously maintain constant vigilance against pathogens by expressing large arrays of 'R genes' (R, resistance). R genes encode putative receptors that interact to the products of 'Avr genes' (Avr = avirulence) expressed by the pathogen during infection (Friedman & Baker, 2007; Jones & Dangl, 2006). In many cases, a single R gene can provide complete resistance to one or more strains of particular pathogen, when transferred to a previously susceptible plant of the same species (Figure 1.9).

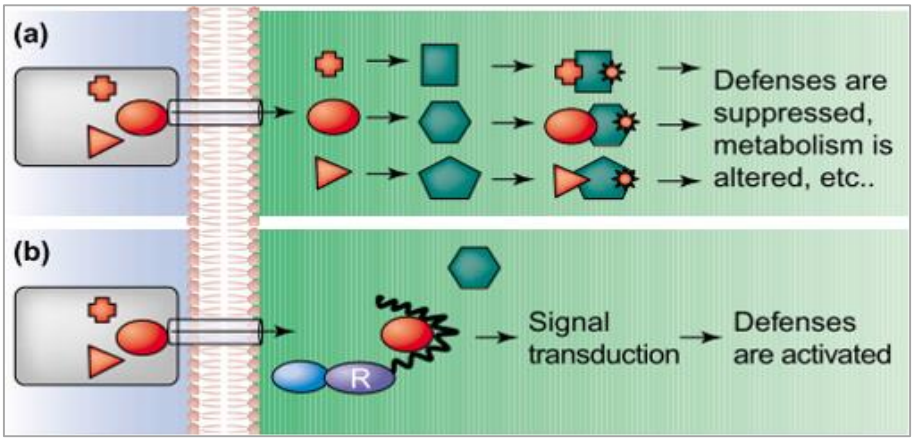


Figure 1.9: Interactions between pathogen Avr proteins and plant R proteins. A hypothetical pathogen (grey) has attached to a plant cell and is expressing virulence proteins (red). These proteins are translocate into the plant and once inside, they target host proteins (green) that control defense responses, metabolism or other plant process that affect pathogen virulence. (a) In this panel, the plant cell does not express an R protein that is capable of recognizing any virulence protein. Thus, the plant cannot detect the pathogen efficiently and defenses are weakly induced. We have the development of the disease. (b) This panel figure the classic receptor–elicitor hypothesis, in which an R protein directly binds a virulence protein. This recognition event activates a complex signal transduction network, which in turn triggers defense responses. Adapted by McDowell & Woffenden, 2003.

1.6.1 Cytoplasmic effectors

There are two groups of oomycete proteins that they could be categorised as cytoplasmic effectors. The first group is the RXLR effectors, which possess an RXLR motif (arginine–any amino acid–leucine–arginine) near their N-terminus that is involved in effector translocation into the host cytoplasm (Dou *et al*.2008; Whisson *et al.,* 2007). The second group is the CRN effectors (also known as the Crinkler effectors), so named because they cause leaf crinkling and necrosis (Stam, Jupe, et al., 2013). It has been reported that CRN deletion mutants identify a C-terminal domain that is sufficient to induce cell death when expressed in the plant cytoplasm (Haas et al., 2009; Sophien Kamoun, 2006; Oliva et al., 2010). These proteins have the LXLFLAK motif (leucine–any amino acid–phenylalanine–leucine–alanine–lysine) that is proposed to be involved in their movement into the host cytoplasm (Schornack *et al.,* 2010). Recent sequencing of *Phytophthora* genomes has revealed that both categories of effectors include large numbers of genes.

Analysis of the genomes of *P. infestans*, *P. sojae* and *P. ramorum* predict that they encode 563, 350 and 350 RXLR, and 196, 100 and 19 CRN effectors, respectively (Haas *et al.,* 2009; Tyler *et al.,* 2006). The large repertoires of predicted cytoplasmic effectors are indicative of their important roles during host infection. Precisely how oomycete cytoplasmic effectors are translocated into the host cytoplasm from the apoplast after

secretion by the pathogen is currently not known, although a mechanism involving plant endocytosis has been suggested (Birch *et al.,* 2009; Dou *et al.,* 2008). Both categories of effectors are modular proteins containing a conserved N-terminal region that includes a signal peptide that directs protein secretion and the RXLR or putative LXLFLAK motifs that direct uptake into the host cytoplasm, and a variable C-terminal domain (Haas *et al.,* 2009). However, although there are similarities in the uptake motifs, RXLR-mediated entry of oomycete effectors into plant cells is independent of any other pathogen component (Dou *et al.,* 2008) but the uptake of malarial parasite effectors is not.

1.7 Plant Immunity

The environment is full of dangerous microorganisms, the survival of higher eukaryotic organisms depends on efficient pathogen sensing and rapidly mounted defence responses. Such protective mechanisms are found in all multicellular organisms and are collectively referred as innate immunity. Though plants, in contrast to vertebrates, do not possess an adaptive immune system, their innate immune system effectively protects them from a wide range of different phytopathogenic microorganisms such as bacteria, viruses, fungi, oomycetes (Akira *et al.,*2006; Mazzotta & Kemmerling, 2011). Pathogenic microbes must access to plant tissues 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. Once the plant interior has been breached, microbes are faced with another obstacle: the plant cell wall, a rigid, cellulose-based support surrounding every cell. Penetration of the cell wall exposes the host plasma membrane to the microbe, where they encounter extracellular surface receptors that recognize "Pathogens or Microbe-Associated Molecular Patterns" (PAMPs or MAMPs) (Chisholm *et al.,* 2006)

Plants have evolved strategies for the perception of pathogens. First, on the cell surface, conserved microbial elicitors PAMPs, are recognized by receptor proteins called pattern recognition receptors (PRRs)(Goss *et al.,*2013). Plant PRRs are receptor-like kinases (RLKs) or receptor-like proteins (RLPs), which are localized at the plasma membrane and possess extracellular domain for ligand recognition. The major PRR types carry leucine rich repeats (LRR) or lysine motifs (LysM), while others can carry C-type lectin or EGF-like ectodomain (Trouvelot *et al.,* 2014).

Plants also respond to endogenous molecules released by pathogen invasion, such as cell wall or cuticular fragments called damage-associated molecular patterns (DAMPs). Stimulation of PRRs leads to PAMP-triggered immunity (PTI). The second class of

perception involves recognition by intracellular receptors of pathogen virulence molecules called effectors; this recognition induces effector-triggered immunity (ETI) (Figure 1.10) (Dodds & Rathjen, 2010).

1.7.1 Zig-Zag Model

For many years, view of the plant immune system was represented as a four-phased 'zigzag' model (Figure 1.10).

Phase 1: Plants recognize chemical elicitors, Microbe-Associated Molecular Patterns (MAMPS) derived from non-pathogenic microbes, Pathogen-Associated Molecular Patterns (PAMPS) derived from pathogens and Damage-Associated Molecular Patterns (DAMPS) that are produced by plants upon insect, herbivore or pathogen attack, via transmembrane Pattern Recognition Receptors (PRRs). The recognition leads to the onset of defense mechanisms referred to as pattern-triggered immunity (PTI).

Phase 2: successful pathogens deploy effectors that contribute to pathogen virulence. Effectors can interfere with PTI. This results in effector-triggered susceptibility (ETS).

Phase 3: a given effector is 'specifically recognized' by one of the NB-LRR proteins, resulting in effector-triggered immunity (ETI). Recognition is either indirect, or through direct NB-LRR recognition of an effector. ETI is an accelerated and amplified PTI response, resulting in disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site.

Phase 4: natural selection drives pathogens to avoid ETI either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress ETI. Natural selection results in new R specificities so that ETI can be triggered again (Jones & Dangl, 2006).

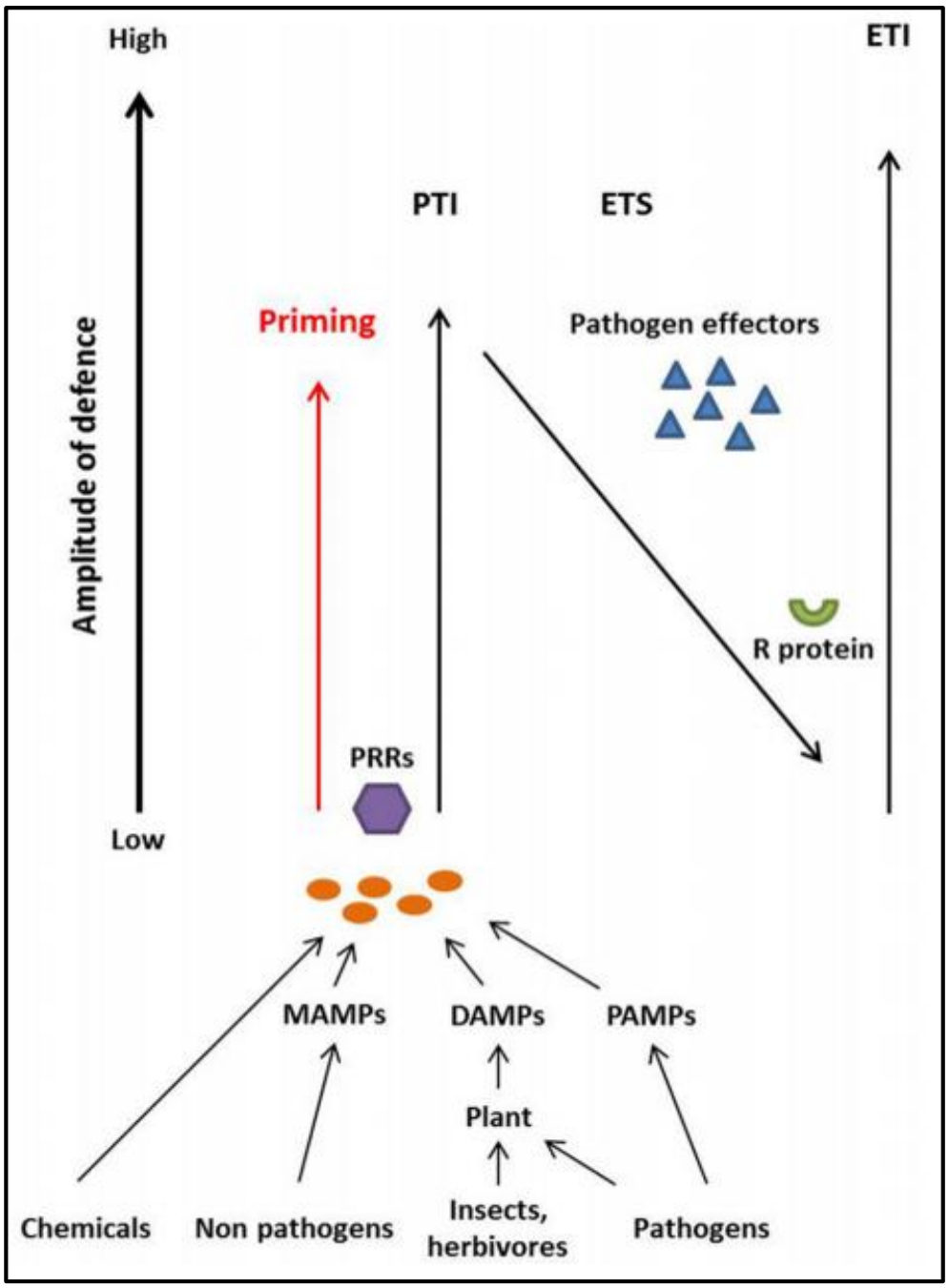


Figure 1.10: A zigzag model in oomycete plant interactions. Treatment of plants with elicitor compounds (chemicals, MAMPs, DAMPs, or PAMPs) in the absence of adapted pathogen leads to priming and/or PTI-based immunity that put plants into an alerted stage of defense that provides some enhanced resistance toward otherwise virulent pathogens via PRRs. The recognition leads to the onset of defense mechanisms referred to as pattern-triggered immunity (PTI). Adapted pathogens secrete effectors that disturb plant defense mechanisms leading to effector-triggered susceptibility (ETS). Plant resistance (R) proteins recognize pathogen effectors and induce effector-triggered immunity (ETI). Adapted from Wiesel et al., 2014.

1.7.2 Pathogens Elicitors

Three well-known PAMPs from bacteria are flagellin (Felix *et al.,* 1999), EF-Tu (Kunze *et al.,* 2004) and peptidoglycan (Willmann *et al.,* 2011). Flagellin is one of the 6 different proteins making up the flagellum, a long thin rotating helical filament used by mobile eubacteria for movement (Chevance & Hughes, 2008). A highly conserved N-terminal fragment of 22-amino acid, named flg22 (Felix *et al.,* 1999), is sufficient to trigger PTI in *A. thaliana* and other plant species. EF-Tu (elongation factor thermo unstable) is the most abundant bacterial protein, which is highly conserved and plays a central role in the elongation phase of protein synthesis in bacteria (Fu *et al.,* 2012). An N-terminal (N-acetylated) 18-amino acid domain of EF-Tu, named elf18, is recognized as a MAMP in *Brassicaceae spp*, but not in other tested plant families (Kunze *et al.,* 2004). Peptidoglycans are made up of strands of alternating N-acetylglucosamine and N-acetylmuramic acid residues linked by β-1-4 bonds (Vollmer *et al.,* 2008), and are only found in bacteria in which they are a major structural component of most bacterial cell walls. The best studied example of a fungal PAMP is chitin, a long linear homopolymer of β-1,4- linked N-acetylglucosamine, which is an essential structural component of the fungal cell wall (Sharp, 2013). Plants are able to recognize chitin, and fragments of four to ten N-acetylglucosamine residues are the most potent inducers of defense (Trouvelot *et al.,* 2014).

One of the first proteinaceous oomycete PAMPs to be identified is a Glycoprotein 42 (GP42) calcium dependent transglutaminase that functions in irreversible protein cross-linking and is abundant in the cell wall of the genus *Phytophthora* (Brunner *et al.,* 2002). Heptaglucoside fragments derived from branched 1,3-1,6-β-glucans, the main polysaccharide components of oomycete cell walls, which trigger defense responses in many Fabaceous plants (Hein, Gilroy, Armstrong, & Birch, 2009).

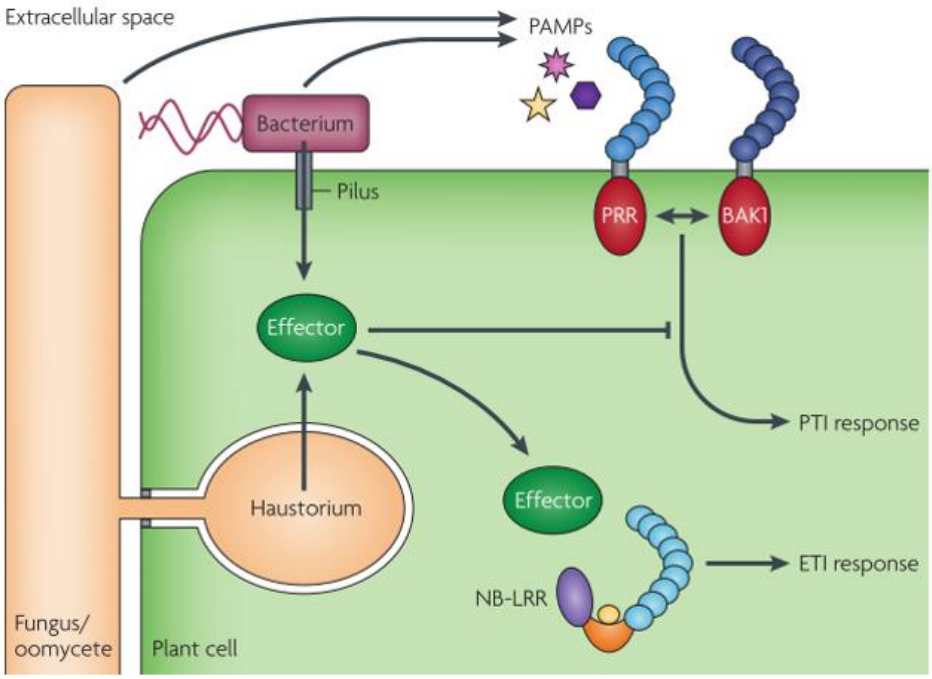


Figure 1.11: The plant immunity. Recognition of pathogen-associated molecular patterns (such as bacterial flagellin) by cell surface pattern recognition receptors (PRRs) promptly triggers PTI leading to basal immunity. Many PRRs interact with the related protein BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) to initiate the PTI signalling pathway. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress basal immune responses. Plant resistance proteins (such as NB-LRR) recognize effector activity and restore resistance through effector-triggered immune responses (ETI). Adapted from (Dodds *et al.* 2010).

1.7.3 Phytophthora Elicitors

The Pep-13 domain from the cell wall elicitor GP42 of *Phytophthora sojae* is required and sufficient to elicit MTI responses in parsley and potato (Brunner *et al.*, 2002). Elicitins, proteins with a sterol binding activity , which are able to elicits necrosis in the genus *Nicotiana* through induction of an HR (Takemoto, Hardham, & Jones, 2005). The *Phytophthora* cellulose-binding elicitor lectin (CBEL), which is thought to cause perturbation of the cell wall cellulose status, there by triggering necrosis and PTI in tobacco and *A. thaliana* (Dumas, Bottin, & Gaulin, 2008). Necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLP) are recognized in dicots and it has been shown that these proteins trigger a variety of defense responses in *A. thaliana* (Qutob *et al.*, 2006). Similarly, *P. infestans* INF1 elicitin causes an HR response in *Nicotiana benthamiana* (Sophien Kamoun, Huitema, & Vleeshouwers, 1999) that is dependent on the receptor-like kinase SERK3/BAK1 which, as a central regulator of innate immunity in plants, is required for

multiple resistance responses, including those mediated through FLS2 (Chaparro-garcia *et al.*, 2011).

1.7.4 Elicitors suppressing PTI

To suppress PTI during infection, *Phytophthora*, like other plant pathogens, secretes extracellular and intracellular effectors into plants. Some extracellular effectors encode protease or glucanase inhibitors to prevent, respectively, host protease or glucanase activity in the apoplast (Wiesel *et al.*, 2014). Intracellular effectors contain the canonical RXLR motif and contain an N-terminal signal peptide and a C-terminal effector activity site (Birch et al., 2009). The modes of action of RXLR effectors in promoting virulence are diverse. For example, it has recently been shown that the *P. infestans* RXLR effector PexRD2 interacts with the kinase domain of the host MAPKKKε to perturb PTI signaling pathways and to yield ETS responses (King *et al.*, 2014) . The RXLR effector PITG_03192, on the other hand, targets two membrane- associated NAC transcription factors that rapidly accumulate following PTI elicitation (Zheng et al., 2014). The effector prevents the release of these NAC transcription factors from the endoplasmic reticulum and subsequent accumulation in the plant nucleus that is typically observed as part of a PTI response. In contrast, the *P. infestans* RXLR effectorAvrblb2 prevents secretion of an immune-associated protease (Bozkurt *et al.*, 2011), whereas two *P. sojae* RXLRs have been shown to act as silencing suppressors (Qiao *et al.*, 2014). One of the best-characterized intracellular RXLR effectors is Avr3a from *P. infestans*. Avr3a interacts with, stabilizes the potato E3 ubiquitin ligase CMPG1, and thus perturbs cell death responses triggered by INF1 and a range of other pathogen elicitors. Avr3a exists in two forms that both suppress INF1 responses but differ in two amino acids that determine recognition by the potato R gene R3 that subsequently triggers ETI (Bos *et al.*, 2010). Finally, several RXLRs from *P. infestans* act redundantly to suppress flg22-mediated signal transduction and early transcriptional changes (Zheng *et al.*, 2014).

1.8 Plant Basal Defences

Disease is actually a relatively rare phenomenon in plants; the majority of plant species are resistant to infection by all isolates of any given microbial species. The ability of an entire plant species to resist infection by all isolates of a pathogen species is termed non-host (or species) resistance. This is the commonest form of disease resistance in plants, and the infrequent change in the range of host species colonised by plant pathogens is indicative of its stability (Ingle, Carstens, & Denby, 2006). Non-host resistance is thought to rely on both preformed barriers, such as the waxy cuticle and cell wall, which physically

impede the growth and spread of the potential pathogen, and on the induction of the basal defence system mounted in response to the recognition of non-self by the plant (Qutob *et al.,* 2006)

Induction of PTI in response to PAMPs or DAMPs occurs in both host and non-host plant species and is based on basal defense mechanisms. Studies of the effects of PAMPs and DAMPs point to a stereotypical response, indicating that signaling converges to a common defense response. This is exerted trough a time course of events following PRR activation.

1.8.1 Very Early Responses

Among the earliest and most easily recordable physiological responses to MAMPs and DAMPs in plant cell cultures, starting after a lag phase of ∼0.5–2 min, is an alkalinization of the growth medium due to changes of **ion fluxes** across the plasma membrane (Boller & Felix, 2009; Chisholm *et al.,* 2006). These changes include increased influx of H^+ and Ca^{2+} and a concomitant efflux of K^+; an efflux of anions, in particular of nitrate, has also been observed (Wendehenne *et al.* 2002). The ion fluxes lead to membrane depolarization. PAMPs and DAMPs are known to stimulate an influx of Ca^{2+} from the apoplast and cause a rapid increase in cytoplasmic Ca^{2+} concentrations, which might serve as second messenger to promote the opening of other membrane channels (Blume *et al.* 2000;Lecourieux *et al.* 2002), or to activate calcium-dependent protein kinases (Boudsocq *et al.* 2010).

Another very early response to PAMPs and DAMPs, with a lag phase of ∼2 min, is the **oxidative burst** (Mersmann *et al.,*2010). Reactive oxygen species can act as antibiotic agents directly or they may contribute indirectly to defense by causing cell wall crosslinking; in addition, reactive oxygen species may act as secondary stress signals to induce various defense responses. The oxidative burst is an immediate and localized reaction that is believed to have several roles in plant defense (Sharma *et al.,* 2012). The quantities of reactive oxygen species produced can be cytotoxic and thus are expected to be antimicrobial. Reactive oxygen species are thought to have direct (through cytotoxicity) and indirect (through signaling) roles in the plant cell death required for the HR. Reactive oxygen species induce the expression of defense related genes, and are implicated as second messengers that elicit other defense responses, including systemic acquired resistance (SAR) and the HR (Boller & Felix, 2009). SAR is the induction of defense mechanisms at locations remote from the original wound or infection site that serve to prepare the plant to defend itself against new attacks by pathogens. In addition, reactive oxygen species drive the rapid

peroxidase-mediated oxidative cross-linking of cell wall lignins, proteins, and Reactive oxygen species: metabolism, oxidative stress, and signal transduction, thereby reinforcing the wall against enzymatic maceration by the pathogen (Sharma *et al.*, 2012).

1.8.2 Early Responses

An early response to PAMP and DAMP signals is an **activation of Mitogen-Activated Protein Kinase (MAPK) cascades** . The MAPK phosphorylation cascade is a highly conserved signal transduction mechanism that plays a key role in regulating many aspects of growth and development in eukaryotes. In plants, MAPK cascades have been associated with hormonal, abiotic stress, and disease defense responses and with the regulation of the cell cycle (Tena *et al.* 2001). A MAPK cascade consists of a core module of three kinases that act in sequence: a MAPK kinase kinase (MAPKKK) that activates, via phosphorylation, a MAPK kinase (MAPKK), which activates a MAPK (Sheen *et al.*, 2007). Activated MAPKs phosphorylate a number of different target proteins; the majority of targets appear to be transcription factors, but other targets include various protein kinases, phospholipases, and cytoskeletal proteins, all of which effect changes in gene expression and/or physiological responses appropriate to the stimulus in question (King *et al.*, 2014).

Activation of MAPK is accompanied by **changes in protein phosphorylation**. Pulse-labeling of Arabidopsis cells with radioactive phosphate, followed by two-dimensional gel electrophoresis, revealed dozens of proteins that showed increased phosphorylation within minutes of flg22 stimulation (Boller & Felix, 2009). With the advent of technologies that allow large-scale analysis of phosphopeptides, a number of proteins showing elicitor-responsive phosphorylation could be directly identified and their phosphorylation sites determined (de la Fuente van Bentem *et al.*, 2006).

Gene activation. Treatment of Arabidopsis plants with flg22 and elf26 caused the induction of almost 1000 genes within 30 min and the downregulation of approximately 200 genes. The pattern of gene regulation in response to flg22 and elf26 is almost identical, indicating that signaling through FLS2 and EFR converges at an early step (Zipfel *et al.*, 2004, 2006). In fact, other MAMPs such as fungal chitin and endogenous elicitors such as OGA seem to induce a similar set of genes (Feng *et al.*, 2011; Ferrari *et al.*, 2007; Ramonell *et al.*, 2002), which suggests a stereotypical gene activation response to all MAMPs and DAMPs. Interestingly, among the induced genes, RLKs are overrepresented. FLS2 and EFR are included in the induced genes, indicating that one role of early gene induction is a positive feed- back to increase PRR perception capabilities (Zipfel *et al.*, 2006).

1.8.3 Late Responses

Callose deposition. Arabidopsis leaves treated with flg22 and fixed and stained with aniline blue, display a strong accumulation of fluorescent spots thought to represent callose deposits (Ellinger *et al.*, 2013). Although the biological foundation of this response is not clear, it has been used frequently, particularly to characterize pathogen effectors that interfere with MAMP signaling (Figure 1.12) (Chisholm *et al.*, 2006; Jones & Dangl, 2006).

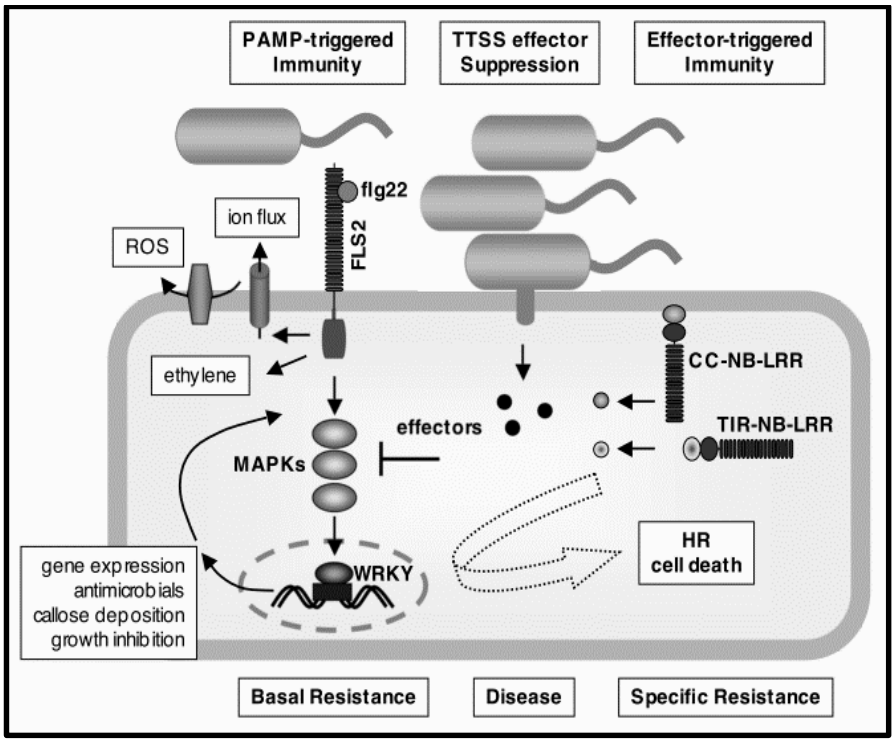


Figure 1.12: Plant immune responses. Perception of flagellin (flg22) by FLS2 elicits ion fluxes, the generation of reactive oxygen species (ROS), production of the stress hormone ethylene and activates a MAP kinase cascade. WRKY transcription factors mediate numerous changes in gene expression, including those encoding components of the flg22/FLS2 pathway itself. Phytopathogenic bacteria inject effector proteins via their type-III-secretion system (TTSS) into the cell where they exert suppressive functions on PAMP- triggered immunity. Additionally, plants use intracellular immune receptors (CC-NB-LRR or TIR-NB-LRR type) that recognize bacterial effector proteins in a plant-cultivar/pathogen-strain specific manner. Effector- triggered immunity appears to be a potentiation of PAMP-triggered responses leading to a rapid localized cell death (HR). Adapted from (Chinchilla *et al.*, 2007).

1.9 The plant cell wall is the first barrier against pathogens

The plant cell wall is an exoskeleton surrounding the external face of the plasma membrane that controls cell shape and allows high turgor pressures to develop. It is responsible for the shape of the cell, it plays a role as a reserve storage and in the intercellular transport control but also has a defence function (Carpita & Gibeaut, 1993).

It is composed of a highly integrated and structurally complex network rich of polysaccharides, including cellulose, hemicelluloses and pectin (Figure 1.13) (Tomassini, *et al.*,2009). It is the first barrier to invading organisms and many of the recognition events of the plant pathogen interactions occur at the cell wall level. Plants pathogens extensively invade the plant tissue cause the degradation of plant cell wall.

The plant cell wall is composed of cellulose fibrils which form an insoluble and inelastic crystalline material, interconnected with high molecular weight hemicellulose (typically xyloglucan or arabinoxylan) molecules.

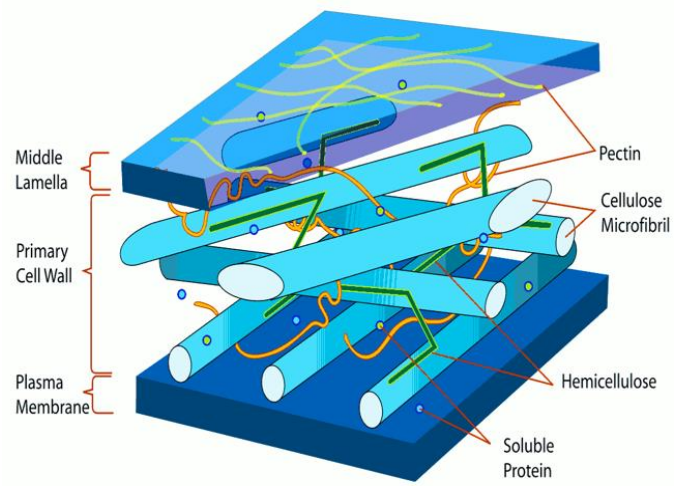


Figure 1. 13: *R*epresentation of the plant cell wall structure

These are embedded in a matrix of pectin, a term to indicate the galacturonic acid-rich fraction of the cell wall. The most abundant component of pectin is rhamnogalacturonan I (RGI), which consists of a backbone of alternating rhamnose and galacturonic acid with various side groups, principally galactans and arabinans. The three major components are homogalacturonan (HG), xylogalacturonan (XGA) and rhamnogalacturonan II (RGII) (Figure 1.14_A). Homogalacturonans are linear 1,4-linked α-D-galactopyranosyluronic acid chains, in which some of the residues carry methyl or acetyl groups (Peaucelle *et al.*, 2012).

Portions of carboxylic acid of pectins confer them the ability to bind calcium and other divalent cations by lateral association of two different chains in a structure known as

"egg- box" (Figure 1.14_B) (Peaucelle *et al.,* 2012). This characteristic structure is responsible for gel formation in the presence of calcium and gives to pectins a specific defence function in the cell wall.

Many pathogens release enzymes such as polygalacturonases and pectate lyases that degrade cell wall polysaccharides; some of their degradation products elicit defensive responses by plants (see below).

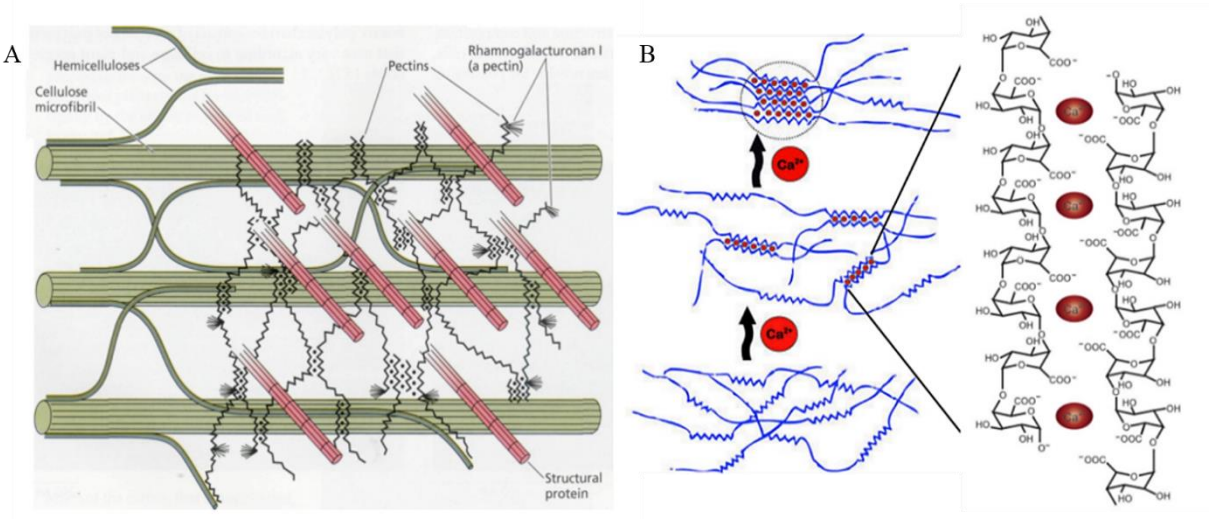


Figure 1.14: A) Schematic diagram of the major structural components of the primary cell wall and their likely arrangement. Cellulose microfibrils are coated with hemi- celluloses (such as xyloglucan), which may also cross-link the microfibrils to one another. Pectins form an interlocking matrix gel, perhaps interacting with structural proteins. Adapted from(Taiz 2010), **B) Egg-box model.** The "glue" effect of pectin is due to the presence of non-esterified homogalacturonan that forms calcium-mediated cross-links.

1.10 Cell Wall Degrading Enzymes (CWDEs) as apoplastic effectors

Recent research has focused on the elucidation of genomic DNA sequences of various species including animals, plants and microbes, which will contribute to genetic healing, increases in crop yields, fermentation, production of valuable substances and many other proposed benefits. The amount of data in fungal DNA databases is increasing, but the databases are sufficiently detailed at present to be utilized. DNA and amino acid sequences of CWDEs are extractable from published genomic DNA sequences by *in silico* comparisons with identified DNA and amino acid sequences.

The protein databases associated with cell wall modification, glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs) and carbohydrate esterases (CEs), are organized and can be accessed at the Carbohydrate-Active Enzymes database

(CAZY) website (http://www.cazy.org/). Out of more than 100,000 non-redundant entries in the database, GHs are at present classified into 133 families based on amino acid similarity (Takeda, 2014).

The ability to penetrate the formidable physical barrier of the plant cell wall is fundamental to successful pathogen invasion of plants and is facilitated by the secretion of CWDEs by the pathogen. Pathogen-encoded cell wall degrading enzymes may be considered to be effectors because they clearly manipulate plant structure and function to aid infection (Adrienne R. HardhamA, 2010). These extracellular effectors degrade a wide range of complex and cross-linked polysaccharides and glycoproteins. Pathogen CWDEs function not only in plant penetration but also in the release of nutrients for pathogen use (Blackman *et al.,* 2014). Many different microbial enzymes that catalyse the degradation of the cell wall components have been described (Donèche, 2002). In addition, some species produce different isozymes with specific activities. To degrade pectin, plant pathogens produce different types of pectinases during the infection process that are classified by their substrates and mode of action on the pectin polymer such as polygalacturonases (PGs), pectate lyases (PLs), pectin methylesterases (PMEs), and cellulases (Bellincampi, Cervone, & Lionetti, 2014). The first proof that CWDEs can be involved in pathogenesis was reported for the bacterium *E. chrysanthemi*. A directed mutation in *pel*B gene, encoding a pectate lyase resulted in a strain with reduced macerating capability. (Ried and Collmer, 1988).

1.10.1 Pectin Methyl Esterases (PMEs)

The activities of pectin methyl esterases from both plants and pathogens and the degree and pattern of pectin methyl esterification are critical for the outcome of plant–pathogen infections (Lionetti *et al.,* 2010). PMEs, which remove methyl esters from pectin (Figure 1.15), are controlled by PME inhibitor proteins (PMEIs) either during growth and development (Bellincampi *et al.,* 2014) and during plant–pathogen interactions (Lionetti, Cervone, & Bellincampi, 2012). The biochemical and structural bases of the enzyme/inhibitor interaction have been elucidated (A. Di Matteo *et al.,* 2005). *A. thaliana* over expressing PMEIs have a lower level of PME activity, a higher degree of pectin esterification and a concomitant reduced susceptibility to *B. cinerea* and *Pectobacterium carotovorum* (Lionetti *et al.,* 2007; Raiola *et al.,* 2011).

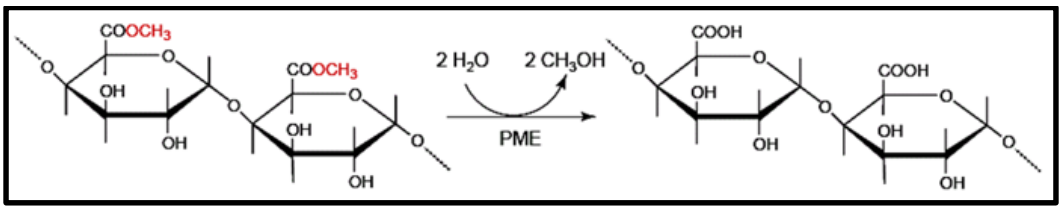


Figure 1.15: The methyl esterification of pectin is mainly controlled by pectin methyl esterases (PME). PME activity, which catalyzes the de-methyl esterification of the C6 linked methyl ester group of HG.

1.10.2 Pectate Lyase (PL)

Pectate Lyase, catalyse the eliminate cleavage of the de-esterified pectin, which is the major component of the primary cell wall of many plants. The backbone of the pectic polysaccharides is built of blocks of α-1,4 linked polygalactosyluronic acid and rhamnosyl residues. Cleavage by PL requires the presence of calcium ions and generates oligosaccharides with unsaturated galacturonosyl residues at their non-reducing ends (Figure 1.16) (Rondriguez *et al.,* 2002).

1.11 Polygalacturonase (PG)

Polygalacturonases play a critical role since their action on pectin makes other cell wall components accessible to other CWDEs. Consequently, as a strategy to optimize the action of CWDEs, PGs are often the first enzymes secreted by pathogens growing on the plant cell walls (Desiderio *et al.,* 1997). PGs in plants play important roles in processes such as growth, fruit softening, root formation, organ abscission and pollen development. They are poly [1,4-α-D-galacturonide] glycanohydrolases (EC 3.2.1.15) and they have the ability to hydrolyse the α-1,4-glycosidic linkages between galacturonic acids in homogalacturonans. Plant pathogens, produce two types of PGs *Exo*PGs and *Endo*PGs (Cook *et al.*, 1999). *Exo*PGs release monomers in a processive fashion from the nonreducing end of the substrate polymer. *Endo*PGs cleaves homogalacturonan by binding at random sites along the length of the substrate, catalysing the hydrolysis of a glycosidic linkage (Figure 1.16). The PGs then dissociates from the products of the reaction and is then available for another random cleavage of the substrate. This mode of cleavage results in the production of a mixture of oligomers, ranging in degree of polymerisation (DP) from monomers to the maximum DP of the starting polymer (Cervone *et al.*, 1989). Polygalacturonase genes and enzymes have been isolated and characterized from a number of prokaryotic and eukaryotic species. PGs exist in a variety of isoenzymatic forms that

differ in their stability, specific activity, pH optimum, substrate preference and types of oligosaccharides released such as molecular mass, pI, kinetic constants, mode of action, catalytic conserved residues, structural aspects and their interactions with inhibitors. (Niture, 2008).

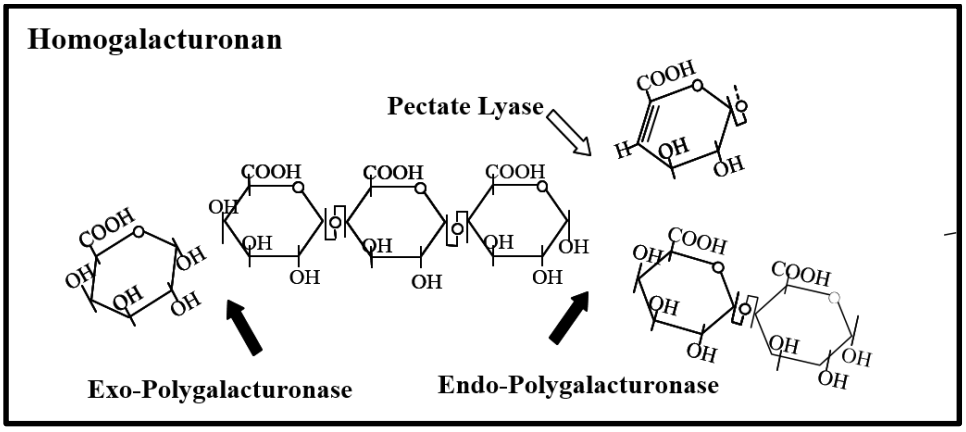


Figure 1.16: The enzymes hydrolyzing the pectic substancesare pectinases, including **EndoPolygalacturonase** (endoPG) that cleaves homogalacturonan by binding at random sites along the length of the substrate, **ExoPolygalacturonase** (exoPG) release monomers in a processive fashion from the nonreducing end and **Pectate Lyase** catalyse the eliminate cleavage of the de-esterified pectin.

1.11.1 Structure of Polygalacturonase

During the last few years, the structures of several polygalacturonase enzymes have been determined by x-ray crystallography. From the fungus *Aspergillus niger* (*An*PGII) (van Santen *et al.,* 1999), from the bacterium *Erwinia carotovora* (Pickersgill *et* al.,1998) and the PG from *Fusarium moniliforme* (*Fm*PG) (L Federici *et al.,* 2001). The sequence identity between *Fm*PG and *An*PGII is 43.5%, and the two proteins maintain a β-helix fold with the same number of turns, the same length and position of β-strands, and the same number and position of disulphide bridges. The two proteins are almost completely superimposable. The architecture consists of a right-handed parallel β-helix, resulting in the tandem repetition of 10 coils, each formed by three or four β-strands (Figure 1.17) (Federici *et al.,* 2001).

Sequence alignment analysis of more than 100 PGs analyzed including bacterial, fungal, oomycetes and plant enzymes confirmed that there are four strictly conserved sequence segments 178NTD, 201DD, 222GHG and 256RIK (according to the *A. niger* PG II sequence numbering). In the 178NTD conserved segment, Asn^{178} and Thr^{179} are strictly conserved residues. The second segment 201DD is also conserved in all PGs with specific

amino acid residues neighbouring at both sides of this dipeptide. The third segment 222GHG is also highly conserved: 95% conserved Gly^{222} followed by two very conserved His^{223} and Gly^{224}. The forth conserved segment 256RIK contains a highly conserved (87%) Ile^{257} in addition to the strictly conserved Lys^{258} and Arg^{256}(Federici *et al.*, 2001; Götesson *et al.*,2002; Palanivelu, 2006).

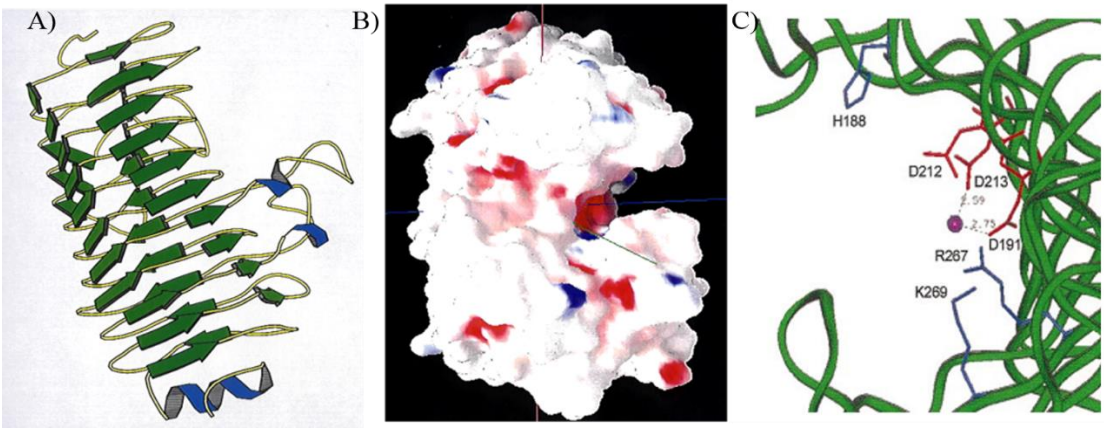


Figure 1.17: Structure of *Fm*PG. (A) MOLSCRIPT representation of the right-handed parallel β -helix, consisting of 10 coils each made up of three or four β–strands.(B) Electrostatic potential surface representation. The model is oriented to highlight the putative active site. Negative charges are shown in red, positive charges in blue. C) Overview of the active site of *Fm*PG. According to the proposed mechanism of action, three aspartic acids catalyze the reaction; D212 D191 and D213. K269 and R267 are necessary for substrate binding and are involved in binding to PGIP-2 together with H188.Adapted from Federici et al., 2001.

1.11.2 Polygalacturonase as virulence factor

Targeted mutagenesis of PG-encoding genes has demonstrated that PG is a virulence factor of several plant pathogenic fungi. Disruption of *Bc*PG1 (*Botrytis cinerea* polygalacturonase 1) reduces the virulence of *B. cinerea* on host plants (Have *et al.*, 1998) Mutation in the PG gene reduced the ability of *Alternaria citri* to cause black rot symptoms in citrus and the maceration of potato tissues (Isshiki *et al.*, 2001). Gene replacement mutants of *Claviceps purpurea*, which lack PG activity, are nearly nonpathogenic in rye (Volpi *et al.*, 2013) .

However, genetic evidence obtained by targeted inactivation does not always support an essential role of PG in fungal pathogenicity. Disruption of *en*pg-1, which encodes the major extracellular PG produced by *Cryphonectria parasitica* in culture, demonstrated that this *endo*PG is not required for the expression of *C. parasitica* virulence in American chestnut stems (Gao, Choi, & Shain, 1996). In *Cochliobolus carbonum*, targeted inactivation of both the *Exo*- and *Endo*PG genes resulted in a nearly complete loss of PG activity.

However, the double mutant was still pathogenic in maize (Scott-craig *et al.,* 1998). Disruption of pg5, which encodes *Endo*PG, has no detectable effect on the virulence of *Fusarium oxysporum* f. sp. *lycopersici* in tomato (García-Maceira *et al.,* 2001). A possible explanation for these results is the presence of multigene families of *Endo*PG in fungi, each gene performing defined biological tasks in the infection process. Studies indicated that *B. cinerea*, that cause gray mold, contains a family of 6 PG genes that are differentially expressed during interaction with plants and when grown in different carbon sources (Donèche, 2002). *Bc*PG recombinant proteins heterologously expressed by *Pichia pastoris* differ in several aspects, including specific activity, substrate preference, and mode of action. In addition, analysis by infiltration of recombinant proteins indicated the differential activity of these *Bc*PG in causing necrosis in plants (Kars *et al.*, 2005). *Sclerotinia sclerotiorum* secrets a set of *endo*PG that differ in their catalytic properties and expression patterns. Of these *endo*PG, *ss*pg1 (*S. sclerotiorum* polygalacturonase 1), *ss*pg2, and *ss*pg3 are up-regulated during colonization in healthy plant tissues, *ss*pg5 was induced in the final phase of maceration, and *ss*pg6 and *ss*pg7 were expressed constitutively throughout the infection process (Kasza *et al.*, 2004; Li *et al.*, 2004). As well, *Chondrostereum purpureum*, which causes silvery leaf disease on apple, contains a multigene family of *endo*PG and is the first basidiomycete reported to have an *endo*PG gene family (Williams & Benen, 2002).

1.11.3 Role of PGs

The ability of a plant to respond defensively against an invading pathogen depends on its perception (recognition) of the pathogen. This information then must be transmitted from the infected cells to adjacent plant cells. PGs are thought to be important during the early stages of plant pathogenesis, thus acting as pathogenicity factors, is that these enzymes spread through the host tissue in advance of the invading fungal mycelia. This results in hydrolysis of the pectic components present in the primary plant cell walls and in the middle lamellas, leading to cell wall degradation and tissue maceration (Mertens & Bowman, 2011). The degradation of the plant cell wall also provides the fungus with a nutrient source needed for growth of the invading fungus (Talbot, 2010). EndoPGs initiate the production of elicitors for signal transduction known as oligogalacturonides (OG) from degradation of homogalacturonan polymer of pectin (Cervone *et al.*, 1987, 1989). Degradation of pectin by endoPGs in the presence of PGIPs gives rise to the production of elicitor-active OGAs of 10-15 residues in size. EndoPGs are potential signalling molecules that elicit plant defense responses. Phytoalexin and proteinase inhibitor production β-1,3- glucanase production,

synthesis of PR proteins, lignin synthesis and necrosis are examples of defense responses elicited in plant tissue by fungal PGs (Ferrari *et al.*, 2013).

1.11.4 PGs in Phytophthora spp.

Phytophthora genomes typically contain large multigene families encoding Polygalacturonase (Götesson *et al.*, 2002; Wu *et al.*, 2008). *P. infestans* encodes an endopolygalacturonase gene and it was one of the first putative virulence genes of *Phytophthora* identified using EST databases.(Torto, Rauser, & Kamoun, 2002). *Phytophthora cinnamomi* contains a large *endo*PG gene family with 19 members(Götesson, *et al.* 2002). Not much is known, however, about the proteins encoded by each of these genes. Also *Phytophthora parasitica* contain a family of 10 members of PGs which they have been cloned and analyzed finding that they encode functional *endo*PG and they have distinct biological functions in planta and some are induced during plant infection (Wu *et al.*, 2008; Yan & Liou, 2005).

1.12 Polygalacturonase inhibiting proteins (PGIPs) and their role in plant defense

Plants have developed different systems to defend themselves from pathogens. When phytopatogenic fungi secrete PG to breech the cell wall during the early stages of infection, in the wall is present a leucine rich repeat protein (LRR) which inhibits PG activity. This protein, called polygalacturonase inhibiting protein (PGIP), interacts with PG, slows down its pectin degrading activity and promotes the formation of active elicitors (OGs) (Aziz, Heyraud, & Lambert, 2004). The occurrence of PGIP has been reported in a variety of dicotyledonous plants and in the pectin–rich monocotyledonous plants such as onion and leek (Lorenzo, Ovidio, & Cervone, 2001).

The structure of the isoform 2 of *Phaseolus vulgaris* PGIP (*Pv*PGIP2) has been solved (Di Matteo *et al.*, 2003) showing that the central LRR domain consists of a set of 10 tandemly repeating units, each derived from modification of a 24-amino acids leucine-rich peptide. The LRR element matches the consensus GxIPxxLxxLxxLxxLxLxxNxLx and has regularly spaced Leu residues and conserved Gly, Pro, and Asn in the 1st, 4th and 21st position (De Lorenzo *et al.*, 1994). A long parallel β- sheet (B1) occupies the concave inner side of the structure. The β-sheet B1 corresponds to the predicted β-sheet where the residues

determining the affinity and specificity of PGIP2 are known to reside (Leckie *et al.*, 1999). An additional extended parallel β-sheet (B2) characterizes the fold of PGIP2 (Figure 1.18).

Figure 1.18: a) Representation of the crystal structure of PGIP2 LRR sequence of PGIP2. A) signal peptide, B) N-terminal of the mature protein,C) Modules LRR, D) C-terminal. The frame indicates the presumed region involved in the interaction with the PG. Di Matteo *et al.*, 2003

Plants have evolved different PGIPs with specific recognition capabilities against the many PGs produced by fungi (De Lorenzo *et al.*, 2001). Consistent with their role in defence, PGIPs are constitutively expressed at low levels and their expression is induced in response to several stress stimuli. Interestingly, oligogalacturonides are capable of inducing PGIP expression, suggesting the existence of a feed-forward mechanism for the accumulation of oligogalacturonides (Ferrari *et al.*, 2013).

In many cases it has been proven that the PGIP has a direct role in plant defence, transgenic *Arabidopsis* plant overexpressing PGIPs exhibit enhanced resistance to *B. cinerea*(Ferrari *et al.*, 2003a) . Like many other defence proteins, PGIPs are encoded by gene families and in *Arabidopsis* two tandemly duplicated genes (*At*PGIP1 and *At*PGIP2) are coordinately upregulated in response to *B. cinerea* infection, through separate signal transduction pathways(Ferrari *et al.*, 2003b). In many plants, PGIP is constitutively present and ready to interact with PGs to form active elicitors.

1.13 PG-PGIP interaction

The PG-PGIP interaction is considered a paradigm that describes some of the recognition events determining plant immunity (Federici *et al.*, 2006). A low resolution structure of the complex formed by *Pv*PGIP2 and PG from *Fusarium phyllophilum* (*Fp*PG) and *Collecotrichum lupine* Polygalacturonase 1(*Clu*PG1) and PvPGIP2 was solved by

Small-Angle X-ray Scattering (SAXS)(Benedetti *et al.*, 2011; 2013). This allowed to pinpoint the residues involved in the *Fp*PG- *Pv*PGIP2 interaction and explained the competitive inhibition played by the inhibitor on *Fp*PG. Furthermore it has been shown that a few PGIP residues, sometimes only one, are critical for a stable PG-PGIP interaction (Casasoli *et al.*, 2009; D'Ovidio *et al.*,2004).

1.14 Oligogalacturonides

Well studied plant- derived elicitors include oligogalacturonides (OGs), which are structural components of plant cell walls and are released upon partial degradation of homogalacturonan by microbial polygalacturonases during infection or by plant polygalacturonases induced upon wounding (Ferrari *et al.*, 2013). When the polygalacturonase inhibiting proteins modulate the activity of the polyagalcturonase, the OGs are produced (Figure 1.19). OGs are considered the classic examples of DAMPs that are generated by the host cell during the infection process(De Lorenzo *et al.*, 2011; Galletti *et al.*, 2009).

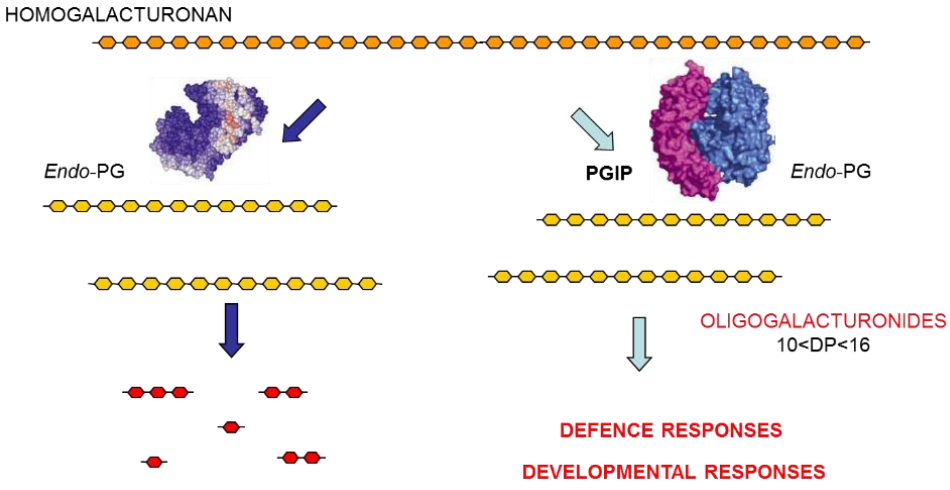


Figure 1. 19: Model for the OG accumulation during pathogen infection.

1.14.1 Oligogalacturonide-induced responses involved in plant defense

Plant cell wall- derived OGs are recognized by wall-associated kinase 1 (WAK1) receptor-like kinases, with an extracellular domain containing epidermal growth factor motifs, a transmembrane domain and an intracellular Ser/Thr kinase domain (Anderson et al., 2001) and subsequent signaling is jasmonic acid (JA) salicylic acid (SA) and ethylene (ET) pathways independent (Brutus *et al.*,2010; Ferrari *et al.*, 2013). A number of different

biological responses to OGs have been reported, and the particular response observed depends on the plant species, the bioassay, and the chemical structure of the OG used (Pogorelko *et al.,* 2013).

One of the earliest events occurring upon DAMP or MAMP perception is the phosphorylation of mitogen-activated protein kinases (MAPKs) (Droillard *et al.,* 2002). A MAP kinase cascade is triggered upon OG perception in *A. thaliana,*and MPK3 and MPK6 are phosphorylated. However, the importance of these signaling events remains elusive and it has been shown, for example, that lack of MPK3 increases basal susceptibility to *Botrytis cinerea* but elicitor-induced resistances are not affected. In contrast, MPK6 is necessary for OG-induced resistance but does not play a role in basal resistance toward *B. cinerea* (Galletti, Ferrari, & De Lorenzo, 2011).

Transcript profiling of seedlings treated with either OGs or flg22, indicates an extensive overlap of responses, at least at the early times after treatment(Denoux *et al.,* 2010). Both OGs and flg22 trigger a robust oxidative burst mediated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase *At*RbohD, which is at least partially responsible for the subsequent production of callose(Galletti et al., 2008; Jie Zhang et al., 2007) by the callose synthase POWDERY MILDEWRESISTANT4(Nishimura, 2003).

OGs are not only involved in defence but also in plant growth and development. One of the first described effects, the induction of tomato fruit ripening through the induction of ethylene, was later shown to be mediated by OGs in the size range of DP 4–6 and not 10–15 (Simpson *et al.,* 1998) Auxins, and in particular indole-3-acetic acid (IAA), are crucial for plant growth and development (Leyser, 2002). OGs affects plant growth and development in an antagonistic manner to that of the auxin (Spiro, Bowers, & Cosgrove, 2002) recently, have been subsequently shown to inhibit auxin-induced root formation in tobacco and *Arabidopsis* leaf explants as well as in thin cell-layer explants (Ferrari *et al.,* 2013; Savatin *et al.,* 2014).

A model of PGIP as a component of the cell surface signalling system that leads to the formation of elicitor-active oligogalacturonides is presented in (Figure 1.20). In this model it is shown that PGIP not only inhibits PGs activity by slowing down pathogen penetration, but also avoids the depolymerization of homogalacturonan allowing the formation of OGs with a degree of polymerization between 10 and 15. These OGs belong to the class of non-specific elicitors and induce defence responses in many plant species and genotypes (Ferrari *et al.,* 2013).

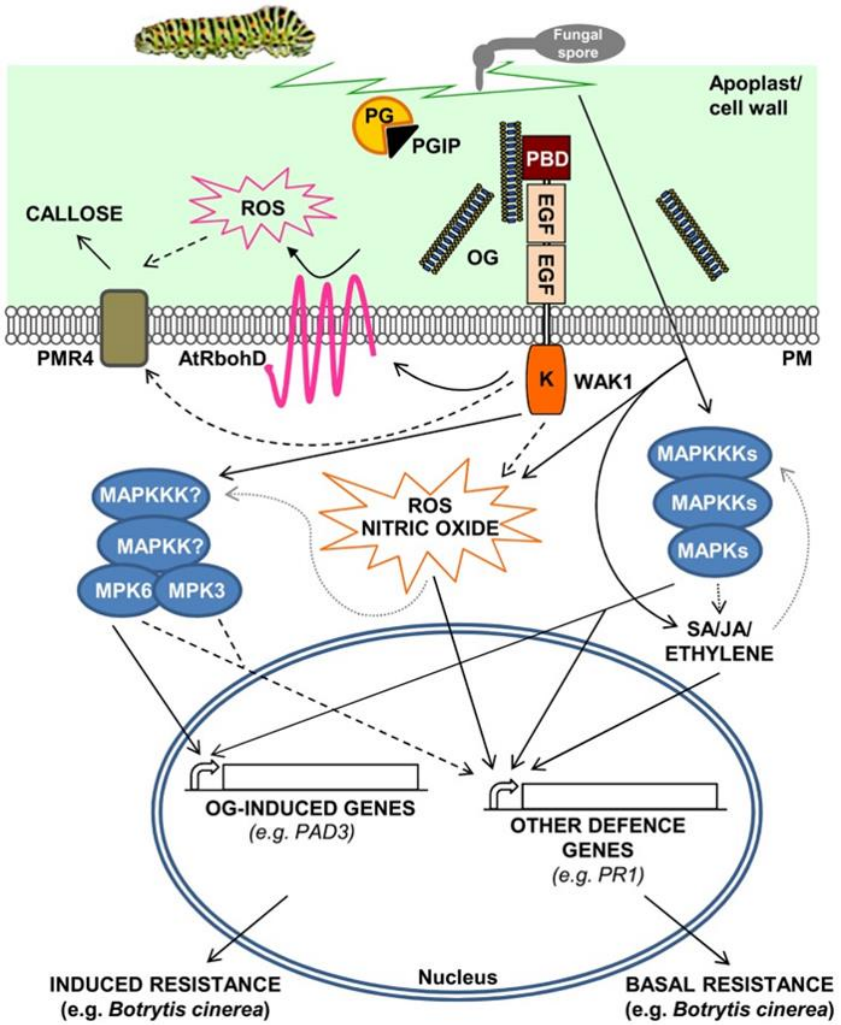


Figure 1.20: Model for the activation of plant defense responses triggered by oligogalacturonides (OGs). OGs are released from the cell wall after degradation of homogalacturonan by mechanical damage or by the action of hydrolytic enzymes such as PGs, secreted by pathogens. PGIPs in the apoplast modulate PG activity, favoring the accumulation of elicitor-active OGs, which function as DAMPs. OGs are perceived byWAK1 and trigger defense responses such as ROS accumulation through the activation of the NADPH oxidase AtRbohD, nitric oxide production, callose deposition, and MAPK-mediated activation of defense gene expression. Pathogen invasion or mechanical damage also cause an increase of JA, SA, and ethylene levels, mediated by MAPK cascades, triggering defense responses independently of OGs. DAMP- and hormone-mediated defense responses result, respectively, in induced and basal resistance toward necrotrophic pathogens, such as Botrytis cinerea. Dashed lines indicate hypothetical cascades; dotted gray lines indicate oversimplification of the complex and still partially uncharacterized roles of MAPKs in the regulation of hormone and ROS synthesis/response Ferrari *et al.*, 2013.

1.15 Reverse Genetics

1.15.1 Gene silencing

Since oomycetes have not been traditional experimental models, tools for their functional genomics are still at an early stage although reasonably efficient gene transfer procedures are established The increasing amount of sequence and data available for *Phytophthora spp*.(Haas *et al.,* 2009; Lamour *et al.,* 2012) prompts the development of genetic tools to explore the functions of novel genes that are identified. Nowadays, with more than 100 species recognizes and with destructive diseases caused on thousands of plant species, *Phytophthora* remains an active subject of research and a nagging problem to farmers and growers(Kamoun, 2000; Kroon *et al.,* 2012).

RNA silencing emerged to be an important approach for down regulation of target genes in *Phytophthora*. Internuclear gene silencing was reported in *P. infestans* (West *et al.,* 2008; West *et al.,* 1999). The introductions of sense, antisense, and hairpin constructs were all subsequently confirmed to induce gene silencing, enabling gene function studies in *Phytophthora* (Ah-Fong *et al.,* 2008). Using *inf1* as a target, Ah-Fong *et al.*, (2008) compared three method including PEG treatment of protoplasts, zoospore electroporation, and microprojectile bombardment and they found that hairpin vectors combined with protoplast transformation was the highest including methods for transient and stable gene silencing (knock-down). Transformation and gene silencing has been successful also for other of *Phytophthora spp*. *P. infestans* (Ah-Fong e*t al.,* 2008; Judelson & Brett, 1993.), *P. sojae* (Qutob, Kamoun, & Gijzen, 2002), *P. palmivora* (Vijn & Govers, 2003), and P. *parasitica* and *P.capsici* (Bailey, Mena, & Herrera-estrella, 1991) *P. cinnamomi* (Mitter *et al.,* 2011).

Methods for reverse genetics involving gene disruption are not currently feasible in oomycetes, due to low rates of homologous recombination during transformation and the diploid nature of the vegetative stage. Ttransformants exhibited only a partial knock-down they are a lack of being stable or inheritable characters (Ah-Fong *et al.,* 2008). Another disadvantage of gene knock-down is that, as it requires only a short sequence, genes other than those targeted might be silenced. This causes unexpected changes in gene expression patterns (off- target effects). Testing for the possibility of off-target effects is simpler for phytopathogen species for which complete genome sequence data are available but remains elusive for those phytopathogens whose genomes have not been sequenced (Bhadauria *et al.,* 2009)

1.15.2 CRISPR-Cas9

Technology based on the prokaryotic CRISPR (clustered regularly interspersed short palindromic repeats)-Cas9 system is completely revolutionizing genome engineering. During the last few years, CRISPR-Cas9 editing has been successfully employed in a multitude of organisms. CRISPR-Cas is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. The CRISPR/Cas systems are divided into three main types (I, II and III) on the basis of the identity and organisation of genes within a Cas locus. (Reeks, Naismith, & White, 2013) RNAs and a distinctive array of repetitive elements (direct repeats). These repeats are interspaced by short variable sequences derived from exogenous DNA targets known as protospacers, and together they constitute the CRISPR RNA (crRNA) array. Within the DNA target, each protospacer is always associated with a protospacer adjacent motif (PAM), which can vary depending on the specific CRISPR system (Figure 1.21).

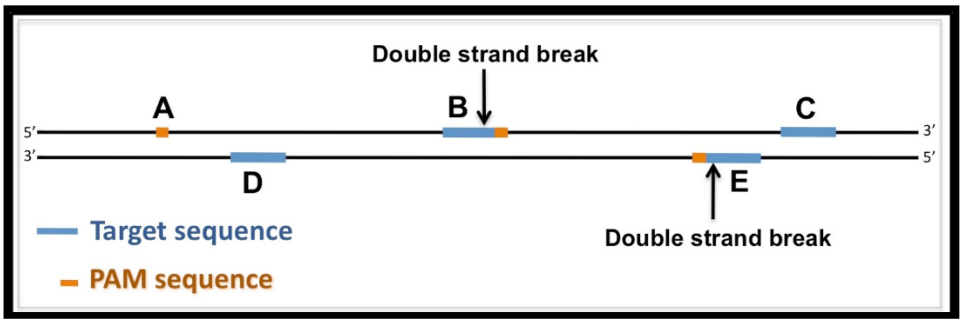


Figure 1. 21: For Cas9 to successfully bind to DNA, the target sequence in the genomic DNA must be complementary to the gRNA sequence and must be immediately followed by the correct protospacer adjacent motif or PAM sequence. The PAM sequence is present in the DNA target sequence but not in the gRNA sequence. Any DNA sequence with the correct target sequence followed by the PAM sequence will be bound by n the figure, the target sequence is followed by the PAM sequence at two separate locations (B and E). Cas9 will only cut at B and E. The presence of the target sequence without the PAM following it (C and D) is not sufficient for Cas9 to cut. The presence of the PAM sequence alone (A) is not sufficient for Cas9 to cut. *www.addegene.org*

The Type II CRISPR system is one of the best characterized (Chylinski *et al*., 2014), consisting of the nuclease Cas9. The crRNA array that encodes the guide RNAs and a required auxiliary trans-activating crRNA (tracrRNA) that facilitates the processing of the crRNA array into discrete units. Each crRNA unit then contains a 20-nt guide sequence and a partial direct repeat, where the former directs Cas9 to a 20-bp DNA target via Watson-Crick base pairing.(F Ann Ran, *et. al*, 2013) In nature, the Cas9 system requires three components: 1) an RNA (~20 bases) that contains a region complementary to the target

sequence (crRNA); 2) an RNA that contains a region complementary to the crRNA (tracrRNA); and 3) Cas9, the enzymatic protein component in this complex. A single guide RNA (gRNA) can be constructed to serve the roles of the base-paired crRNA and tracrRNA. The gRNA/protein complex can scan the genome and catalyze a double strand break at regions that are complementary to the crRNA/gRNA (Chhabra, 2014; F Ann Ran *et al.*, 2013; Gaj *et al.*, 2013) (Figure 1.22).

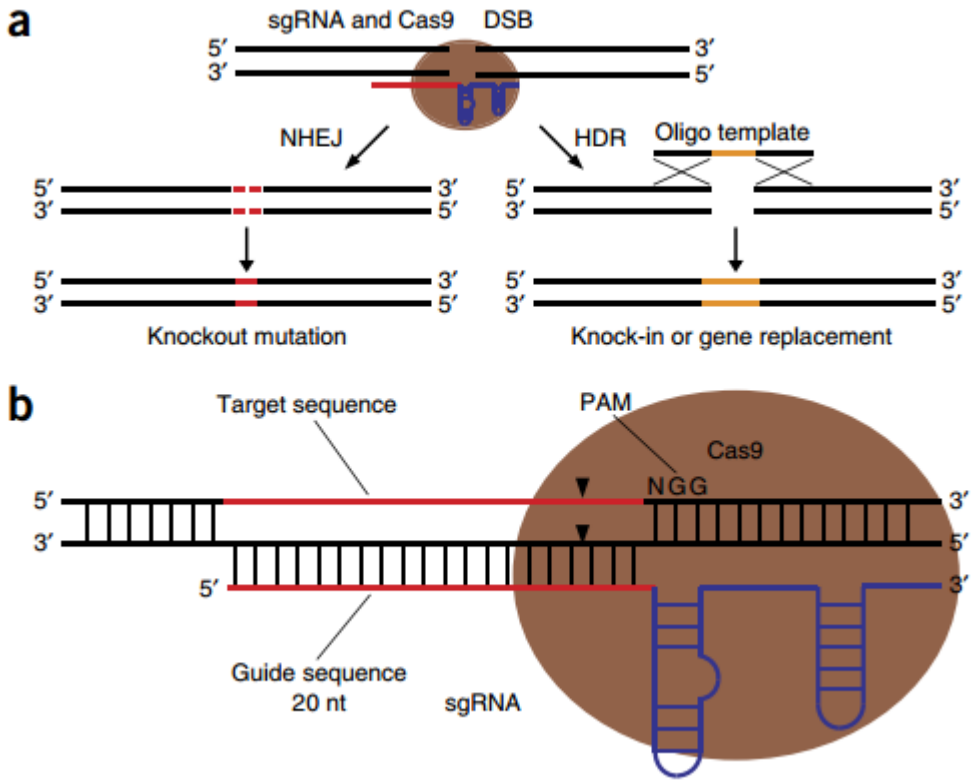


Figure 1. 22: a)The gRNA/Cas9 complex is recruited to the target sequence by the base pairing between the gRNA sequence and the complement to the target sequence in the genomic DNA. For successful binding of Cas9, the genomic target sequence must also contain the correct Protospacer Adjacent Motiff (PAM) sequence immediately following the target sequence. The binding of the gRNA/Cas9 complex localizes the Cas9 to the genomic target sequence so that the wild-type Cas9 can cut both strands of DNA causing a Double Strand Break (DSB). A DSB can be repaired through one of two general repair pathways: the Non-Homologous End Joining (NHEJ) DNA repair pathway or the Homology Directed Repair (HDR) pathway. (b) Diagrams illustrating the CRISPR/Cas system composed of Cas9 (brown) and sgRNA (red and blue). The secondary structure of sgRNA mimics that of the crRNA-tracrRNA heteroduplex. The Cas9 HNH and RuvC-like domains each cleave one strand of the sequence targeted by the sgRNA, provided that the correct protospacer-adjacent motif sequence (PAM) is present at the 3′ end. Figure from Shan *et al.*, 2014.

Understanding the complexities of host-pathogen interactions is greatly facilitated by the ability to manipulate host and pathogen genomes by gene disruption or by insertion of genes with new or enhanced functions. Based on the prokaryotic CRISPR (clustered regularly interspersed short palindromic repeats)-Cas9 system which is completely revolutionizing genome engineering. During the past year, CRISPR-Cas9 editing has been implemented in a multitude of model organisms and cell types. While manipulation of the *Phytophthora* genome has been possible for some time, the processes to achieve modifications are not rapid, easy, or routine. Here we report the use of the CRISPR-Cas9 system in *P.capsici* to knock out target genes. This system never have been used in *Phytophthora* genus and it can be a great opportunity to use this system for studying the *Phytophthora* cytoplasmatic or apoplastic effectors repertoires.

2. AIM OF THE THESIS

Plants constantly encounter a diverse range of microorganisms that are potential pathogens, and they have evolved multi-faceted physical and chemical strategies to inhibit pathogen ingress and establishment of disease. Microbes, however, have developed their own strategies to counteract plant defence responses. Recent research on plant–microbe interactions has revealed that an important part of the infection strategies of a diverse range of plant pathogens, including bacteria, fungi and oomycetes, is the production of effector proteins that are secreted by the pathogen and promote a successful infection by manipulating plant structure and metabolism, including plant defense mechanisms(Adrienne R. HardhamA, 2010). *Phytophthora* species are eliciting a growing interest for their considerable economical and environmental impact. Both the initial penetration of the plant surface and the subsequent colonization of plant tissues are facilitated by the action of a range of degradative enzymes (CWDE) that are synthesized and secreted by the invading pathogen. *Phytophthora* genomes typically contain large multigene families encoding CWDEs (Götesson *et al.*, 2002; Wu *et al.*, 2008).

The characterization of the repertoire of genes required for the degradation of the plant cell wall secreted by *Phytophthora spp* is an important area of research for deciphering molecular mechanisms responsible for host plants colonization and infection. Most of the species of *Phytophthora* appear to produce multiple genes families encoding Polygalacturonases (PGs) that differ in isoelectric point, molecular weight, in their regulation and show a differential expression. This multiplicity of PGs may give flexibility to the pathogen, with each enzyme having its own unique properties that contribute to the performance of all the enzymes (Jia *et al.*, 2009).

This work wants to contribute to study the role of the PGs in *P. nicotianae* and *P. capsici*, among the most dangerous pathogens for many plant species: Specific points of this thesis are:

1. Identification of the whole set of the PGs from well-known oomycetes, which present different lifestyles.
2. Comparison of large PG families found in the oomycetes species using phylogenetic analysis for tracking evolutionary relationships.
3. Primary sequences analysis on identified PGs to detect domains and/or amino acids responsible for PG-PGIP interaction.
4. Characterization of PGs from *P.nicotianae* and *P.capsici. In vitro* analysis were carried on for *P. nicotianae. In vivo* analysis were carried on *P. capsici* including, infection assays on wild type and transgenic tomato plants expressing *Pv*PGIP2, microarray analysis

to determine the expression pattern of the PGs upon infection time course experiments and ectopic expression in *N. bethamiana* to reveal the function of the them.

5. Construction of *P. capsici* mutants for investigate the role of PGs in the pathogenesis, using different approach of reverse genetics.

3. MATERIALS AND METHODS

3.1 Used Organisms

3.1.1 Bacterial strains

Escherichia coli DH5α: supE44 Δ*lac*U169 (Φ80 *lacZ* ΔM15) hsdR17 *rec*A1 *end*A1 gyrA96 *thi*-1 *rel*A1).

Escherichia coli DH10β: F^- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lacZ*ΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ^- (Grant *et al.*, 1990).

Agrobacterium tumefaciens (Smith and Townsend) Conn, strain AGL1: AGL1 genotype is AGL0 (C58 pTiBo542) recA::bla, T-region deleted Mop(+) Cb(R) [AGL0 is an EHA101 with the T-region deleted, which also deletes the aph gene] (Lazo *et al*., 1991). EHA101, genotype C58 pTiBo542; T-region::aph, Km(R); A281 derivative harboring pEHA101, T-DNA replaced with *npt*II, elimination of T-DNA boundaries uncofirmed, super-virulent (Hood, Helmer, & Fraley, 1986).

3.1.2 Yeast strain

Pichia pastoris X-33 (Invitrogen)

3.1.3 Oomycete species

Phytophthora nicotianae isolate 329

Phytophthora capsici LT1534

Phytophthora capsici LT6535

Phytophthora capsici OP97

3.14 Plant species

Solanum lycopersicum cv moneymaker,*Nicotiana bentamiana*

3.2 Culture media for Escherichia coli

3.2.1 Luria-Bertani Medium

Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

3.2.2 Low Salt Luria-Bertani Medium (LSLB Medium)

Tryptone	10 g/L
Yeast extract	5 g/L
NaCl	5 g/L

3.2.3 SOC Medium

Bactotryptone	20 g/L
Yeast extract	5 g/L
NaCl	5 g/L
KCl	0,2 g/L
$MgCl_2$	1 g/L
Glucose	7,2 g/L

Ampicillin 100 g / mL and zeocin 25 g / mL were eventually added, at room temperature after sterilization carried out at 121 °C for 15 minutes.

3.3 Culture media for *Pichia pastoris*

3.3.1 Yeast Extract Peptone Dextrose Medium

Yeast extract	10 g/L
Pepton	20 g/L
Glucose	20 g/L

3.3.2 Yeast Extract Peptone Dextrose Sorbitol Medium

Yeast extract	10 g/L
Peptone	20 g/L
Glucose	20 g/L
Sorbitol	1 M

3.3.3 YTG/2 Medium

Yeast extract	10 g/L
Tryptone	10 g/L
Glucose	20 g/L

Zeocin 100 µg / mL was eventually added, at room temperature after sterilization at 121 °C for 15 minutes. The solid media for yeast and bacteria were prepared by agar to a final concentration of 2%. Where it was required, antibiotics were added at 50 °C prior media solidification.

3.4 Culture media for *Phytophthora* species

3.4.1 V8 medium

V8 juice (Campbell)	200 mL/L
$CaCO_3$	1 g/L
ß-sitosterol	300 mg/L (used for sporulation media)
Agar	20 g/L (if solid medium)

3.4.2 Pea Broth

125 g of boiled and filtered peas / L

Sterilization is carried out at 121 °C for 15 minutes

3.4.3 Minimal medium

$Na_2HPO_4 \cdot 7H_2O$	12.8g/L
KH_2PO_4	3 g/L
NaCl	0.5 g /L
NH_4Cl	1 g/L
Yeast extract	5g/L
Agar	20 g/L (if solid medium)

After sterilization were added glucose at final concentration 2.5% or citrus pectin (Sigma) 1% as carbon source.

Each prepared medium was transferred to a flask, plugged with cotton and sterilized in an autoclave at 121 °C for 15 min. Prior to pouring the agar media into sterilized petri dishes, antibiotics (if needed) were added to each medium.

3.4.4 ALB (Amended Lima Bean) medium

K_2HPO_4	1.0 g/L
KH_2PO_4	1.0 g/L
KNO_3	3.0 g/L
$MgSO_4$	0.5 g/L
$CaCl_2$	0.1 g/L
Sorbitol	5.0 g/L
Glucose	5.0 g/L
Vitamin stock	2.0 ml/L
Trace elements	2.0 ml/L
Yeast extract	2.0 g/L
Lima bean extract	250 ml/L
Mannitol	5.0 g/L

Sterilize this solution by autoclave 20 min. at 120°C, and after add under sterile conditions trace elements and vitamin stock. Stock at 4°C.

3.4.4.1 Lima bean extract:

Autoclave 150 g of frozen lima beans in 1 liter of distilled H_2O for 30 min. Strain through two layers of cheesecloth. Adjust at 1 liter, stock by 500 ml and autoclave again.Stock at 4°C. Keep frozen until needed.

3.4.4.2 Vitamin stock:

Biotin	0.20 mg/300 mL
Folic acid	0.20 mg/300 mL
l-inositol	12.0 mg/300 mL
Nicotinic acid	60.0 mg/300 mL
Pyridoxine-HCl	18.0 mg/300 mL
Riboflavin	15.0 mg/300 mL
Thiamine-HCl	38.0 mg/300 mL
Coconut milk	50.0 ml/300 mL

Filter coconut milk on miracloth and autoclave 20 min. 120°C. After, prepare vitamin solution in 150 ml of distilled water and filter under sterile conditions with Millex®-GP 0.22 µm (Millipore) in sterile bottle. Add 50.0 ml of coconut milk sterile (cold) and 100 ml of distilled H_2O sterile. Stock this solution at -20°C.

3.4.4.3 Trace elements:

$FeC_6H_5O_7 \times 3H_2O$	215 mg/400 mL
$ZnSO_4 \times 7H_2O$	150 mg/400 mL
$CuSO_4$	30 mg/400 mL
$MnSO_4 \times H_2O$	15 mg/400 mL
H_3BO_3	10 mg/400 mL
MoO_3^-	7 mg/400 mL

Sterilize this solution by autoclave 20 min. at 120°C. Stock at Room Temperature

3.5 RNA extraction

The extraction and subsequent handling of RNA from *Phytophthora* species was performed using handling procedures designed to avoid contacts with RNase present in all biological materials. RNA was isolated from 1g of frozen mycelium from *Phytophthora* spp (RNeasy Plant Mini Kit; Qiagen Inc., Valencia, CA, USA) and treated afterwards with DNAse (Ambion, Foster City, CA, USA) to remove genomic DNA contamination, in accordance with the instructions of the manufacturers. To test for genomic DNA contamination, PCR using primers specific for *P capsici* Tubulin, was performed on the extracted RNA.

3.6 DNA Methods

3.6.1 Extraction of genomic DNA (gDNA) from P. nicotianae and P. capsici

The mycelium collected by filtration or centrifugation of the liquid ninimal culture medium (§ 3.4.3) containing glucose as carbon source, was homogenized in a sterile mortar with liquid nitrogen to obtain a fine powder. The homogenate was resuspended in 1 mL of extraction buffer (Tris-HCl pH 6,8 50 mM; EDTA 20 mM; Sarcosyl 2%; Urea 8M; NaCl 100 mM) every 15 mg of used mycelium. The homogenate was treated with a volume of phenol-chloroform 1: 1 and centrifuged at 5000 rpm for 15 minutes using the 5415 centrifuge (Eppendorf). The supernatant was collected in sterile 2 mL eppendorf tubes and, precipitated with 0.1 volumes of 3 M sodium acetate pH.5.8 and 2 volumes of 100% ethanol and placed at -70 ° C for at least 1 h. The pellet was washed three times with 70% ethanol and centrifuged for 30 minutes at 13000 rpm. The pellet of DNA and RNA is resuspended in a small volume of distillated water. The obtained DNA was treated with RNase (Promega) and analyzed by electrophoresis on 1% agarose gel. DNA was quantified with a spectrophotometer at a wavelength of 260 nm. The determination of the concentration was carried out by applying the following formula:

[A260] x diluition factor x 50 = X ng/µL

3.6.2 Extraction and purification of plasmid DNA from E. coli strains

A single bacterial colony was inoculated in 10 mL of LSLB (§3.2.2) or LB (§3.2.1) medium supplemented with (or w/o) antibiotic and incubated at 37 °C until reaching the stationary phase of growth. The culture was centrifuged for 2 minutes at 12,000 xg and the pellet was used for plasmid purification using a commercial kit (NucleoSpin® Plasmid-MACHEREY-NAGEL, Germany) following the manufacturer's instructions

3.6.3 Polymerase Chain Reaction (PCR)

The reaction of chain polymerization was performed with Thermo-iCycler (BioRad). The reaction consists of a series of identical cycles, each characterized by three phases with different temperatures. In the first phase, the reaction mixture is heated to 94 °C, at this temperature the double helices are completely separate. In the second phase the temperature is lowered to allow pairing of oligonucleotide primers to complementary sequences present in the DNA mixture. This annealing temperature is a variable parameter that can determine the specificity of the PCR. In the next stage the temperature is raised to 72 ° C to allow the DNA polymerization. At the end of these cycles are needed 7 minutes 72 ° C to fully stop

the polymerization reaction. PCR was carried out in tubes of propylene (0.2 mL) at final volume of 25µL in the following way:

COMPONENTS	**VOLUME**	**FINAL CONC.**
DNA 0,2 - 2 ng/µL	1-10 µL	-
PCR buffer 10X	2,5 µL	1x
dNTP mix (100mM each) 10X	2,5 µL	2 mM
Primers (20 µM each) 40X	0,5 µL	5 µM
Taq DNA polimerase	0.2 µL	1 U
Deionized ultrapure H_20	to 25 µL	

3.6.4 Reverse Transcriptase-PCR (RT-PCR)

cDNA was synthesized using 1µg of total RNA, using a commercial kit and primer "Super- Script™ II cDNA synthesis kit and Oligo dT primer" (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's instructions.

3.6.5 Single colony PCR

A single bacterial colony was picked with a sterile toothpick and placed in a 0.2 mL tube containing 25 µl of a PCR reaction mix (§3.6.3). The reaction mixture was boiled for 10 minutes to allow the lysis of the cells. Subsequently, the suspension was cooled to room temperature and was added 1 U of Ampli Taq polymerase. The reaction mixture was subjected to the cycles of PCR amplification under the general conditions (§3.6.3).

3.6.6 Real Time PCR

Expression levels of the genes were analyzed using real-time, quantitative PCR as a validation of microarray results. The cDNA samples were diluted to 10ng/µl. Gene-specific primers designed by using GenScript Real-Time PCR (TaqMan). All real-time PCR reactions were performed using the ABI 7700 sequence detection system (Perkin-Elmer Applied Biosystems) and the amplifications were done using the SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions were composed of 50ºC for 2 min followed by an initial denaturation step at 95ºC for 10 min, 45 cycles at 95ºC for 30s, 60ºC for 30s and 72ºC for 30s. The experiments were carried out in triplicate for each data point. The relative quantification in gene expression was determined using the $2^{-\Delta\Delta Ct}$ method

(Livak & Schmittgen, 2001). Using this method, we obtained the fold changes in gene expression normalized to an internal control gene, and relative to one line (calibrator).

3.6.7 PCR purification

Amplified PCR fragments were purified using a commercial kit (NucleoSpin® Gel and PCR Clean-up - MACHEREY-NAGEL, Germany) following the manufacturer's instructions

3.6.8 Restriction analysis of DNA

The restriction analysis was performed in a reaction mixture consisting of: 200-2000 ng of DNA, 1 unit of restriction enzyme/µg of DNA, specific reaction buffer at 1X final concentration, distilled water to 20 µL. The reaction mixture, after gentle stirring, was centrifuged at maximum speed for 30 seconds and incubated for 1 hour at the temperature required for the specific restriction enzyme. Subsequently, the DNA subjected to restriction was analyzed by electrophoresis on 1% agarose gel and purified.

3.6.9 Ligation reaction

The ligation reaction was carried out by incubating the reaction mixture at 4 °C for 12 h. The ligation reaction was performed using an insert: vector molar ratio 1: 1 or higher. The ligation mixture of 10 µL was prepared in the following way:

Plasmid:	X ng	**Ligase buffer10×**	1 µL
Insert:	Y ng	**T4 DNA Ligase (Promega)**	1U
Ultrapure H_2O to 10 µL			

3.6.10 Competent cells of bacterial strain

The bacterial strains were inoculated onto LB-agar (§3.2.1) and growth overnight. A single colony of bacterial strain was used to inoculate 5 mL of LB medium and growth until reaching the stationary phase. The culture was tranferred in a 1 L Erlenmeyer flask containing 250 mL of LB. The bacterial culture was incubated at 37 ° C for three hours until reaching an optical density of 0.3 OD (λ = 600 nm), e.g. exponential phase. The bacterial suspension was transferred into a bottle of beckman sterile 250 mL and then was centrifuged at 4000 xg for 15 minutes at 4 °C. The bacterial pellet was washed twice with cold 10% glycerol and than resuspended in 2 mL of the same solution. The cells, were dispensed in sterile eppendorf pre-cooled and subsequently frozen in liquid nitrogen and stored at -80 °C.

3.6.11 Electroporation of competent cells from bacterial strains

Competent cells of bacterial strains were transformed by electroporation using a "MicroPulser Electroporator" (BioRad). The ligation mixture containing 10 ng of plasmid was added to an aliquot of 50 µL of competent cells of *E. coli* or *A. tumefaciens*. Electroporation was performed according to the parameters set by the manufacturer. After heat shock, the mixture of bacteria was inoculated in 1 mL of SOC (§3.2.3) in a 15 mL sterile tube. The rapid addition of SOC is very important for the "recovery" of the transformants, after undergoing the electric shock. The cell suspension, transferred in suitable culture tubes, was incubated at 37 °C for 1 h under stirring. Subsequently, the bacteria were plated on Petri dishes in LB supplemented with antibiotics and incubated overnight at 37 °C. The mixture was spread on LB agar plates containing 50 µL each of the antibiotics ampicillin, kanamycin zeocin, streptomycin, rifampicin and carbenicillin, (100 mg/mL) and incubated at 37 – 28 °C for 12 or 96 hours (last timing was used for *A. tumefaciens* AGL1).

3.6.12 P. pastoris competent cells

A single colony of *P. pastoris* was inoculated in 10 mL of YPD medium (§3.3.1) in a sterile 50 mL tube and incubated at 28 °C for 12 hours. Next day, 500 µL of the culture were used to inoculate 500 mL of fresh and steril YPD medium. The yeast culture was incubated at 28 ° C for a few hours until reaching an optical density of 1.3-1.5 at OD (λ = 600 nm). Subsequently, the culture was centrifuged at 1,500 xg for 5 minutes at 4 ° C and the cell pellet was resuspended in 500 mL of sterile water beforehand cooled; then it was centrifuged at 1,500 xg at 4 ° C for 5 minutes and the cells were resuspended in 250 mL of cold sterile water. The cell suspension was centrifuged at 1,500 xg at 4 ° C for 5 minutes and the pellet resuspended in 20 mL of 1 M sorbitol again, the cell suspension was centrifuged at 1,500 xg at 4 ° C for 5 minutes and the pellet resuspended in 1 mL of 1 M sorbitol. The competent cells were dispensed into aliquots of 100 µL in 1.5 mL sterile tubes and stored at -70 °C.

3.6.13 Electroporation of P. pastoris competent cells

The electroporation was performed with gene pulser apparatus X-cell (Bio-Rad). For this purpose, 80 µL of electrocompetent cells were added to 5 µL of distillate water containing 1-5 micrograms of linearized plasmid with *Avr*II. The mixture was transferred into a cuvette of 0.2 cm previously cooled in ice. The conditions used for the electroporation are:voltage 2000 V, capacitance 25 uF, resistance 200 Ω.

Immediately after electroporation was added 1 ml of 1 M sorbitol previously cooled in ice. The content of the cuvette was transferred into a tube containing 2 mL of YPD medium and incubated for 2 hours at 28 °C without shaking. Subsequently, the culture was centrifuged at 1,500 xg and the pellet was plated om Petri dishes containing YPDS medium (§3.3.2) supplemented with zeocin 100 µg/mL.

3.7 Cloning Vectors

3.7.1 pGAPZα A, B, C

The plasmid vectors pGAPZαA, B, C of 3.1 Kb (Fig. 3.1) contain the origin of replication of pUC plasmid; the *ble* gene which confers resistance to Zeocin, used for transformants selection of both *E. coli* and of *P. pastoris*; the constitutive promoter of the glyceraldehyde 3-phosphate dehydrogenase (GAP), the transcription control elements AOX1, TEF1, EM7 and CYC1. Downstream the GAP promoter there is a region containing the sequence encoding α-factor (Figure 3.2) from *Saccharomyces cerevisiae* useful for the secretion of the protein into the culture medium, a multiple cloning sites with the possibility of merge the myc epitope and the 6xHis tag sequences at the C-terminus of the protein. (Manual pGAPZα A, B, and C Invitrogen, 2008)

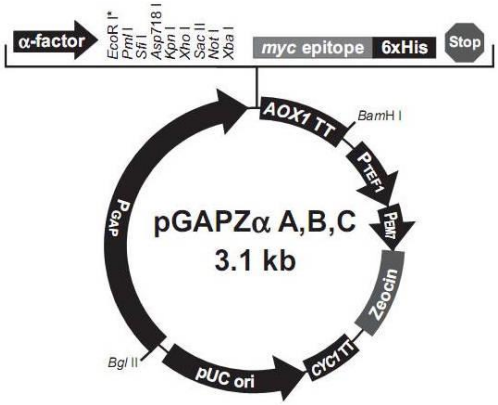


Figure 3. 1: The figure summarizes the features of pGAPZ A (2884 bp), pGAPZ B (2882 bp), and pGAPZ C (2883 bp) vectors. The complete nucleotide sequences for pGAPZ A, B, and C are available for downloading from our web site at www.invitrogen.com.

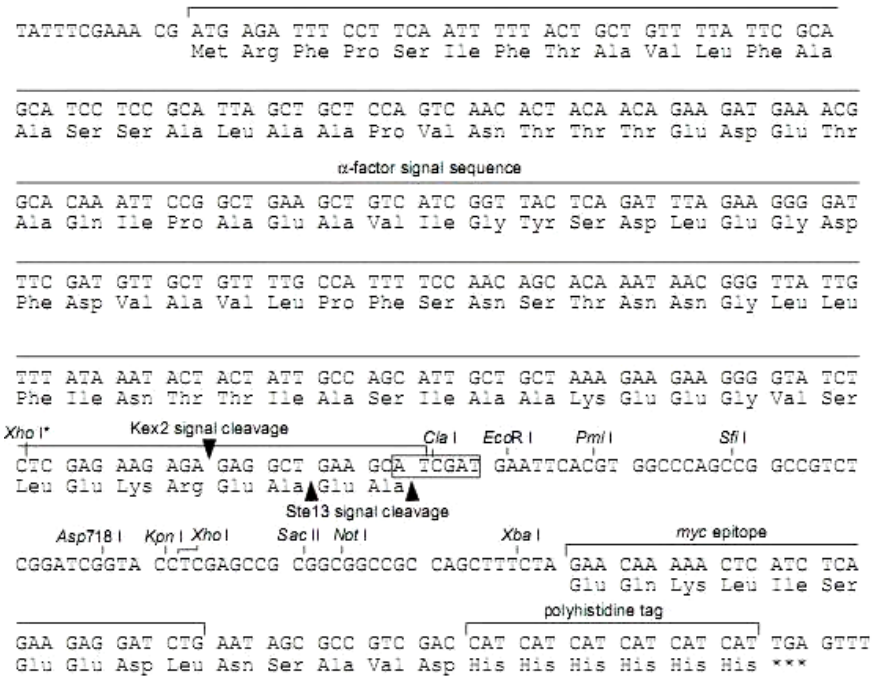


Figure 3. 2: Nucleotide sequence of the plasmid vector PGAPZαA (Invitrogen), are shown the sequences related to α-factor, multiple cloning site and the myc epitope and 6xHis tag.

3.7.2 pENTR™/D-TOPO®

The pENTR D-TOPO (Figure 3.3) is a plasmid used for directionally clone a blunt-end PCR product into a vector for entry into the Gateway® System. Features of the vectors include: 1) attL1 and attL2 sites for site-specific recombination of the entry clone with a Gateway® destination vector; 2) Directional TOPO® Cloning site for rapid and efficient directional cloning of blunt-end PCR products; 3) *rrn*B transcription termination sequences to prevent basal expression of the PCR product of interest in *E. coli*; 4) Kanamycin resistance gene for selection in *E. coli*; 5) pUC origin for high-copy replication and maintenance of the plasmid in *E. coli*.

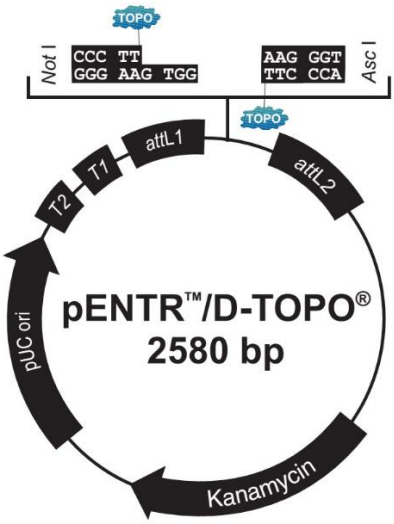


Figure 3. 3: Schematic representation of pENTR™/D-TOPO® plasmid. Main features summatized on text

3.7.3 pK7WG2

The destination vectors pK7WG2 was used for the transient expression of the PGs in *N.bethamiana* the vector is available with the plant selectable marker genes: the neomycin phosphotransferase II (*npt*II), which confer resistance against kanamycin. The selectable marker is under transcriptional regulation of the nopaline synthase (nos) promoter and nos terminator and is placed at the left side of the T-DNA. For overexpression of a DNA sequence, the GATEWAY™ site was placed between the promoter and the terminator of the cauliflower mosaic virus (CaMV) 35S transcript because that promoter is highly active in most plant cells of transgenic plants. Downstream of the 35S promoter part, the tobacco mosaic virus (TMV) Ω leader ensures efficient translation of the inserted coding sequences(Figure3.4) (Karimi, Inzé, & Depicker, 2002)

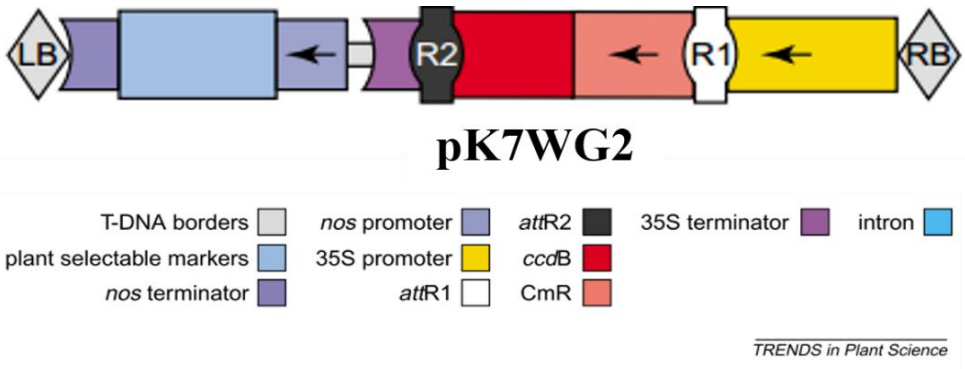


Figure 3. 4: Schematic representation of pK7WG2 plasmid. Main features summatized on text.

3.7.4 pTOR

pTOR plasmid contains the constitutive promoter of *Bremia lactucae ham*34 and terminator flanking a multiple cloning site, and a gene expressing npt for G418-resistance (Figure 3.5) (Torche 2004).

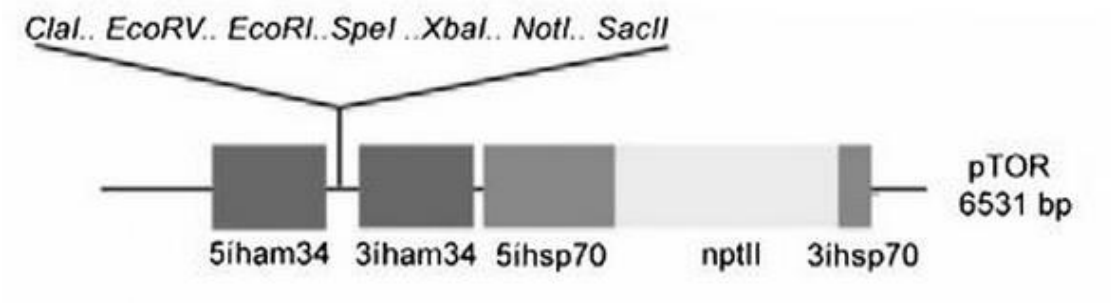


Figure 3. 5: Schematic representation of pTOR plasmid Main features summatized on text

3.7.5 pSTORA

NPT-based gene silencing plasmid. Contains an additional promoter and terminator flanking the Ste20 intron from *P. infestans*, plus rare-cutting cloning sites, to facilitate the construction of hairpin genes (Figure 3.6).

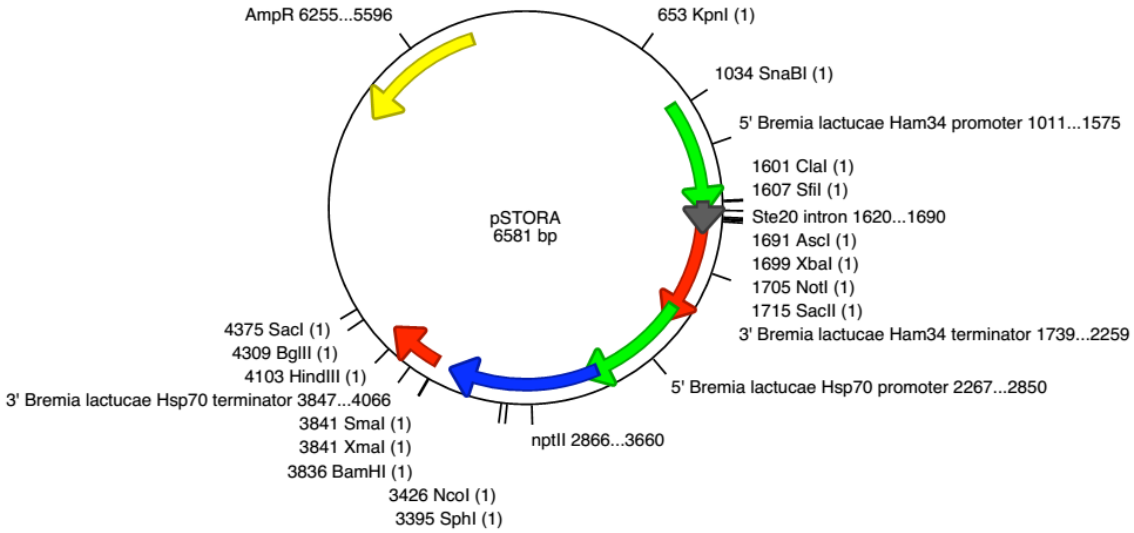


Figure 3. 6: Schematic representation of pTOR plasmid Main features summatized on text

3.7.6 pT7-gRNA (#46759)

pT7-gRNA was purchased from the addgene (plasmid #46759). This plasmid contain the T7 promoter which is specific for the transcription *in vitro* of the DNA downstream. pT7-gRNA contains the resistance gene for the Ampicillin.

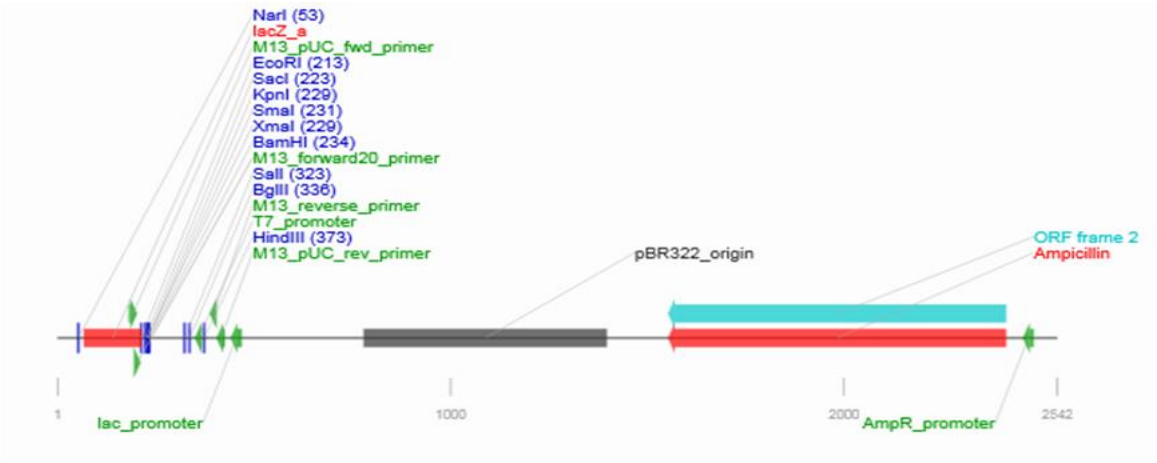


Figure 3. 7: Schematic representation of pT7-gRNA (#46759) plasmid Main features summatized on text

3.7.7 hCas9 (#41815)

hCas9 was purchased from the addgene (plasmid #41815). Express human codon optimized Cas9 nuclease for genome engineering bearing a C-terminal SV40 nuclear localization signal.

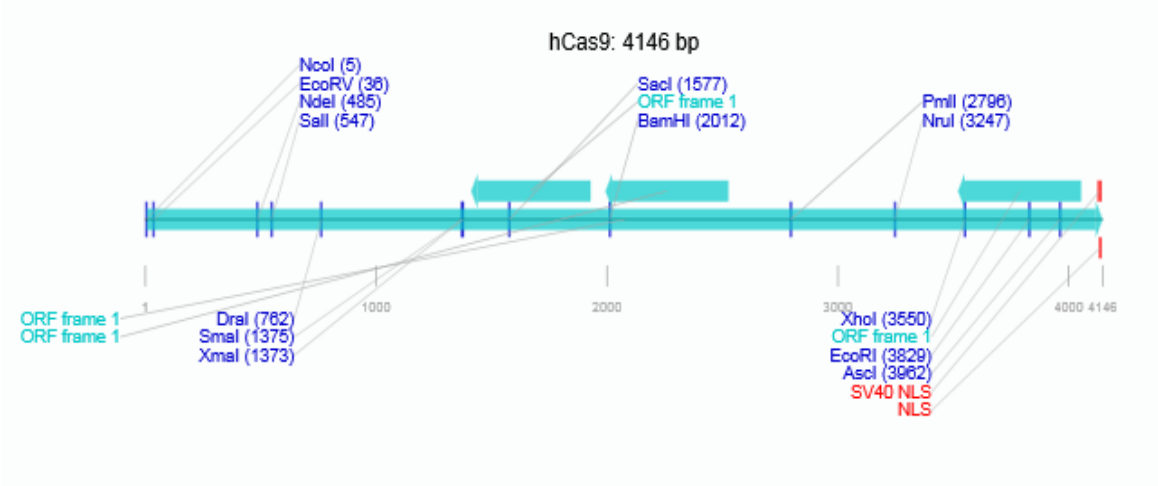


Figure 3. 8: Schematic representation of hCas9 (#41815) plasmid Main features summatized on text

3.8 Expression of polygalacturonase genes

3.8.1 P capsici and P. nicotianae strains and growth conditions

P. nicotianae isolate 329 was obtained from the INRA (Sophia-Antipolis) *Phytophthora* collection. *P. capsici* LT1534 was available in Huitema's laboratory. Both these *Phytophthora* strain were grown in petri dishes on V8 agar medium (§ 3.4.1) in a dark climate chamber at 25 °C for 4 days. To induce zoospore from *P. capsici,* after 4 days in the dark was grown under standard light at 22 °C for 3 days. To release zoospores, plates were flooded with ice-cold distilled water. Spores were harvested from sporulating mycelia by dislodging the sporangia with a sterile glass spreader. To induce zoospores from *P. nicotianae*, mycelia were cultivated for 1 week in V8 liquid medium (§ 3.4.1) at 24 °C under continuous light. This material was macerated and incubated for 4 days on steril water supplemented with 2% agar. The zoospores were released by the following heat shock treatment: incubation at 4°C for 1 h followed by incubation at 37°C for 30 min. Water (10 ml) was added in between incubations

3.8.2 Transient Expression of PcPGs in N.benthamiana

N. benthamiana plants with fully expanded leaves were used for the agroinfection assays. Plants were cultured and maintained in a greenhouse with an ambient temperature of 22 to 25°C and high light intensity.

Transient expression: pK7WG2 plasmids containing PG inserts were transformed into *Agrobacterium tumefaciens* strain AGL1. Transformants were grown on LB medium containing Rifampicin and Spectinomycin to maintain each plasmid. For each construct, a single colony was grown overnight and resuspended in infiltration buffer (10 mM MgCl, 150 uM Acetosyringone) to an OD of 0.5 for necrosis. The buffer was mixed 1:1 with buffer containing Agrobacterium expression silencing suppressor P19 and infiltrated in *N. benthamiana* leaves. Plants were grown in a glasshouse under 16 hours light and set at 26uC by day and 22uC by night.

3.8.3 Lesion Phenotypic Assays

For each PG construct, were infiltrated in same leaf in the left the empty vector control pK7WG2 and on the right the different PG construct. Infiltration was repeated 3 times. The level of cell death was scored after 3 days. *P. capsici* growth assays were done on leaves that were fully infiltrated with *Agrobacterium* strains carrying PGs constructs. Two days after infiltration, leaves were drop inoculated with two 10 mL droplets of zoospore

solution ($1x10^5$ spores per mL). Lesion diameters were measured 2 days post inoculation (DPI).

3.8.4 Tomato infection

Solanum lycopersicum cv 'Moneymaker' plants were grown in controlled growth chambers at 22°C, with a photoperiod of 16 hours, supplemented by artificial light. The third leaf from the top of every plant was detached and placed upside-down in humid transparent plastic trays in a controlled incubator with the same settings as in the growth chamber. Leaves were drop inoculated wirh $1x10^4$-$1x10^5$ zoospores. Diameter of infected lesions were measured starting from 2 days post inoculation (DPI)

3.8.5 Induction of the polygalacturonase genes of P.nicotianae

To investigate the effect of different carbon source on the expression of polygalacturonases of *P. nicotianae* the strain was grown on a minimal medium (§3.4.3) containing 2.5% glucose and in minimal medium containing 1% of pectin from apple (SIGMA76282) for 14 days at 28 °C. The relative mRNA level of the PGs in each treatment was measured by RT-PCR (§3.6.4).

3.9 Vector construction for transformation *P.capsici*

3.9.1 Vector construction for the Cas9

Cas9 (4140 bp) (Mali, *et al.,* 2013) was amplified from the vector hCas9 using the primers FwClaCas9 5'ATGCATATCGATACCATGGACAAGAAGTACTCCATTG3' and RvCas9Not 5'ATGCATGCGGCCGCTCACACCTTCCTCTTCTTCTT3'. The primers have incorporated in the N and C terminal of Cas9 restriction enzymes ClaI and NotI respectively (PROMEGA) for the expression in pTOR plasmid. PCR were carried out with initial denaturing of five minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for 4 minutes and then a final extension at 72° C for fifteen minutes using 10ng of DNA from the purified plasmid. Once verified the sequence, the PCR fragment was cloned in the expression vector pTOR. Cas9 and 1µg pTOR were digested with ClaI and NotI in a double digestion in a standard reaction following the manufacturer's instructions

3.9.2 Generation of Customized Guide RNA Expression Constructs.

The sequence of hmp1 of P. capsici was amplified from the cDNA using the primers PcHmp1Fw_5'ATGGTGCTTCGTGCGGTTA3' and PcHmp1Rv_5'

CTTAGGAGGCTTCATGACC 3' corresponding to the sequence from genome database P. capsici LT1534 v11.0 http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=Phyca11. With these primers, I amplified a 1.4 kb of the gene. PCR were carried out with the following steps: initial denaturing of five minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 54°C for 45 seconds and 72°C for two minutes and then a final extension at 72° C for fifteen minutes using 10ng of cDNA from *P.capsici* as template. The pT7-RNA plasmid, that contain the gRNA, was used for express the target sequence using the primers gRNABglIIFw 5'GATCTGGTGCCGGAAATGTCTGCAGG3' and gRNASalIRv 5'TCGACCTGCAGACATTTCCGGCACCA 3', corresponding to the target sequence of P*chmp1* were diluted to 10 μM each in 1× NEBuffer 3 (New England Biolabs) in a total volume of 20 μL. Oligonucleotides were then annealed with the following procedure: A 5-min denaturing step at 95 °C, followed by cooling to 50 °C at−0.1 °C per second, pausing at 50 °C for 10 min, and cooling to 4 °C at−1 °C per second., The resulting DNA was digested with BglII and SalI. The same digestion was also made to the pT7-gRNA plasmid (Addgene plasmid#46759). One microliter of the annealed oligonucleotides was mixed with 400 ng of the guide RNA (pT7-gRNA) cloning vector. A total of 2 μL of the reaction was then used for transformation of DH5a elettrocompetent cells. 1μg of the template DNA was digested with BamHI (PROMEGA) and purified using a QIAprep column. gRNA was generated by in vitro transcription using T7 RNA polymerase (MEGAshortscript T7 kit Invitrogen). After in vitro transcription, the gRNA (~100 nucleotides long) was purified with Ethanol precipitation. The size and quality of resulting gRNA was confirmed by electrophoresis.

3.9.3 Preparation of cultures for protoplasting

Inoculate plates with *P. capsici*. Typically, fifteen to twenty 150 mm plates of V8 media are inoculated with three plugs and incubated inverted at 25°C. Sporangia are normally harvested from such plates after 7 days. When the mycelia has grown 90% across the plate (if starting from plugs), you harvest sporangia with 50 mL of cold water. Remove chunks of mycelia or agar from the spore suspension by pouring through a 60 μm nylon mesh. Divided the sporangia in 10 bottles on 250 mL of ALB (amended Lima bean) media. Add additional water and ALB to a final concentration of 2 to 5 x $10^{\wedge 5}$ asexual sporangia per ml. Add 50 μg/ml ampicillin. 50 μg/ml vancomycin, and 4 ml per liter nystatin suspension (Sigma). lncubate, at room temperature in the dark.

3.9.4 Isolation of protoplasts and DNA treatment

Harvest the young germlings by pouring the culture through 60 µm nylon mesh. Protoplasts were prepared as described by (Judelson *et al* 1993) by digesting mycelium in osmoticum (1 M mannitol, 7 mM $MgSO_4$) containing 5 mg/mL Lysing Enzymes (Sigma L-1412 from *Tricoderma harzianum* and 2.5 mg/mL cellulase (Sigma C-8546 from *T. reesei*). Digesting mycelium was shaken gently (70 rpm) at room temperature for 45 min, protoplasts filtered through miracloth and harvested by centrifugation at 700 g for 5 min, and washed four times in osmoticum before use. Protoplasts were then counted on a haemocytometer (Fig. 3.9) and diluted to10^5/mL. Assuming 5% regeneration of protoplasts, this concentration and volume of protoplasts should result in approximately 100 regenerated protoplast lines from a volume of 20 µL.

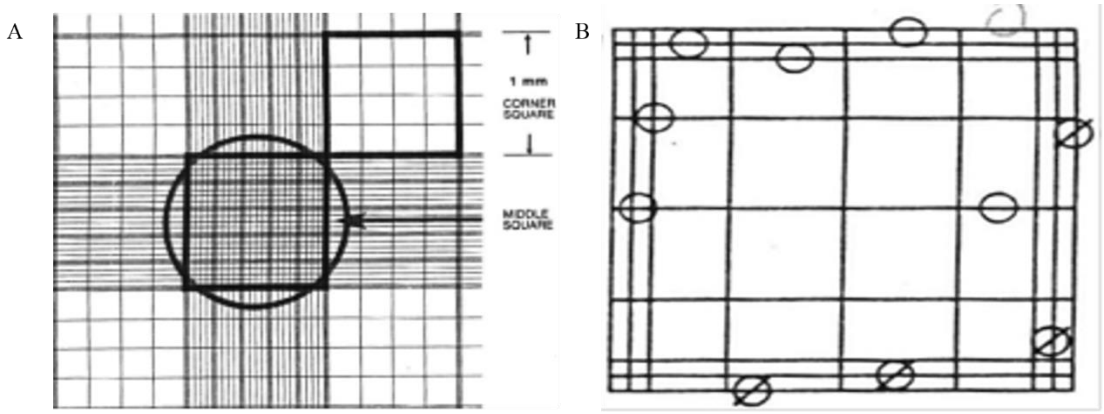


Figure 3. 9: Schematic figures representing the improved Neubauer chamber. A) standard chamber; B) The large square in the center is subdivided into 25 group squares of 0.2 mm sides. Each group square consists of 16 mini squares with 0.05 mm sides, each having an area of 0.0025 mm^2 wHere zoospores were counted.

3.10 Protein Methods

3.10.1 Cultures of P. pastoris

A single colony of *P. pastoris* was inoculated into 5 mL of medium YTG/2 (§3.3.3) in a sterile 50 mL tube and incubated at 28 °C for 12 hours. The next day, 500 µL of the culture were used to inoculate 50 mL of fresh medium YTG/2. The yeast culture was then incubated at 28 °C for 12 hours. The following day 30 mL of the culture in the stationary phase were used to inoculate 1 L of fresh medium YTG/2. The culture was then incubated at 28 °C for 96 hours. After this incubation time, the culture was centrifuged at 12,000 rpm for 15 minutes at room temperature and the supernatant was used for the subsequent steps of purification and characterization of the expressed protein.

3.10.2 Cultures of Phythophthora strains

A slice of 1 cm^2 of *P. nicotianae* mycelium was inoculated in a 1 L Erlenmeyer flask containing 250 mL of minimal liquid medium (§3.4.3). The culture was grown as described in (§3.8.5). The supernatant was used for the subsequent purification steps of the expressed protein.

3.10.3 Dialysis and concentration of P. pastoris liquid cultures by ultrafiltration

The culture medium of *P. pastoris*, about 1 L, was dialysed and concentrated using the system "Vivaflow 200" (Sartorius), following the manufacturer's instructions (Fig. 3.6). Schematically, in a first phase the volume of the medium was concentrated to the final volume of 100 mL and then, flushed with approximately 500 mL of 20 mM CH3COONa pH 4.6. At the end were obtained 100 mL of medium YTG/2 in acetate buffer

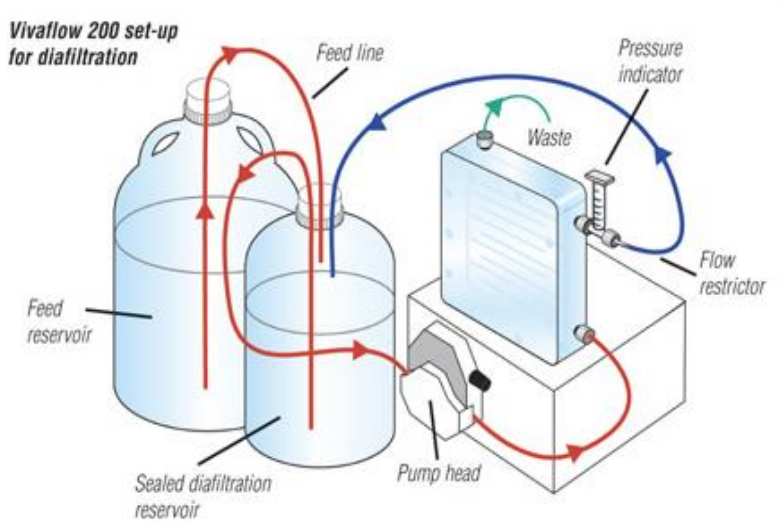


Figure 3. 10 Schematic representation of the system of concentration and dialysis by ultrafiltration "Vivaflow 200".

3.10.4 Ionic exchange columns

Dialyzed proteins were mixed with a suspension of diethylaminoethyl (DEAE) cellulose, an anion exchange matrix, (DE52, Whatman, UK) pre-equilibrated with 20 mM Na acetate pH 4.6. The diethylaminoethyl cellulose (DEAE) consists of a matrix of agarose to which it is covalently bound diethylaminoethyl group [$-CH_2-CH_2-N + H- (CH_2-CH_3)_2$, pK ~ 8.5]. The not-absorbed proteins were loaded on a S-Sepharose column (Amersham), a cation exchange chromatography column, pre-equilibrated with 20 mM Na acetate pH 4.1. Elutions were carried out using a manual step gradient of NaCl (0 to 1 M) at a flow rate of

1 ml/min in 20 mM Na acetate pH 4.1. Fractions that showed the highest activity were pooled, concentrated and dialyzed against glycerol and stored at -20 °C. The fraction with highest activity was analized.

3.10.5 Protein concentration assay

Total protein were determined according to Bradford (Bradford, 1976), and Bovine Serum Albumin (Sigma) was used as standard. Polygalacturonase was determined by densitometric method on SDS-PAGE using Image j software (Schneider *et al.*, 2012).

3.10.6 Electrophoresis of proteins

This method allows the separation of proteins of different molecular weight by their migration through the mesh of a polyacrylamide gel under the action of an electric field. Proteins were analyzed on 10% denaturing polyacrylamide minigel according to the method of Laemmli (Laemmli, 1970). The protein bands were visualized by silver staining (Blum *et al.*, 1987) and Blue Coomassie R 250 (Sigma) staining. Mr of the protein was determined on 10% SDS-PAGE using perfect protein ladder (EURx) as weight reference. Before loading, the samples were incubated at 100 ° C for 5 minutes, and electrophoresis was carried out by applying a constant voltage of 120 mV.

3.10.7 Agar Diffusion Assay for polygalacturonase activity

*Endo*PG enzymatic activity was assayed as described in (An aliquot of crude filtrate from *P. nicotianae*/*P. pastoris* cultures was loaded in 20-ml wells made in 30 ml plates of solid substrate (0.5% polygalacturonic acid, 0.8% agarose, 100 mM CH_3COONa) and incubated o/n at 28 °C to allow diffusion. After adding 6 M HCl, degradation haloes revealed *endo*PG enzymatic activity. PG activity was expressed as agarose diffusion units, with 1 unit defined as the amount of enzyme producing a halo of 0.5 cm radius (external to the inoculation well). The inhibition assay was performed loading the cell free medium and *Pv*PGIP2 in the same well and incubated o/n at 28°C to allow diffusion. The agarose inhibition unit was defined as the amount of PvPGIP2 causing 50% inhibition of 1 agarose diffusion unit at pH 4.7

3.10.8 Reducing end groups assays for polygalacturonase activity

Most commonly used enzyme assays are based on measuring “reducing ends” upon the hydrolysis reaction. Moreover, this analysis enables quantification of the degree of polymerization of cellulosic substrates. So called “reducing ends” are free aldehyde groups

in a sugar, that will be oxidized to carboxylic group, in the presence of oxidizing agents like: p-hydroxybenzoic acid hydrazide or copper sulphate. This reaction is typically followed spectrophotometrically due to the color change of the oxidizing agent.

3.10.8.1 Determination of polygalacturonase activity by PAHBAH (p-hydroxy benzoic acid hydrazide) assay

The assay is based on the determination of reducing sugars produced by degradation of the substrate (sodium salt of polygalacturonic acid). This method allows the determination of reducing sugars present in a solution, through a colorimetric reaction (Lever, 1972). The reaction is carried out by para-hydroxybenzoic acid hydrazide (PAHBAH) in alkaline solution with the reducing groups present in the sample. The yellow color that develops from the reaction after boiling is due to the formation of osazones and is indicative of the presence of reducing groups (Fig. 3.7).

Aliquot 1,5mL of PABA solution (50 mL of solution is made by mixing 10 mL of 5% PAHBAH + 40 mL of 0,5M NaOH) in glass tubes (3 for each sample "T_0", "T_{15}", "T_{30}" and 4 for the standard curve). Set up the reaction mix in eppendorf tube as follow: 1% polygalacturonic acid 100 µL, 0,5M CH3COONa pH 4,5 30 µL, polygalacturonase X µL, H_20 (distillated or deionized) up to 300 µL (the substrate must be the last component of the mix). Vortex the eppendorf tube and take an aliquot of 40 µL from the reaction mix and put it in the glass tube marked T_0; vortex it and put T_0 on the bench, Incubate the reaction mix in waterbath at 30°C. After 15 min repeat T_0 step and put the aliquot in the tube marked T_{15} and same procedure for T_{30}. After the last sample, standard curve has been prepared as follow: "BL" = 1,5 mL PABA; "St 2,5" = 1,.5 mL PABA + 2,5 µL of 10 mM galacturonic acid; "St 5" = 1,5 mL PABA + 5 µL of 10 mM galacturonic acid; "St 10" = 1,5 mL PABA + 10 µL of 10 mM galacturonic acid. All glass tubes (samples and standard curve) are incubate for 10'on a waterbath at 95°C. Samples are cooled at toom temperature and readed at 410nm. The blank for the standard curve will be "BL" while the blank for samples will be "T_0".

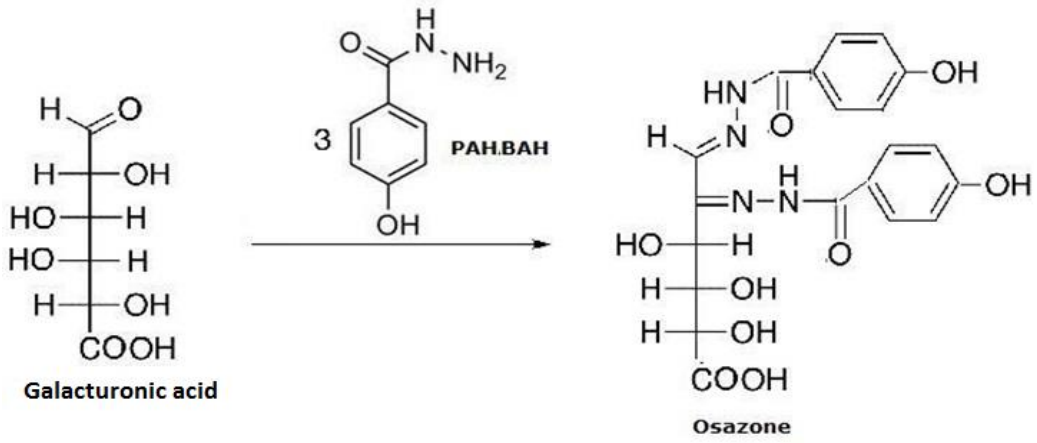


Figure 3. 11: Schematic representation of the colorimetric reaction on PAHBAH assay for reducing sugars.

The enzymatic activity was calculated in U/mL[1] using the following formula:

$$\frac{\boldsymbol{OD_{sa}}}{\boldsymbol{V_{aliquot}}} \boldsymbol{x} \frac{\mathbf{1}}{\overline{\boldsymbol{OD}}_{\boldsymbol{st/\mu L}}} \boldsymbol{x} \frac{\boldsymbol{C_{st}}}{\boldsymbol{t}} \boldsymbol{x} \frac{\boldsymbol{V_{mix}}}{\boldsymbol{V_{enz.}}} = \boldsymbol{U/mL}$$

$\boldsymbol{OD_{sa}}$ = sample reading at 410nm; $\boldsymbol{V_{aliquot}}$ = withdrawn aliquot in µL at T_0, T_{15} e T_{30}; $\overline{\boldsymbol{OD}}_{\boldsymbol{st/\mu L}}$ = average reading (µL) of standard; $\boldsymbol{C_{St}}$ = standard concentration in mM; $\boldsymbol{t}$ = withdrawn time (T_{15} e T_{30}); $\boldsymbol{V_{mix}}$ = volume of reaction mix (300 µL); $\boldsymbol{V_{enz.}}$ = utilized enzyme (in µL).

3.10.8.2 Determination of polygalacturonase activity by Nelson-Somogyi assay

Copper reagent A, was prepared by dissolving 12 grams of potassium sodium tartrate, 24 grams of sodium carbonate, 16 grams of sodium hydrogen carbonate, 144 grams of sodium sulfate and 4 gram of sodium benzoate in ca. 500 mL of deionized water. The solution was heated until all chemicals were dissolved. The final volume was adjusted to 800 mL with deionized water. Copper reagent B, was prepared by dissolving 4 grams of copper sulfate pentahydrate and 36 grams of sodium sulfate in ca. 150 mL of deionized water. The solution was heated until all chemicals were dissolved. The final volume was adjusted to 200 mL with deionized water. 25 grams of ammonium molybdate was dissolved in 450 mL of deionized water. 21 mL of concentrated sulfuric acid was added into the molybdate solution. 3 gram of sodium arsenate dibasic heptahydrate was dissolved in 25 mL of deionized water. Finally, the color reagent was prepared by mixing the acidic molybdate

[1] one unit of activity and is defined as the amount of enzyme which produces 1 microequivalent of reducing groups per minute at 30 °C, using 0.5% (weight / volume) of polygalacturonic acid (sodium salt) as the substrate to the optimal pH.

solution with the arsenate solution. Galacturonic Acid (0,01M) standard was prepared 0, 1, 2.5, 5 and 10µl. 100 µL of sample or standard was mixed with 80 µL of copper reagent (A) and 20 µL of copper reagent (B) in an eppendorf tube. The mixture was thoroughly mixed before being heated in a boiling water bath for 10 minutes. The mixture was then cooled. 100 µL of color reagent was then added to the mixture and mixed well. 950 µL of deionized water was also added to the mixture and mixed well. Once all carbon dioxide had escaped from the solution, absorbance was measured against a blank at 520 nm (Norton, 1944). Definition and calculation of polygalacturonase activity has been previously shown at §3.9.8.1.

3.11 Characterization of *P. nicotianae* polygalacturonases

3.11.1 Activity

For testing the activity of each polygalacturonase, a solution containing 100 µL of 3% galacturonic acid 30µL of 50mM Na-acetate buffer (pH 5.0) and 100 µL of *Pn*PG in a volume of 300 µL was prepared in 1,5 mL Eppendorf tube. The reaction mixture was placed in watherbath and heated at 30 °C in a time course experiment withdrawing 40 µL at 0, 15, 30, 45 and 60 minutes as described at §3.9.8.1 and §3.9.8.2.

3.11.2 Optimum of pH

*Pn*PGs activity was assayed as a function of pH, in 50 mM acetate buffer (pH 3.0–6.0) and Tris–HCl (pH 6.5–7.5), at 30 °C with 1% lowesterified citrus pectin (Sigma) as substrate.

3.10.3 Effect of the temperature on PnPGs activity

Fifteen mL test tubes, each containing 100 µL of 3% galacturonic acid, 30 µL of 50 mM Na-acetate buffer (pH 5.0) and 100 µL of *Pn*PG in a volume of 300 µL. The tubes were incubated in a range of temperatures between 30-70 °C for 20 min. The *Pn*PGs activity was determined by measuring the reducing sugar released as a result of the hydrolysis of polygalacturonic acid using a Nelson somogyi reagent at 520 nm.

3.11.4 Thermal stability

The purified enzymes were incubated at different temperatures ranging between 30 and 60°C for various periods of time up to 2 h. After cooling, the residual activities were determined by Nelson somogyi reagent at 520 nm. *Pn*PGs, without thermal treatment was

used as control. The deactivation rate of the enzyme was calculated using a first-order expression (Naidu and Panda, 2003):

$\frac{dE}{dt} = -K_d E$ So that $ln\left(\frac{E_t}{E_0}\right) = -K_d t$

The K_d (deactivation rate constant or first order rate constant) values were calculated from a plot of time (t) versus ln (E_t /E_0) at a particular temperature. The time required for the enzyme to lose half of its initial activity, is given by:

$$t_{\frac{1}{2}} = \frac{ln2}{K_d}$$

3.11.5 Kinetics

The Michaelis constant (K_M) and Vmax of the purified *Pn*PG5, *Pn*PG7 and *Pn*PG9 were determined by measuring the reaction velocities as activity (U/mL) at various concentrations of polygalacturonic acid (mg/mL) at 40 ºC for 5 min.The data were plotted according to Lineweaver-Burk plot (1/[S] versus 1/V) or Michaelis-Menten curve ([S] versus V).

3.12 *In silico* analysis of Polygalacturonase

Databases: Phyca11 scaffolds, gene models and proteins, were obtained from the *Phytophthora capsici* sequencing consortium website (http://genome.jgi-psf.org/Phyca11/Phyca11. home.html). Databases for other oomycete species were obtained from their original sources: http://www.broadinstitute.org /annotation/genome for Phytophthora infestans and Phytophthora parasitica genome.wustl.edu for Hyalopernospora arabidopsidis, http://pythium.plantbiology.msu.edu for *Pythium ultimum, P. vexans and P. aphanidermatum*, http://www.polebio.scsv.ups-tlse.fr/aphano for *Aphanomyces euteiches, A. astaci and A. stellatus*.

The sequences identified were used for phylogenetic analysis on a protein multiple alignment comprising the Glyco hydro 28 domain (Pfam accession number PF00295) extracted using the Pfam database search facility (Finn *et al.*, 2014). The alignments were generated using HMMalign aligning the domains against the Pfam seed alignment using the Pfam profile HMM. Columns not in the domain were removed from the alignment. Model selection was done in TOPALi v2 (Milne *et al.*, 2009) and the substitutuion model of protein evolution was selected based on the BIC criterion. Phylogenetic trees were estimated using MrBayes. Two analysis were run for 1,000,000 generations following with a burn-in of 1000 generations The potential scale reduction factor values was less than 1.2 for all parameters suggesting good convergence of the two runs (Gelman *et al.*, 1995).

4. RESULTS

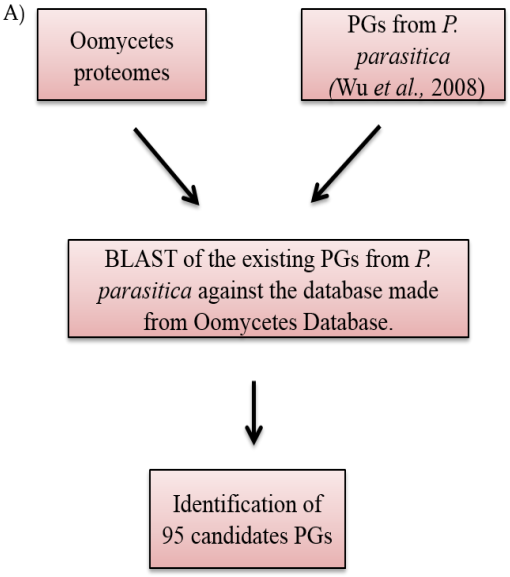
4.1 *In silico* analysis of the Polygalacturonase (PGs): Identification and Phylogenetic analysis

4.1.1 Identification of the PGs from different species of Oomycetes.

Basic Local Alignment Search Tool (BLAST) was carried out for the research of new PGs from different oomycetes species, using as probe the sequences of ten, already identified and characterized PGs from *P. nicotianae* (Wu *et al.*, 2008). This approach contributed to identify a multigene family encoding PGs, composed of 19 members, from the genome of *P. capsici*, recently published. The figure 4.1_A summarizes the pipeline used. This pipeline was applied for the identification of PGs from different oomycetes proteomes database, with different lifestyle. In details, in this analysis were included the hemi-biotrophic *P. infestans*, three destructive necrotrophic species of plant pathogens such as *Pythium aphanidermatum, P. vexans and P. ultimum*, three species of *Aphonomycetes*, including both, plants and animals pathogens (*A. astaci*, *A. stellatus*, *A. euteiches*), and the obligate biotroph *Hyaloperonospora. Arabidopsidis*, (Coates & Beynon, 2010).

In total 95 candidate PGs were identified from the oomycetes databases; twenty-three PGs for *P. infestans*, nineteen PGs for *P. capsici*, ten new PGs were identified in *P. nicotianae* genome, not identified by Wu *et al.*, 2008. Five PG isoforms were identified for *P. aphanidermatum* and *P. vexans*, only three for *P. ultimum* and *H. arabidopsidis*. No genes encoding PGs were identified for *A. astaci* and *A. stellatus*, which are animal parasite species, but in *A. euteiches*, the plant parasitic specie, eighteen PGs are present. (Figure_4.1B).

A)



B)

species	genome size (Mb)	total genes	PGs
P. infestans	240	18140	23
P. capsici	64	19805	19
P. parasitica	71	20822	20
P. ultimum	37.6	15323	3
P. vexans	33.8	11958	5
P. aphanidermatum	35.9	12312	5
A. astaci	75	20170	0
A.stellatus	176	14096	0
A. euteiches	50	20555	18
H. arabidopsidis	77.8	14321	3

Figure 4. 1*:* PGs identification. A) Pipeline for identify the PGs from *P. capsici* the proteins were extracted from the proteomes databases. Identified PGs from *P. parasitica* were used as a template. B) Table showing the species, the genome size, the total number of the genes and identified PGs for each species under investigation.

4.1.2 Phylogenetic analysis

In order to understand the relationship between the PG proteins from *Phytophthora spp* with the PGs from other oomycetes identified from the analysis described above, a phylogenetic analysis was performed and constructed a phylogenetic tree based on the HMM (Hidden Markov Model) multiple sequence alignments (Yoon, 2009). This approach is useful in modeling biological sequences, such as proteins. Typically, a biological sequence consists of smaller substructures with different functions, and different functional regions often display distinct statistical properties. PG proteins from fungi such as *Fusarium phyllophilum* (*Fp*PG) *Aspergillus niger* (*An*PG), *Colletotrichum lupini* (*Cl*PG) and *Sclerotium rolfsii* (*Sr*PG) were used as outgroup and the PGs from *Phytophthora* are well separated into a distinct groups. Analyzed PGs from *P. infestans*, *P. capsici and P. nicotianae* shows intensive gene duplication (Figure 4.2) especially for the PGs from *P. infestans*. *Phytophtophora* gene duplications are also well known on other expanded gene families such the RXLR and CRN effectors. Gene duplication is a fundamental process in the evolution of species and is believed to play a leading role in the creation of sequence variability and novel gene functions (Jianzhi Zhang, 2003). Based on the tree analysis, by event of separation and speciation, the *Phytophthora* PGs are separated into a 4 groups; PGs encoded from *Aphanomycetes and Pythium* are separately clustered from other analyzed oomycetes and the three PGs from *H. arabidopsidis* are mixed with the PGs from *Phytophthora*.

Within the *Phytophthora* clusters several enzyme characteristics were analyzed for finding common characteristics (e.g. enzyme dimensions, presence of glycosylation sites, isoelectric point and enzyme activity), only in the group N° 2 are clustered together all the heavily glycosylated PGs.

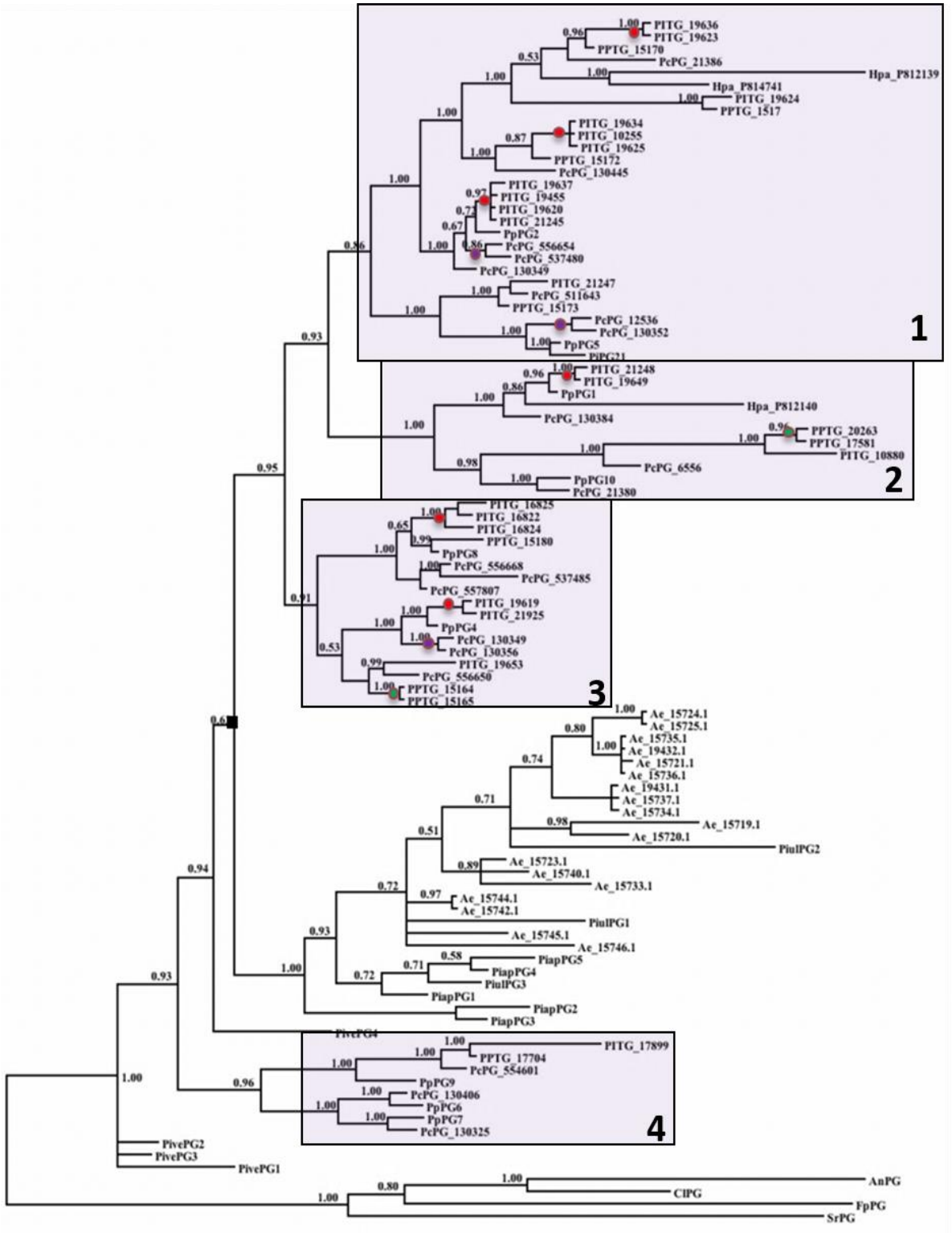


Figure 4. 2: Phylogenetic tree containing all sequences of Oomycetes PGs identified from our pipeline. *P. infestans* (PITG*) P. capsici* (*Pc*PG), *P. nicotianae* (*Pp*PG), *H.Arabidopsis* (*Hpa*), *P. ultimum* (*Piul*PG), *P. vexans* (*Pive*PG), *P. aphanidermatum* (*Piap*PG) and *Aphanomycetes euteiches* (*Ae*). Bootstrap support values of 0.5 or higher are indicated. Sequence from fungus *Aspergillus niger*(*An*PG), *Fusarium phylophillum* (*Fp*PG), *Sclerotium rolfsii (SrPG)*and *Colletotrichum lupini*(*Cl*PG) PGs were used as an outgroup. ● Gene duplication in *P.infestans* ● gene duplication in *P.capsici,* ● gene duplication *P.nicotianae.* ■ Event of speciation between *Phytophthora spp* and the others oomycetes as *Aphanomycetes* and *Pythium.* In the box 2 are indicated the hyperglycosylated PGs.

4.1.3 Sequence analysis of the Polygalacturonase from P. capsici and P.nicotianae

The predicted sequences for *P.capsici* and *P.nicotianae* obtained from the Pipeline were used for further analysis. The sequences were converted to FastA format and then analyzed by ClustalW, alignment program available at ExPASy site (www.expasy.org). PGs have been studied in detail from a large number of sources including bacteria, plant, fungi (ten Have *et al.* 2001; Blackman, Cullerne & Hardham 2014; Götesson *et al.* 2002; Hong *et al.* 2013; Kalaitzis, Solomos & Tucker 1997). All the analyzed PGs show four highly conserved motifs, including the PGs from *P. cinammomi* and the ten PGs previously identified from *P. nicotianae,* (Götesson *et al.,* 2002; Wu *et al.,* 2008). Among these four regions, two are *"catalytic regions"* (G/QDD and G/SHG) and are strictly conserved. A second set of highly conserved domains are *"substrate-binding regions"* (NTD and RIK) (Palanivelu, 2006).

Amino acid multi-alignment showed that all PGs from *P.capsici*, *P nicotianae* identified by previous analysis contain the highly conserved region NTD_QDD_G/SHG and RIK (gray blocks in Figure 4.2).

Since PG-PGIP interaction is considered a paradigm that describes some of the recognition events determining plant immunity (Misas-Villamil & van der Hoorn 2008) and, unlike for other CWDE inhibitors that engage many contacts in the interaction with their partners (Payan *et al.,* 2004; Raiola *et al.,* 2004; Matteo *et al*., 2005) only a few residues, sometimes only one, are critical for maintaining the stability of the PG-PGIP interaction (Leckie *et al.,* 1999; Casasoli *et al.,* 2009). The sequence of *F. phyllophilum* (*Fp*PG) was added to emphasize the amino acids that were found to be essential for the interaction with the *Pv*PGIP2 (Benedetti *et al.*, 2013). The results from the multiple alignment relieved that, none of the motifs and/or aminoacids are presents on the *P.capsici* and *P.nicotianae* PGs sequences, considered at this analysis, except for the Lysine at the position 360 (K^{360}) and the Serine at the position 362 (S^{362}) of *Fp*PG. K^{360} there is in 5 PGs from *P. capsici* *Pc*PG_537485, *Pc*PG_556668, *Pc*PG_130356, *Pc*PG_130349 *Pc*PG_537480 and 2 from *P. nicotianae* *Pn*PG8 and *Pn*PG4. The S^{362} is present in 8 PGs from *P. capsici* *Pc*PG_130356, *Pc*PG_130349, *Pc*PG_556650, *Pc*PG_554601, *Pc*PG_130406, *Pc*PG_130325, *Pc*PG_130352, *Pc*PG_12536 and 5 in *P. nicotianae* *Pn*PG_7, *Pn*PG_6, *Pn*PG_17704, *Pn*PG_15171 and *Pn*PG_15164(red blocks in Figure 4.3).

```
PcPG130325      --PAGVTLDLSDVADGTTIEFTGTTTFE-TSKWEGPLILLTGTDLTVGGTG--TLDGQGD 55
PpPG7           --PAGVTLDLSDVADGATIEFTGTTTFE-TSTWEGPLILLTGTDLTVGGTG--TLDGQGD 55
PcPG130406      --PAGVTLDLSDLKSGANVVFSGTTTFG-KKKWSGPLVLLTGTKLTVSGSG--TLDGQGA 55
PpPG6           --PAGVTLDLSDLKSGANVVFSGTTTFG-TKKWSGPLVLLTGTKLSVSGSG--TLDGQGA 55
PcPG554601      --PASTTLDLTKVAKGATITFEGTTTFE-QAEWEGPLILLTGTDLTVAGTG--TLDGQGD 55
PpPG17704       --PASTTLDLTKVANGATITFEGTTTFE-YAEWEGPLILLTGTDLTVTGTG--TLDGQGD 55
PpPG9           --PKGVTLDLTDIADGATITFEGTTTFG-EEEWDGPLILLTGNDLTVTGSG--TLDGQGS 55
PcPG130384      --PAGVMLDLTNVTEGASIKFQGTTTFG-PKLWEGPLIKLTGKDLTVSGPG--TLDGQGS 55
PpPG1           --PAGVMLDLTNVTDGANIKFQGTSTFG-PKLWAGPLIKLTGNDLTVTGPG--TLDGQGA 55
PcPG21380       --PAGVTLDLSNVTSGAQIQFQGTTTFG-QKLWDGPLLKLKGTNLSVTGPG--TLDGQGS 55
PpPG10          --PAGVTLDLSNLTSGAHIHFQGTTTFG-QKLWDGPLLKLKGTDLSVTGPG--TLDGQGS 55
PcPG6556        --PAGVTLNFTKIQDNATVSFRGVTTFG-QMLWDGPLVKLQGNNLTVTGPG--TLDGQGA 55
PpPG17581       --PAGVMLNLTNLHNNTRISFHGTTTFG-KMLWAGPLVELQGNNLTVTGPG--TLDGQGA 55
PcPG511643      --PAGVTLDLSKARAGATIEFTGTTTFG-TAKWEGPLVRVSGNSLTVKGSG--TLDGQGS 55
PpPG15173       --PAGVTLDLSRTKNGATITFTGTTTFG-TAKWDGPLVRVSGNSLTVQGSG--TLDGQGS 55
PcPG130352      --PAGVTLDLSKAKDGATINFTGTTTFG-TAKWAGPLVSITGNSLTVKGSG--TLDGQGD 55
PcPG12536|      --PAGVTLDLSKAKDGATINFTGTTTFG-TAKWAGPLVSITGNSLTVKGSG--TLDGQGD 55
PpPG5           --PAGVTLDLSKAKNGATINIAGTVTFG-TQKWAGPLLSISGNSLTVKGSG--TLDGQGD 55
PcPG556654      --PAGVTLDLSKTKSGATITFTGTTTFG-TAKWEGPLVLLGGNSLTVKGSG--TLDGQGA 55
PcPG537480      --PAGVTLDLSKTKSGATITFTGTTTFG-TAKWEGPLVLLGGNSLTVKGSG--TLDGQGA 55
PpPG2           --PAGVTLDLSKAKSGATITFTGTTTFG-TAKWEGPLVLLGGNSLTVKGSG--TLDGQGA 55
PpPG3           --PAGVTLDLSKAKSGATITFTGTTTFG-TAKWEGPLVLLGGNSLTVKGSG--TLDGQGA 55
PcPG21386       --PAGVTLDLSKAKKGAIIEFLGTTTF-------------------------------- 25
PpPG15170       --PAGVTLDLSKAKKGAIIEFLGTTTFG-TQKWAGPLVLVGGTDLTVKGSG--TLDGQGM 57
PcPG130445      --PAGVTLDLSAAKTGATIEFLGTTTFG-TQKWEGPLVRVSGTDLTVKGSG--TLDGQGA 55
PpPG15171       --PAGVTLDLSRAKNGATIQFSGTTTFG-TLKWNGPLVTLSGTDITVKGNG--KLDGQGA 55
PpPG15164       --PAGVTLDLTKTKSGATIEFTGTTTFG-TKTWEGPLVLLSGSDLTVKGSG--TLDGQGA 55
PpPG15165       --PAGVTLDLTKTKSGATIEFTGTTTFG-TKTWEGPLVLLSGSDLTVKGSG--TLDGQGA 55
PcPG556650      --PAGVTLDLTKTKSGATIEFTGTTTFG-TQTWEGPLVLLSGSDLAVKGSG--TLDGQGD 55
PcPG130349      ------------------------------------TGSDLTVKGSG--TLDGQGS 18
PcPG130356      --PAGVTLDLSKAKTGATIEFTGTTTFG-TAKWDGPLVLLTGSDLTVKGSG--TLDGQGS 55
PpPG4           --PAGVTLDLSKAKTGATIEFTGTTTFG-TAKWDGPLVLLTGSSLTVKGSG--TLDGQGS 55
PcPG556668      --PAGVTLDLSKAKTGANIKISGTVTFG-QKKWAGPLVLLGGSNLKVSGSG--TLDGQGS 55
PpPG8           --PAGVTLDLSKAKTGANIKISGTVTFG-EKKWAGPLVLLGGTNLKVSGSG--TLDGQGA 55
PcPG537485      --PAGVTLDLSKAKTGANIKISGTVTFG-QKKWAGPLVLLGGSNLKVSGSG--TLDGQGS 55
AnPG            --PAGTTLDLTGLTSGTKVIFEGTTTFQ-YEEWAGPLISMSGEHITVTGASGHLINCDGA 57
ClPG            --PAGTTLDMTGLKSGTTVTFQGKTTFG-YKEWEGPLISFSGTNININGASGHSIDCQGS 57
FpPG            --PTGKQLDLSSLQNDSTVTFKGTTTFATTADNDFNPIVISGSNITITGASGHVIDGNGQ 58

PcPG130325      WYWQ----QGTSIDRP-VFFRMSKVIS-STVSDFTIKNSPYRTFSVLSCESTTISGLTLD 109
PpPG7           WYWE----QGTSIDRP-VFFRISKVIS-STVSDFTIKNSPYRTFSILSCESTKISGLTLD 109
PcPG130406      WYWK----QGTSITRP-VFFRMSKVIS-STVKGFNIKNSPYRTFSILSSQSTTVSGLTLD 109
PpPG6           WYWK----QGTSITRP-VFFRMSKVIS-STVKGFMIKNSPYRTFSILSSQSTTISGLTLD 109
PcPG554601      LYWK----KGTSITRP-VFFRLKRVMS-STVKGFNIKNSPYRTFSILDSEDTTVSGLTLD 109
PpPG17704       LYWK----KGTSITRP-VFFRLKRVMS-STVKGFNIKNSPYRTFSILDSEDTTVSGLTLN 109
PpPG9           WYWE----KGTSISKP-VFFRLKKVLS-STLENFNIKNSPYRTFSILDCEDTTLTGLTLD 109
PcPG130384      WYWP----QGQKVTRP-VFFRLSRVAN-SKLSGFSIKNMPFRTFSILSSNYTTISELLID 109
PpPG1           WYWP----QGQNITRP-VFFRLSRVAN-SKLSGFTIKNMPFRTFSILSSNYTTISELTID 109
PcPG21380       WYWP----QGQNVTKP-VFFKLNRVVN-STLSGFNLVNVPYRTFSVGNSNYTTIAGLTLN 109
PpPG10          WYWP----HGQNVTKP-VFFKMNRVIN-STLSGFNLINVPYRTFSIGNSNYTTITGLTLN 109
PcPG6556        WYWP----QGQKVDKP-VFFKLNRVVS-STLSGFTLLNMPYRTFSVGNSNYTTITGLTLN 109
PpPG17581       WYWA----QARNLTKPRWLFKMYKVAD-STISGLNVLNMPFHAFAVDSCNYTTITGVTVN 110
PcPG511643      WYWK----QGTSITRP-VFFKLQNVIG-STVSGFTLKNSPFRTFSIVTCIDTTVSGLTLD 109
PpPG15173       WYWK----QGTSITRP-VFFKLQNVVG-STVSGFTLKNSPFRTFSIVTSRDTTISGLTLD 109
PcPG130352      WYWK----QGESITRP-VFVKLQNVVG-SNISGFTVKNSPFRTFSVVTCQQTTITGLTLD 109
PcPG12536|      WYWK----QGESITRP-VFVKLQNVVG-SNISGFTVKNSPFRTFSVT------------- 96
PpPG5           WYWK----QGESITRP-VFVKLQNVVG-SNISGFTVKNSPFRTFSIVTCQQTTLSGLTLD 109
PcPG556654      WYWK----QGQSITRP-VFFRLQNVVG-STLSGFTLKNSPFRTFSIVTSKQTTLSGLTLD 109
PcPG537480      WYWK----QGQSITRP-VFFRLQNVVG-STLSGFTLKNSPFRTFSIVTSKQTTLSGLTLD 109
PpPG2           WYWK----QGQSITRP-VFFRLQNVVG-STLSGFTLKNSPFRTFSIVTSKQTTLSGLTLD 109
PpPG3           WYWK----QGQSITRP-VFFRLQNVVG-STLSGFTLKNSPFRTFSIVTSKQTTLSGLTLD 109
PcPG21386       ----------------VFFRLQNVLS-STVSGFTIKNMPFRTFSIVTSKDTTLSGLTID 67
PpPG15170       WYWK----QGQSITRP-VFFRLQNVLS-STVSGFTIKNMPFRTFSIVTSKDTTLSGLTID 111
PcPG130445      WYWK----QGQSITRP-VFFKLQNVIS-STVS------------DIVTCKDTTLTGLTID 97
PpPG15171       WYWK----QGQQITRP-VFFQVHKVIH-STISGFTIMNAPYRTFSIVDSMYTTLTGLTLD 109
PpPG15164       WYWK----QGTSISRP-VFFRLHAVTG-STLSGFTLKNSPFRTFSILNSEKTTISGLMLD 109
PpPG15165       WYWK----QGTSISRP-VFFRLHAVTG-STLSGFTLKNSPFRTFSILNSEKTTISGLMLD 109
PcPG556650      WYWK----QGTSISRP-VFFRLHAVTD-STLSGFTLKNSPFRTFSILNSEKTTISGLTLD 109
PcPG130349      WYWK----QGTSISRP-VFFRMNKVLS-STVSGFTLKSMPYRTFSVLNSQKTTISGLTLD 72
PcPG130356      WYWK----QGTSISRP-VFFRMNKVLS-STVSGFTLKSMPYRTFSVLNSQKTTISGLTLD 109
PpPG4           WYWK----QGTSISRP-VFFRMNKVLS-STVSGFTLKNMPYRTFSILNSQKTTISGLTLD 109
PcPG556668      WYWK----QGTSISRP-VFFRLHAVTG-STLSGFKIKNSPYRTFSILNSEKTTITGLTLD 109
PpPG8           WYWK----QGTSISRP-VFFRLHGVTG-STLSGFKIKNSPYRTFSILNSEKTTISGLTLD 109
PcPG537485      WYWK----QGQSITRP-VFFRLQNVLS-STVSGFTIKNMPFRTFSIVTCKDTTLSGLTID 109
AnPG            RWWDGKGTSG--KKKPKFFYAHGLDS--SSITGLNIKNTPLMAFSVQ-ANDITFTDVTIN 112
ClPG            RWWDSKGSNGG-KTKPKFFYAHSLKS--SNIKGLNVLNTPVQAFSINSATTLGVYDVIID 114
FpPG            AYWDGKGSNSNSNQKPDHFIVVQKTTGNSKITNLNIQNWPVHCFDITGSSQLTISGLILD 118
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PcPG130325      SSAGDDVAK---------NTDGFDLSRNTDVTISGNTIYNQDDCLAMQSSTNTVFSSNTC 160
PpPG7           SSDGDNVAK---------NTDGFDLSKNTGVTISGNTIYNQDDCLAMQSSTNTIFSSNTC 160
PcPG130406      SSDGDGSAK---------NTDGFDLSRNTGVTITGNKIYNQDDCLAMQSSTNTVFSSNTC 160
PpPG6           SSDGDDLAK---------NTDGFDLSKNTDITITGCKIYNQDDCLAMQSSTNTVFSSNTC 160
PcPG554601      SKAGDNVAK---------NTDGFDLSRNLRLTISGCTVYNQDDCLAMQSSNDTTFTGNTC 160
PpPG17704       SKAGDNVAK---------NTDGFDLSRNLRLTISGCTVYNQDDCLAMQSSNDTTFTGNTC 160
PpPG9           SSAGDGTAK---------NTDGFDLSRNVRLTISDCTVYNQDDCLAMQSSNDTTFSGNTC 160
PcPG130384      SRAGNGSAK---------NTDGFDLSRNDHVTITNNRVYNQDDCLAMQSSSNTVFSNNYC 160
PpPG1           SRAGNGSAK---------NTDGFDLSRNNHVTITNNRVYNQDDCLAMQSSSNTVFSNNYC 160
PcPG21380       STAGNGVAK---------NTDGFDLSKNDHFTITGNRIYNQDDCLAMQSSTNTVFSNNYC 160
PpPG10          STAGNGIAK---------NTDGFDLSKNDHFTITGNRIYNQDDCLAMQSSTNTVFSNNYC 160
PcPG6556        SSAGNGVAK---------NTDGFDLSKNDHMTITKNRIYNQDDCLAMQSSTNTLFSYNYC 160
PpPG17581       SSAGNGVAK---------NTDGFDLDKNDHVTITKNRIFGQDDCLAMQSSTNIVFSDNYC 161
PcPG511643      SSSGNGIAK---------NTDGFGLTKNDHVTISGNKIFNQDDCLAMQSSTNTFFRNNYC 160
PpPG15173       SSSGNGIAK---------NTDGFGLTKNDHVTISGNKIFNQDDCLAMQSSTNTFFRNNYC 160
PcPG130352      SSAGNGLAQ---------NTDGFDMTKNDHVTITGNKIYNQDDCLAMQSSTNTIFSNNLC 160
PcPG12536|      ----------------------------------------------------------- 
PpPG5           SSAGNGRAQ---------NTDGFDMTKNDHVTITGNKIYNQDDCLAMQSSTNTIFSNNLC 160
PcPG556654      SSAGNGLAK---------NTDGFDLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNLC 160
PcPG537480      SSAGNGLAK---------NTDGFDLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNLC 160
PpPG2           SSAGNGLAK---------NTDGFDLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNLC 160
PpPG3           SSAGNGLAK---------NTDGFDLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNLC 160
PcPG21386       SRAGNGLAK---------NTDGFGLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNYC 118
PpPG15170       SRAGNGLAK---------NTDGFGLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNYC 162
PcPG130445      SRAGNGIAK---------NTDGFDLTKNDHITITGNKIYNQDDCLAMQSSTNTVFSNNYC 148
PpPG15171       SRAGNGIAT---------NTDGFDLSCNDHITITGNTIYNQDDCLAMQSSTNTFFTNNHC 160
PpPG15164       SSAGDGTAK---------NTDGFDLSRNDGVTITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PpPG15165       SSAGDGTAK---------NTDGFDLSRNDGVTITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PcPG556650      SSAGDGTAK---------NTDGFDLSRNDGVTITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PcPG130349      ASAGDGTAK---------NTDGFDLSGNNGVSITGNKIYNQDDCLAMQSSTNTVFSNNYC 123
PcPG130356      ASAGDGTAK---------NTDGFDLSGNNGVSITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PpPG4           ASAGDDLAK---------NTDGFDLSGNDYVTITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PcPG556668      SSAGNGLAK---------NTDGFDLSRNNYVTITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PpPG8           SSAGDGTAK---------NTDGFDLSKNDYVTITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
PcPG537485      SSAGNGLAK---------NTDGFDLTKNNHITITGNKIYNQDDCLAMQSSTNTVFSNNYC 160
AnPG            NADGDTQGG--------HNTDAFDVGNSVGVNIIKPWVHNQDDCLAVNSGENIWFTGGTC 164
ClPG            NSAGDSAGG--------HNTDAFDVGSSTGVYISGANVKNQDDCLAINSGTNITFTGGTC 166
FpPG            NRAGDKPNAKSGSLPAAHNTDGFDISSSDHVTLDNNHVYNQDDCVAVTSGTNIVVSNMYC 178

PcPG130325      SGGHGISIGSIGGSSVSSSDTVSGLTVKNNKIVDSVNGIRIKTIIDDYGEVTDVTYTDNK 220
PpPG7           SGGHGISIGSIGGSSVSSSDTVSGLTVKNNKIVNSVNGIRIKTIIDDYGEVTDVTYMDNT 220
PcPG130406      SGGHGISIGSIGGSSITSSDTVSGLTVKNNKIVDSVNGIRIKTIIDLTGKVTDVTYTDNT 220
PpPG6           SGGHGISIGSIGGSSISSSDTVSGLTVKNNKIVDSVNGIRIKTIIDLTGKVTDVTYTGNT 220
PcPG554601      SGGHGISIGSLGGDSVTESDIVSGLTVKNNKIIDSVNGIRIKTIAGKQGTITGASYTDNT 220
PpPG17704       SGGHGISIGSLGGDLVNKSDIVSGLTVKNNKIIDSVNGIRIKTIIGKHGTVTGASYTGNT 220
PpPG9           SGGHGISIGSLGGDSVTESDIVSGLTVKNNKIVDSVNGIRIKTIIDLYGTITGATYTDNT 220
PcPG130384      SGSHGISIGSLGGPEQNVNTTVSNLLVKDNTIVNSDNGIRIKTIIGLKGLVTNATYLNNR 220
PpPG1           SGSHGISIGSLGGPEQNVNTTVSGLLVKDNTIVNSTNGIRIKTIIGLKGLVTNVTYTNNR 220
PcPG21380       SGGHGISIGSLGGDKQDSSTTVAGLLVKGNTIVDSDNGIRIKTIIGLKGLVTDASYINNK 220
PpPG10          SGGHGISIGSLGGDKQDSSTTVAGLLVKGNTIVNSDNGIRIKTIIGLQGLVTDASYIDNK 220
PcPG6556        CGSHGISIGSLGGPEQNSSTTVDGLTVTGNTIVNSTNGIRIKTIIGLKGLVTNAVYTNNK 220
PpPG17581       CGTHGISIGSLGGPEQNASTTVDGLTVTGNTIVNSTNGIRIKTIIGLKGLVTNAVYTNNE 221
PcPG511643      SGSHGISIGSLGGNAVDQSTTVTGLMVEGNTIANSDNGLRIKTVIGLKGLVSNVKYINNK 220
PpPG15173       SGSHGISIGSLGGNAVDQSTTVTGLTVEGNTIVNSDNGLRIKTVIGLKGLVSNVKYINNK 220
PcPG130352      SGSHGISVGSIGGAAVDQTTTVSGLTVQGNTITNSDNGLRIKTLIGMKGLVTNVKYINNQ 220
PcPG12536|      -------------------TTVSGLTVQGNTITNSDNGLRIKTLIGMKGLVTNVKYINNQ 137
PpPG5           SGSHGISVGSIGGNAVDETTTVSGLTVQGNTIQNSDNGLRIKAIIGMKGLVTNVKYINNK 220
PcPG556654      CGGHGVSIGSLGGAAVDQGSTVQGLTVQGNTIQDSDNGIRIKTIIGLKGLVSNVKYVNNK 220
PcPG537480      CGGHGVSIGSLGGAAVDQGSTVQGLTVQGNTIQDSDNGIRIKTIIGLKGLVSNVKYVNNK 220
PpPG2           CGGHGVSIGSLGGNAVDQSSTVQGLTVQGNTIQNSDNGIRIKTIIGLKGLVSDVKYVDNQ 220
PpPG3           CGGHGVSIGSLGGNAVDQSSTVQGLTVQGNTIQNSDNGIRIKTIIGLKGLVSDVKYVDNK 220
PcPG21386       CGSHGISVGSLGGNSVDQSTTVQGLIVEGNTIVDSDNGLRIKTIIGLKGLVSDVKYVDNE 178
PpPG15170       CGSHGISVGSLGGNAVDQSTTVQGLTVEGNTIVDSDNGLRIKTIIGLKGLVTDVKYVDNE 222
PcPG130445      CGGHGISIGSLGGNAVDQSTTVEGLTVEGNTIENSDNGIRIKTIIGLKGLVTNVKYLNNE 208
PpPG15171       YNSHGISVGSLGGNAVDKTTTVAGLTVQGNHIHDSDNGLRIKTIIGLKGLVKDVKFIDNN 220
PpPG15164       SGGHGISIGSLGGDSVTSSDTVSGLTVTGNSIINSVNGIRIKTIIGLKGLVSNVKYTNNK 220
PpPG15165       SGGHGISIGSLGGDSVTSSDTVSGLTVTGNSIINSVNGIRIKTIIGLKGLVSNVKYTNNK 220
PcPG556650      SGGHGVSIGSLGGDSVTSSDTVSGLSVTGNTIVNSVNGIRIKTIIGLQGLVSNAKYTNNK 220
PcPG130349      SGGHGISIGSLGGSTVSSSDTVSGLTVSGNTIVNSVNGIRIKTIIGLEGLVSNAKYTNNK 183
PcPG130356      SGGHGISIGSLGGSTVSSSDTVSGLTVSGNTIVNSVNGIRIKTIIGLKGLVSNAKYTNNK 220
PpPG4           SGGHGISIGSLGGSTVSSSDTVSGLTVSGNSIVNSVNGIRIKTIIGLKGLVSNVKYSNNK 220
PcPG556668      SGGHGISIGSLGGTAVNQGSTVQGLTVKGNTIVNSVNGIRIKTIVDLKGLVSDVTYTDNK 220
PpPG8           SGGHGISIGSLGGSSVSSADTVKGLTVKGNTIVNSVNGIRIKTIVDLKGLVSDVTYTNNK 220
PcPG537485      SGGHGISIGSLGGTAVNQGSTVQGLTVKGNTIVNSTNGLRIKTIVDLKGLVSDVTYTDNK 220
AnPG            IGGHGLSIGSVGDRS---NNVVKNVTIEHSTVSNSENAVRIKTISGATGSVSEITYSNIV 221
ClPG            SGGHGLSIGSVGGRS---DNTVKTVTISNSKIVNSDNGVRIKTVSGATGSVSGVTYSGIT 223
FpPG            SGGHGLSIGSVCGKS------DNVVDGVQFLSSQVVNSQNGCRIKSNSGATGTINNVTYQNIA 235
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PcPG1J0325      SGWTFSGIDVSG--DTGSCSGEPSGVSC 302
PpPG7           SGWTFSGIDVSG--GTGSCNGEPSGISC 302
PcPG130406      SGWSFSGIDVTG--STGSCSGEPSSVDC 302
PpPG6           SGWTFSGIDVSG--STGSCSGEPSSVNC 302
PcPG554601      SGWTFSGITVKG--SAGSCSGEPSGVKC 302
PpPG17704       SGWTFSGITVKG--DSGSCSGEPSDVKC 302
PpPG9           SGWTFSDITVTG--DTGNCSGEPSGVGC 302
PcPG130384      SNWEFKNIAVNAT-NIGQCKGGPSNVQC 303
PpPG1           SNWEFKNVAVNAT-NIGKCQGGPSNVQC 303
PcPG21380       SNWEFKNVAVNAT-NNGQCKGDPSNVLC 303
PpPG10          SNWEFNNIAVNAT-NVGQCNGGPSNVQC 303
PcPG6556        SNWNFKNLDVTAS-SNGNCTGEPSNIQC 303
PpPG17581       SNWDFKNLDVVVS-SNGNCTGEPSNIQC 304
PcPG511643      SNWKFSGITASG--AKGVCKGQPSGINC 302
PpPG15173       SNWKFSGISVSG--AKGVCKGQPSGITC 302
PcPG130352      SGWTFKGITVNG--AKGSCKGQPAGITC 302
PcPG12536|      SGWTFKGITVSG--AKGSCKGQPAGITC 207
PpPG5           SGWTFKGITVSG--AKGTCKGQPNGITC 302
PcPG556654      SDWTFSGIEVSAS-ATGKAVGQPNSIDV 303
PcPG537480      SSWSFSGIEVSAS-KTGKAVGQPNSLNV 303
PpPG2           SDWTFSGIQVSAS-ANGKAVGQPNSLDV 303
PpPG3           SDWSFSGIKVSAS-ANGKAVGQPNSVSV 303
PcPG21386       SDWTFSGIDVSAS-VKGTLTGMPNSLAV 261
PpPG15170       SDWTFSGIDVSAS-LKGTLAGMPNNLAV 305
PcPG130445      SAWAFSDVKVSAS-VKGKIAGLPNSLSI 291
PpPG15171       SSWKFSGIQVKAA-AKGKLNGVPGGVAV 303
PpPG15164       SDWTFSGVTVSAS-SKGSCSGQPSSITC 303
PpPG15165       SDWTFSGVTVSAS-SKGSCSGQPSSITC 303
PcPG556650      SDWTFSDITVTAS-SQGSCSGQPSSITC 303
PcPG130349      SNWSLSGITVTAS-KTGSCSGQPSNVKC 266
PcPG130356      SNWSFSGITVTAS-KTGSCSGQPSSISC 303
PpPG4           SDWSFSGITVSAS-KTGTCSGQPSSIDC 303
PcPG556668      SNWKFSGITVKAS-KTGKCSGQPSTVKC 303
PpPG8           SNWKFSGVTVKAS-KTGTCNGQPSTVKC 303
PcPG537485      SNWKFSGITVKAS-KTGKCSGQPSTVKC 303
AnPG            SDWTWDDVKVTGGKKSTACKNFPSVASC 307
ClPG            SNWKWSGVSVTGGKKSTKCSNIPSGSGA 309
FpPG            SGFTFSGNAITGGGKTSSCNYPTNTCPS 321
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Figure 4. 3: Multiple alignment of the C- terminal conserved region of the Polygalacturonase from *P.nicotianae* and *P.capsici* and 3 from fungus *Fusarium phyllophilum* (*Fp*PG) *Aspergillus niger* (*An*PG) and *Colletotrichum lupini* (*Cl*PG) analyzed by ClustalW. All the PGs contain the four conserved region in the catalytic site highlighted in gray blocks. Underlined in red are all the residues that were found important in the interaction with of *Fp*PG with PGIP.

A region that play important role in the interaction of the protein with both the pectin molecule and different substrate is the polyglutamine region. Three protein sequences from *P. capsici*, *Pc*PG_537480, *Pc*PG_21386, *Pc*PG_556654 and four from *P. nicotianae* *Pn*PG_2, *Pn*PG_3, *Pn*PG_15164, *Pn*PG_15165, contain glutamine-rich region after the N-terminal site(excluded from the previous alignment)(Figure 4.4). This was observed also *P. cinnamomi* PGs. (Götesson *et al.*, 2002).

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PnPG_15164      HHKHHQVQNSATSSGSNNQQQQQQQQQQQQQQQQQQQQQQSTNSGSNNAQQQTQTSTTS
PnPG_15165      HHKHHQVQNSATSSGSNNQQQQQQQQQQ----------QQSTNSGSNNAQQQTQTSTTS
PcPG537480      ----------QQQVQQEQQQQQSSTSTSSSGCNLTGTYKKGTDISSCSSVTIGSLTVPA
PcPG556654      ----------QQQVQQEQQQQQSSTSTSSSGCNLTGTYKKGTDISSCSSVTIGSLTVPA
PnPG_2          ----------QQQVQQQQQ-----SSTSSSGCNLTGTYKKGTDISSCSSVTIGSLTVPA
PnPG_3          ----------QQQVQQQQQ-----SSTSSSGCNLTGTYKKGTDISSCSSVTIGSLTVPA
PcPG21386       -----EEQQQQQQDPVQQ---QQQSPTTSGKGCNLTGTYKKGTDISTCSSITVNSLTVPA
```

Figure 4. 4: Alignment showing the PGs from *P.capsici* and *P.nicotianae* that they contain glutamine-rich repeats.

Based on the predicted number of the potential N-glycosylation sites, PGs from the *Phytophthora spp* were classified in two groups. One group consists of PGs slightly glycosylated and one that are considered as heavily glycosylated. The *Pc*PG_12536, *Pc*PG_130352, *Pc*PG_130356, *Pc*PG_130384, *Pc*PG_21380 and *Pc*PG_6556 from *P. capsici* contain 4-5-3-9-10-15 N-glycosylation sites respectively. The rest of the PGs are lightly glycosylated with zero or one glycosylated site.

*Pn*PG_10, *Pn*PG_15174 and *Pn*PG_20263 contains 11-9-14 and 8 glycosylation sites respectively and other PGs ranging from zero to two glycosylation sites. *PI*TG_10880TO and *PI*TG_19649TO identified from *P. infestans*, contains 13 and 9 glycosylation sites respectively and the other PGs ranging from zero to two glycosylation sites (Table 4.1).

The predicted pI values of the proteins, using the Bjellqvist method (Rajamurugan *et al.*, 2012), ranging from 4.21 to 9.68. The majority of the PGs presents neutral or slightly basic pI values (5.52-9.7) (Henrissat, Vegetales, & Grenoble, 1991) in each protein. The pI may influence enzyme specificity for substrates with different charge (Bradford, 1976). A basic pI may confer affinity for negatively charged polygalacturonate. Basic pI may also decrease protein mobility within the cell wall, leading to highly localized wall degradation (Table 4.1).

***P. infestants* PGs**				***P.capsici* PGs**				***P. nicotianae PGs***			
Protein ID	**Length**	**Glycosylatio sites**	**pI**	**Protein ID**	**Length**	**Glycosylation sites**	**pI**	**Protein ID**	**Length**	**Glycosylation sites**	**pI**
PITG_19619T0	294	0	6.42	PcPG_12536	256	4	9.05	PpPG1	391	11	5.2
PITG_19623T0	379	1	9.11	PcPG_130325	361	1	4.21	PpPG2	380	1	8.9
PITG_16825T0	322	1	9.71	PcPG_130349	266	1	8.81	PpPG3	380	1	9.3
PITG_10255T0	380	0	8..93	PcPG_130352	360	5	7.54	PpPG4	362	0	6.2
PITG_19985T0	283	9	9.54	PcPG_130356	362	3	9.03	PpPG5	360	2	5.9
PITG_16824T0	361	1	9.5	PcPG_130384	392	9	5.6	PpPG6	363	0	5.2
PITG_19625T0	373	1	6.88	PcPG_130406	360	0	8.11	PpPG7	358	1	4.1
PITG_19649T0	282	9	9.17	PcPG_130445	403	2	8.9	PpPG8	361	0	9.5
PITG_21245T0	381	3	9.26	PcPG_21380	362	10	5.52	PpPG9	512	2	4
PITG_17899T0	221	1	5.06	PcPG_21386	353	1	9.04	PpPG10	391	9	5
PITG_19653T0	249	0	7.62	PcPG_511643	361	1	8.89	PpPG_15164	452	0	8.43
PITG_19455T0	339	3	9.38	PcPG_537480	651	1	9.32	PpPG_15170	442	0	8.42
PITG_19636T0	158	0	7.72	PcPG_554601	470	1	6.82	PpPG_15171	397	2	8.91
PITG_16822T0	335	1	9.42	PcPG_556650	374	0	6.29	PpPG_15172	371	3	8.97
PITG_21248T0	340	1	6.37	PcPG_556654	385	1	8.84	PpPG_15173	361	2	9.07
PITG_21247T0	362	2	8.9	PcPG_556668	361	1	9.7	PpPG_15174	439	14	5.19
PITG_19624T0	373	2	8.85	PcPG_557807	157	0	9.68	PpPG_15180	467	2	6.44
PITG_10880T0	419	13	6.11	PcPG_6556	413	15	6.07	PpPG_17581	74	0	6.51
PITG_19637T0	254	0	9.15	PcPG_537485	357	1	9.87	PpPG_17704	108	0	9.44
PITG_21925T0	261	0	8.17					PpPG_20263	280	8	5.93
PITG_19620T0	377	3	9.34								
PITG_19634T0	217	0	6.03								
PITG_19621T0	136	2	10.12								

Table 4. 1: Distinct characteristics of the PGs from the species of Phytophthora. The Glycosylation sites and the theoretical pI values were predicted.

All this analysis were carried on PGs of *P. infestans, P. capsici* and *P. nicotianae* identified from previous pipeline (§ 4.1). Only ten PGs from *P. nicotianae* (Wu *et al.*, 2008) one from *P. infestans* and two from *P.capsici* (Li *et al.*, 2012; Sun *et al.*, 2009) were previously characterized (Torto, Rauser, & Kamoun 2002).

4.2 Polygalacturonase from *P.nicotianae*: Expression and Biochemical characterization

4.2.1 Effect of different carbon source in the expression of the PGs in vitro.

To investigate the effect of different carbon source on the expression of the PGs from *P. nicotianae* the strain was grown respectively on a minimal medium containing 2.5% glucose and on minimal medium containing 1% of pectin from apple for 14 days at 28 °C. RNA was isolated from frozen mycelium and treated afterwards with DNAse (Ambion, Foster City, CA, USA) to remove genomic DNA contamination, in accordance with the instructions of the manufacturers. To test for genomic DNA contamination, RT-PCR using primers specific for *Pc*Tubulin (Forward 5'GACTCGGTGCTTGATGTTGTC3' Reverse 5'CCATCTCATCCATACCCTCGCCAG3') were used. Reverse transcriptase PCR was performed on the extracted RNA as reported previously (§3.6.4). The primers used were based on the sequence of the PGs from the previous paper Wu *et al.*, 2008.

Mycelium grown on minimal medium containing glucose did not show any PGs expression. 8 *pnpg* (*pnpg*_1, *pnpg*_2, *pnpg*_3, *pnpg*_4, *pnpg*_5, *pnpg*_6, *pnpg*_7 and *pnpg*_8) PG genes out of 10 are differentially expressed when mycelium is grown on minimal medium containing apple pectin (Figure 4.5 A_B). cDNA was synthesized using 1µg of total RNA, to verify the state of the cDNA the *Pc*tubulin primers were used (Figure 4.5_C).

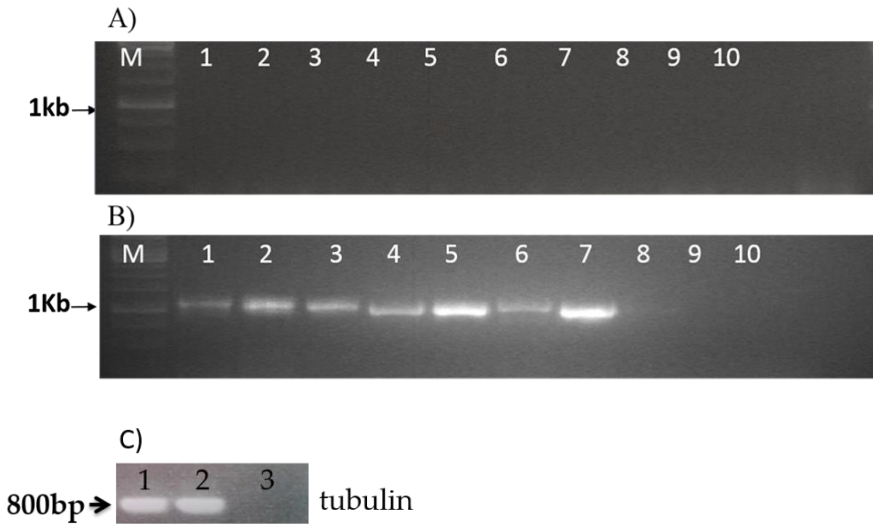


Figure 4. 5*:* RT-PCR for measured the relative mRNA level of the PGs in each treatment. **A)** There is not expression of the PGs in the medium with 2,5% of glucose but **B**) 8 PGs out of 10 are expressed in the medium containing 1% of pectin from apple. **C)** Positive control using the endogenous gene tubulin.

PGs were produced and enzyme activity was confirmed by Agar Diffusion Assay (§3.10.7) 200 µL of *P. nicotianae* colture, grown on crude minimal medium at 27 °C were collected after 24, 72 and 150 h and used for analysis (Figure 4.6). After 12 h of incubation at 30 °C, enzyme activity was detected by halos formation using 6M HCl as revealing agent. Only samples of minimal medium supplemented with 1% pectin show activity.

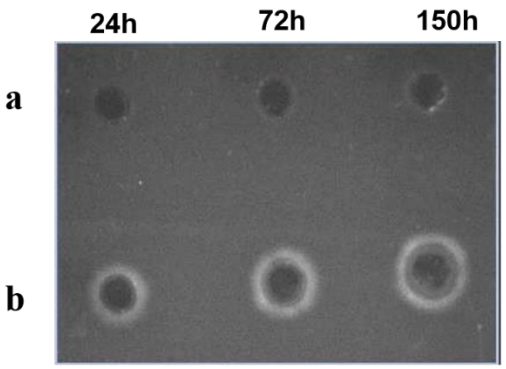


Figure 4. 6: Agar diffusion assay was performed with samples of 200 µL of culture media taken at 24, 72 and 150h. a) Time course experiment, of *P. nicotianae* grown on minimal medium containing 2,5 % of glucose; b) time course of *P. nicotianae* grown on 1% of apple pectin. The halos on row "b" indicates an increasing PG activity. No activity is detected on row "a".

4.2.2 Expression and sequence analysis of the PGs.

Five members of the *Pn*PG family (*pnpg*_2, *pnpg*_3, *pnpg*_5, *pnpg*_7 and *pnpg*_9) were cloned and expressed in *P. pastoris*. The selection of these PGs was based on their expression levels during the *in vitro* induction experiments: low expressions levels for *pnpg*_2 and *pnpg*_3, higher expression levels for *pnpg*_5 and *pnpg*_7 and no expression for *pnpg*_9. The genes were amplified by PCR using cDNA from *P.nicotianae* as template and primers designed according to nucleotide sequences available in GenBank (table 4.2). The product of PCR amplifications were separated by electrophoresis and recovered.

DNA fragments corresponding to the coding sequence of mature protein of *pnpg*_2, *pnpg*_3 *pnpg*_5, *pnpg*_7 and *pnpg*_9 genes were cloned into expression plasmid pGAPZα A (§3.7.1 - Invitrogen) in frame with α factor. The constructed pGAPZα A-*Pn*PG plasmids were used to transform *E. coli* DH5α competent cells by electroporation (§3.6.11). Colonies carrying the zeocin resistance were inoculated on 10 mL of LSLB (§3.2.2) and incubated at 37 °C. Plasmid were purified as described at §3.6.2 and sequenced. Plasmids were linearized with *Avr*II and used to transform *P. pastoris* X-33 competent cells by electroporation. The recombinant PGs were produced in the culture medium and analyzed by agar diffusion assay (§3.10.7) for their activity. All cloned PGs are secreted and active (data not show).

Name	Primer
*Pn*PG2Fw	ATG CATGAATTCTCGCCCATGCTCCGCA
*Pn*PG2Rv	ATGCATTCTAG TACACGTCGAGGCTGTTG
*Pn*PG3Fw	ATGCATGAATTCTCGCCCATGCTCCGC A
*Pn*PG3Rv	ATGCATTCTAGATACACGCTAACGCTGTTG
*Pn*PG5Fw	ATGCATGAATTCCCCATGATCCGCCAAGA
*Pn*PG5Rv	ATGCATTCTAGATAACAAGTGATACCATTCGG
*Pn*PG7Fw	ATGCATGAATTC TCATCGATGATTCGTCAA G
*Pn*PG7Rv	ATGCATTCTAGATAGCAGCTAATGCCACTAG
*Pn*PG9Fw	ATGCATGAATTCAACCAGGCCCAAGGAGG
*Pp*PG9Rv	ATGCATTCTAGATAGCAGCCTACTCCACTC

Table 4. 2: Primers used for amplification of PG genes from *P.nicotianae*. The amplified genes were cloned in pGAPZα A

For the amplification of the ten *pnpg* genes and the sequence analysis of them I designed primers based on the sequence available on GeneBank from the paper Wu *et al.*, 2008. The results have revealed differences on the sequence level for some of the genes: four of the PGs from *P. nicotianae* (*pnpg*_2, *pnpg*_3, *pnpg*_5, *pnpg*_6 and *pnpg*_9) show the 100% of homology with those deposited in GenBank. *pnpg* 4 show a single mutation at the position 368 the amino acid phenylalanine becomes leucine, *pnpg*7 at the position 140 the amino acid threonine becomes lysine and for *pnpg*_8 a single mutation at the position 110 the amino acid lysine becomes glutamic acid. Finally, I found that *pnpg*_1 and *pnpg*_10 present an Internal extra sequence compared with those of *P. parasitca*. Further analysis did not give any evidence of intron sequences on *pnpg*_1 and *pnpg*_10 (figure 4.7).

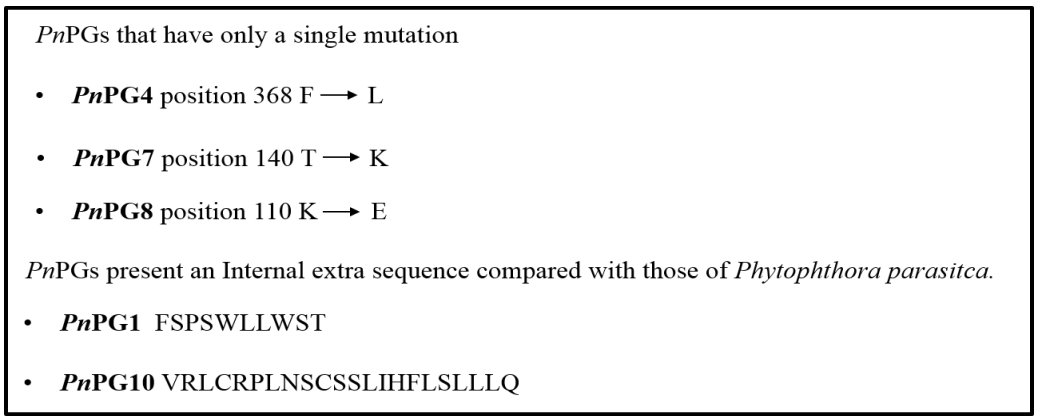


Figure 4. 7: analysis between the sequence amplified and sequenced from *P.nicotianae* with those deposited in GenBank

4.2.3 Characterization of PGs expressed in P. pastoris

The production of the five members of the polygalacturonase from *P. nicotianae* expressed in *P. pastoris* was high after 3 days of cultivation and was increased by stirring at 200 rpm. Each polygalacturonase was purified from the culture broth. Cell free media were concentrated using a Vivaflow 200 and dialyzed against 20 mM Na acetate pH 4.1. Dialyzed proteins were mixed with a suspension of diethylaminoethyl (DEAE) cellulose pre-equilibrated with 20 mM Na acetate pH 4.6.

The not-absorbed proteins were loaded onto a column S-Sepharose pre-equilibrated with 20 mM Na acetate pH 4.1. Elutions were performed manually using a gradient of NaCl (0 to 1 M) in 20 mM Na acetate pH 4.1. Fractions with highest activity showed on agar diffusion assay were further analyzed and by silver stain in polyacrylamide gels (Figure 4.8). Fractions that showed the highest activity were pooled and stored at -20 °C. The purified proteins were quantified by densitometric analysis (§3.10.5) obtaining the following concentrations: 4 ng/µL for *Pn*PG_2, 3 ng/µL for *Pn*PG_3, 25 ng/µL for *Pn*PG_5, 12 ng/µL for *Pn*PG_7 and 9 ng/µL for *Pn*PG_9. The purified *Pn*PGs were further characterized for optimum of pH, effect of temperature on activity, thermal stability and kinetics parameters.

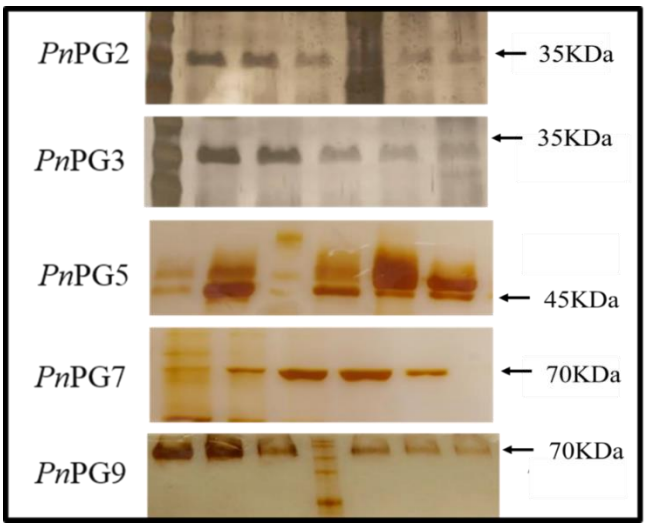


Figure 4. 8: SDS-PAGE analysis of the PGs from *P. nicotianae*, expressed from *P. pastoris*. The fractions with highest activity showed on agar diffusion assay was further analyzed.

The *Pn*PGs activity was determined by measuring the reducing sugar released as a result of the hydrolysis of polygalacturonic acid (20 mg/mL) using a Somogyi Nelson as described §3.10.8.2 (Norton, 1944) reagent at 520 nm and 4-Hydroxybenzoic acid hydrazide (PAHBAH) assay as described §3.10.8.1.

4.2.3.1 Optimum of pH

The optimum of pH experiments were performed with purified enzymes using 40 ng of *Pn*PG_2, 50 ng of *Pn*PG_3, 200 ng of *Pn*PG_5, 1 µg of *Pn*PG_7 and 80 ng of *Pn*PG_9. Results show a peak at pH 6 for *Pn*PG_2, at pH 6,5 for *Pn*PG_3, and 3,5 for the *Pn*PG_5. *Pn*PG_7 and *Pn*PG_9 have the optimum of their activity at pH of 5. *Pn*PG_2, *Pn*PG_3, *Pn*PG_5, *Pn*PG_7 have a shape peak of activity at their optima, *Pn*PG_9 shows a peak of activity at pH 5 but also shows a good activity ranging from 4 to 6,5 (Figure 4.9).

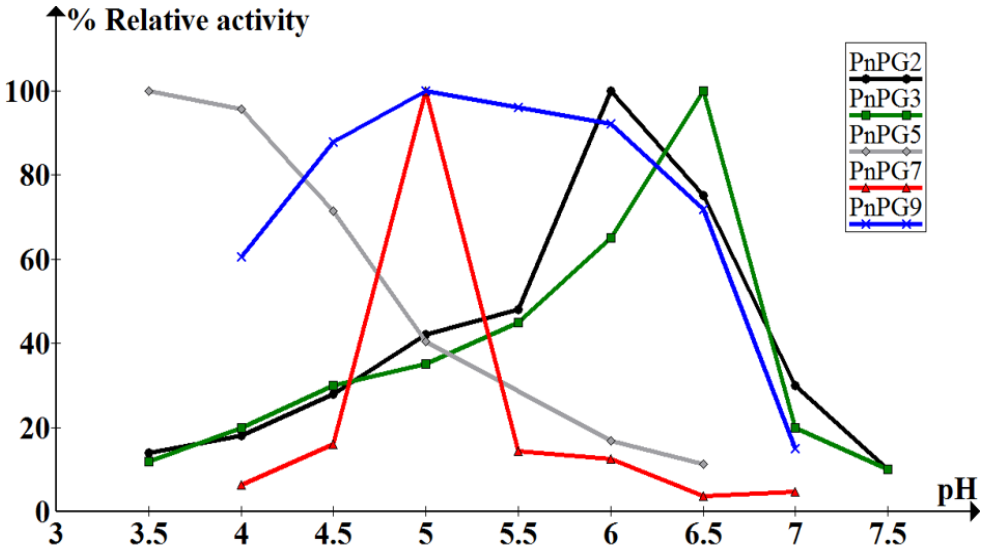


Figure 4. 9: Effect of pH on activity of *Pn*PG_2, *Pn*PG_3, *Pn*PG_5, *Pn*PG_7 and *Pn*PG_9. The assay of and pH optimum were performed by measuring activity of incubating enzymes at pH range of 3.5 - 7.5 as described in Materials and methods §3.9.7 using 1% of polygalacturonic acid from citrus was used as substrate. For relative activity, the highest activity was taken as 100% in assay of optima. Each data point represents the mean of samples assayed in triplicate.

4.2.3.2 Effect of the temperature on *Pn*PGs activity

The effect of the temperature on enzyme activity experiments were performed with purified enzymes using 80 ng of *Pn*PG_2, 90 ng of *Pn*PG_3, 200 ng of *Pn*PG_5, 1 µg of *Pn*PG_7 and 400 ng of *Pn*PG_9. The optimum of the temperature for *Pn*PG_2 and *Pn*PG_3 is 45°C and they show the maximum of their activity. The optimum of the temperature of the *Pn*PG_5 is 50 °C but its activity is still high across the range of the tested temperatures. The optimum of the temperature of the *Pn*PG_7 is 40 °C, is still high at 45°C but rapidly decrease at 50 °C. The optimum of the temperature of the *Pn*PG_9 is 45 °C (Figure 4.10).

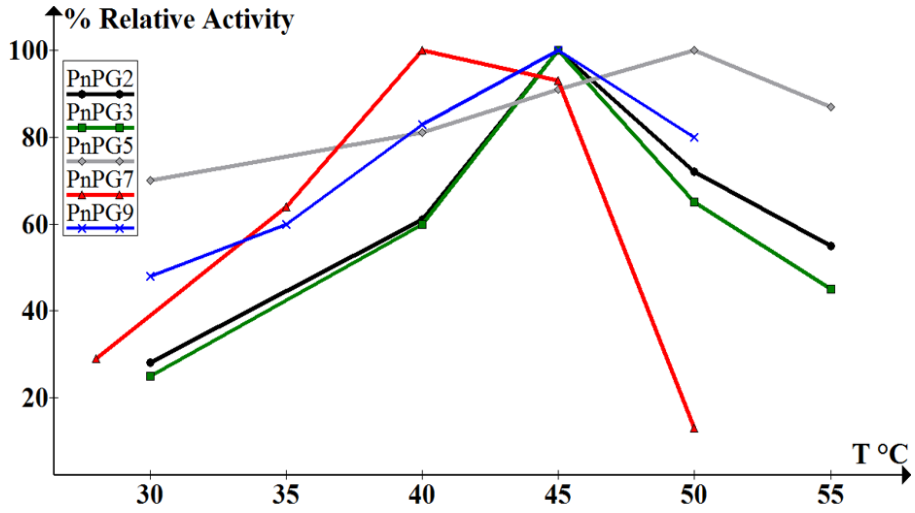


Figure 4. 10: Temperature profiles of the purified Polygalacturonase. activity was determined at a temperature range of 28 - 55°C. The relative activity was plotted against different temperatures. Each data point represents the mean of samples assayed in triplicate.

4.2.3.3 Kinetic analysis of PnPGs

The K_M and Vmax values of the PGs were determined with purified enzymes using 200 ng of *Pn*PG_5, 1 µg of *Pn*PG_7 and 400 ng of *Pn*PG_9, both by nonlinear regression analysis using the Michaelis–Menten equation and by Lineweaver-Burk plot (Figure 4.11). The values of Km and Vmax of all the PGs are summarized on table 4.3. *Pn*PG_2 and *Pn*PG_3 are unstable enzymes and therefore were not analyzed. The *Pn*PG_2 and *Pn*PG_3 activities calculated in previous analysis at 30 °C under optimum of pH are 0.17 U/mL and 0.20 RGU/mL utilizing 200 and 150 ng respectively.

PG protein	**K_M (mg/mL)**	**Vmax (U/mL)**
*Pn*PG5	6.8	22.55
*Pn*PG7	0.17	0.33
*Pn*PG9	1.5	3.55

Table 4. 3 : The **K_M** and **Vmax** values of *Pn*PGs were determined both by nonlinear regression analysis using the Michaelis–Menten equation and by Lineweaver-Burk plot.

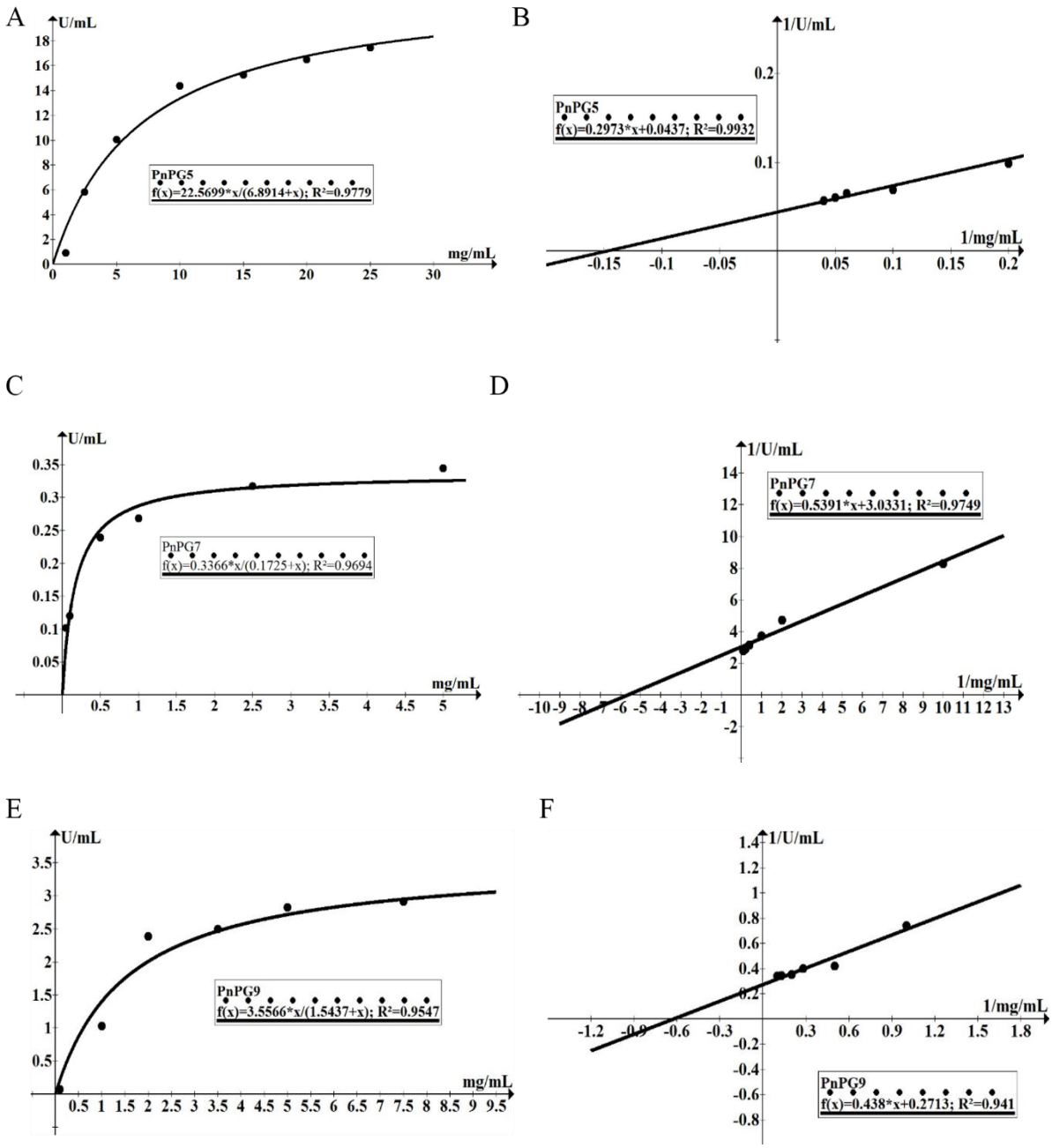
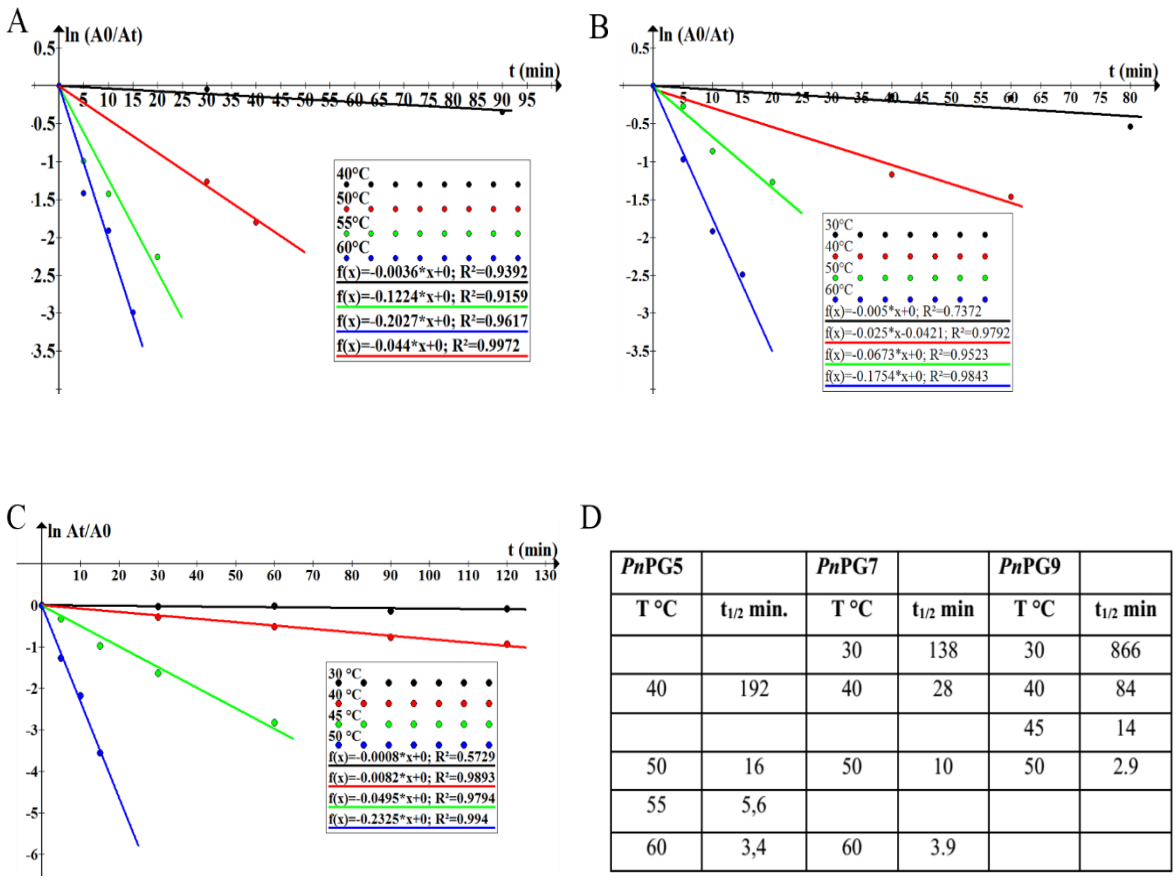


Figure 4. 11: Michaelis-Menten curve of polygalacturonase activity and Lineweaver-Burk plot analysis relating to *Pn*PG_5 (A, B), *Pn*PG_7 (C, D), *Pn*PG_9 (E, F). Analysis were carried out by using polygalacturonic acid concentrations ranging from 0.1 to 25 mg/mL at 30 °C under optimum of pH condition.

4.2.3.4 Thermal inactivation of PnPGs

Thermal stability experiment were performed with purified enzymes using 200 ng of *Pn*PG_5, 1 µg of *Pn*PG_7 and 400 ng of *Pn*PG_9 as described §3.10.4. The extent of deactivation of an enzyme is measured by the deactivation rate, which is proportional to the active enzyme concentration, and thus K_d (the deactivation rate constant) is considered as a proportionality constant. The deactivation rate and half-life were studied at different temperatures as shown in figure 4.12. *Pn*PG_2 and *Pn*PG_3 are unstable enzymes and therefore were not analyzed.



D

*Pn*PG5		*Pn*PG7		*Pn*PG9	
T °C	$t_{1/2}$ min.	T °C	$t_{1/2}$ min	T °C	$t_{1/2}$ min
		30	138	30	866
40	192	40	28	40	84
				45	14
50	16	50	10	50	2.9
55	5,6				
60	3,4	60	3.9		

Figure 4. 12: A) Thermal inactivation kinetics of *Pn*PG_5, *Pn*PG_7 and *Pn*PG_9. The samples were incubated at various temperatures **A) *Pn*PG_5 :**40 °C; 50 °C; 55 °C; 60 °C **B) *Pn*PG_7:**30 °C; 40 °C; 50 °C; 60 °C and *C)* ***Pn*PG_9:**30 °C; 40 °C; 45 °C; 50 °C, prior the addition of substrate, followed by cooling in an ice bath. The activity at zero time was taken as 100% activity. **D)** Elaborated data for showing the time required for the enzyme to lose half of its initial activity as described §3.10.4

4.2.5 Inhibition assays

Plants produced distinct PGIP isoforms that display different specificity of recognition for different PGs produced by various phytopathogens (D'Ovidio *et al.*, 2004). Inhibition capability of *Pv*PGIP2 against the purified PGs from *P. nicotianae* was measured by agar diffusion assay as percent of the PGs activity against different amount of *Pv*PGIP2 in a range between 0,05 – 5µg. None of the analyzed PG exhibits any capability to interact with *Pv*PGIP2 (data not show). *P. vulgaris* encodes a family of four members of PGIP with distinct regulation and distinct specificity, i.e., ability to interact and inhibit PGs from different fungal source. For this reason, the ability of the total PGs from *P. nicotianae* to interact with the bulk (total PGIPs) of PGIP from pods was tested, but even in this case there is no evidence of interaction, since the activity of the PGs has not decreased (Figure 4.13).

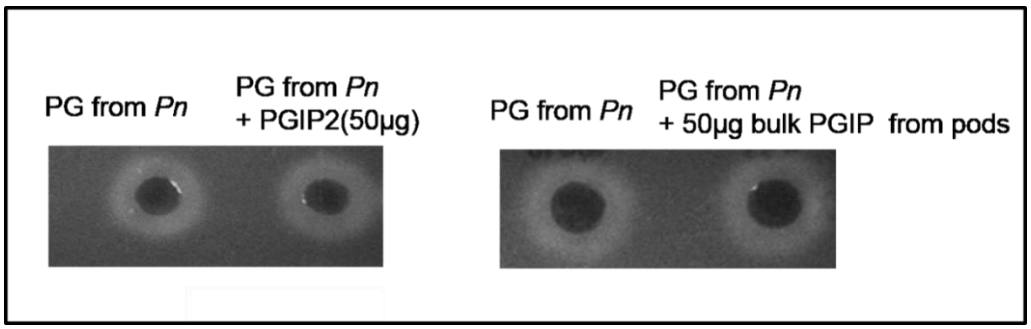


Figure 4. 13: Inhibition activity of *Pv*PGIP2 (A) and bulk PGIPs (B) by agar diffusion assay against total *Pn*PGs produced by *P.nicotianae* grown on minimal medium containing 1% of pectin.

4.3 *In vivo* Analysis of the Polygalacturonase expression from *P.capsici*

4.3.1 Infection of tomato plants overexpressing PGIP with P. capsici zoospores.

The importance of PGIPs in resistance against different phytopathogens is well established. There are many examples of transgenic plants expressing a PGIP which are more resistant to various pathogens (Ferrari *et al.,* 2003).Recently Borras-Hidalgo *et al.,* 2012. propose the use of *Pv*PGIP2 as a powerful way of engineering a broad-spectrum disease resistance. Authors shown that *Pv*PGIP2 from *Phaseolus vulgaris* protects tobacco against an important *Rhizoctonia solani* and two oomycetes (*Phytophthora parasitica var. nicotianae* former classification of *P. nicotianae* and *Peronospora hyoscyami f. sp. tabacina*). Same approach was used to test whether plants of tomato *Solanum lycopersicum* overexpressing *Pv*PGIP2 are more resistant to *P. capsici*. *S. lycopersicum* WT and transgenic plants were grown in controlled growth chambers at 22°C, with a photoperiod of 16 hours, supplemented by artificial light. 25 detached leaves of each, WT and transgenic lines were drop inoculate with 1×10^4/ mL zoospores incubate in a growth chamber at 24°C for 6 days. Transgenic lines had significantly less damage on the leaf tissue compared to the control (Figure 4.14) after 2 and 3 days post inoculation dpi. The difference between the WT and the transgenic lines became less significance going ahead with the infection. Results confirmed by measuring the lesions diameter of the infection were measured after 2, 3 and 6 dpi (Figure 4.15).

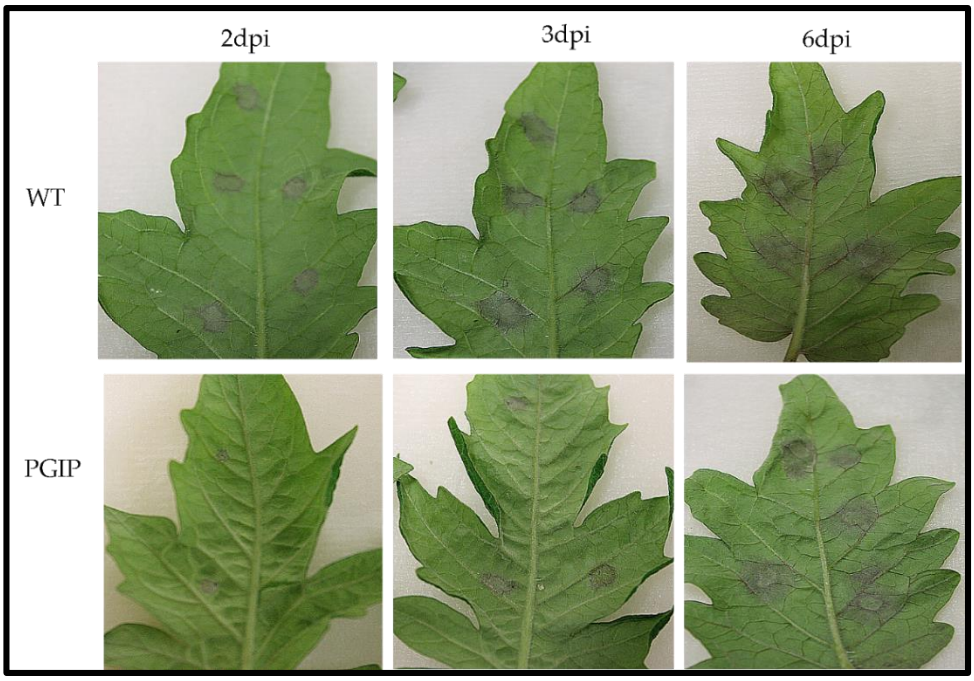


Figure 4. 14: Symptoms appearing on tomato WT and tomato overexpressing *Pv*PGIP2 after inoculation *P. capsici*. Detached leaves of WT and transgenic lines, were drop inoculate with 1 × 10^4/mL zoospores. The lesions diameter of the infection were measured after 2, 3 and 6 days after inoculation.

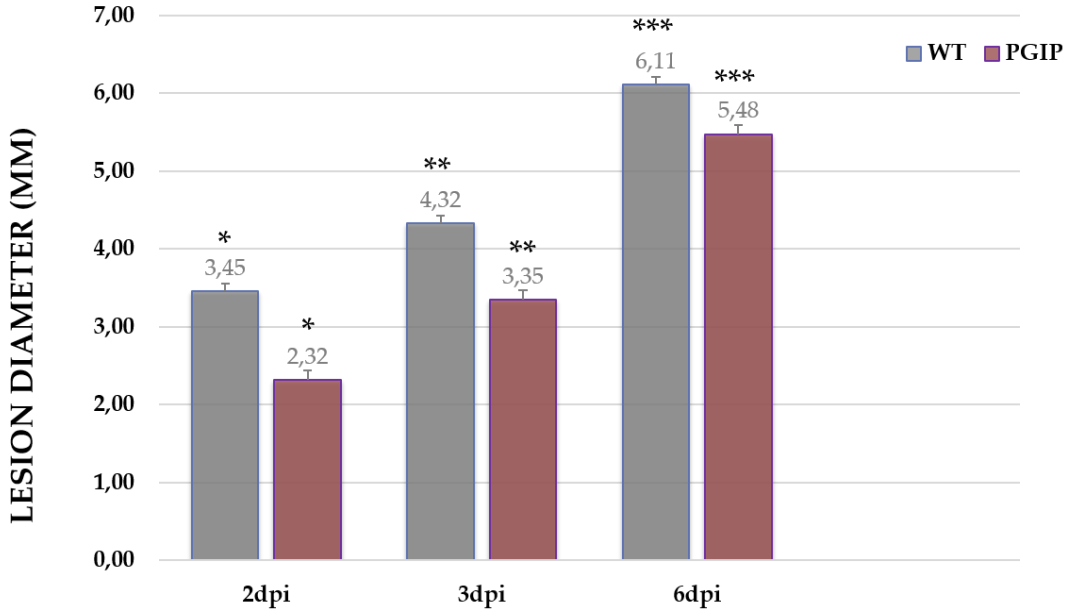


Figure 4. 15: Plants of tomato *Solanum lycopersicum* overexpressing *Pv*PGIP are more resistance to *P. capsici*. 25 detached leaves of each WT and transgenic lines, were drop inoculate with 1 × 10^4/ml zoospores. The lesions diameter of the infection were measured after 2, 3 and 6 days after inoculation. Transgenic lines had significantly less damage on the leaf tissue compared to the control. Asterisks indicate statistically significant differences, according to Student's t test *Statistically relevant with t-test value $8.88E^{-9}$, **Statistically relevant with t-test value $7.77E^{-6}$, ***Statistically relevant with t-test value 0.094. The difference between the WT and the transgenic lines became less significance going ahead with the infection.

4.3.2 Gene expression analyses

To investigate the expression profiles of the PGs from *P. capsici*, an already available microarray gene expression datasets was used. The expression datasets were generated in Dr. Huitema's lab from a *P. capsici* tomato infection time series (Jupe *et al.,* 2013). The preparation of the infection for the microarray is described as follow: detached leaves from tomato *Solanum lycopersicum* were inoculated with four 20 µL droplets of the zoospore solution. In addition to samples taken during the infectious stages (0, 8, 16, 24, 48 and 72h), were taken also three samples of sporangia/zoospores (Spor), germinating cysts (GC), and mycelia (Myc) grown *in vitro*. Spor (taken at 0 hpi) and GC (taken at 16 hpi) were sampled from the same inoculum/sporangial suspension, differing only in harvesting times. They were collected from 10 mL of sporangial suspension after an incubation time of 1 h (Spor) and 16 h (GC) at 22 °C.

The mycelia were grown in 1 mL pea broth (§3.4.2), harvested 48 hpi. All samples were placed in the controlled incubator with the same settings and conditions as the leaf samples, and harvested after centrifugation for 2 minutes at 1,200 × g. After the supernatant was removed, the pellets were collected and frozen in liquid nitrogen (Jupe *et al.,* 2013).
Analysis of the microarray data shows the fourteen PGs that are expressed from *P. capsici* during infection. The five genes not present in the microarray data are: *pcpg*_21380, *pcpg*_6556, *pcpg*_12536, *pcpg*_130445 and *pcpg*_130356. From the sequence analysis shown above (4.1.3) there is not any correlation sequence function to explain why this genes are not expressed apart from *pcpg*_12536, which lacks catalytic site.

The expressed PGs were located in two panel. In panel A), are distributed all the PGs with highest expression levels compared to panel B). All the PGs display the typical expression pattern of the polygalacturonase, high levels of expression at the very early times points, (0-8h post infection). Only two PGs do not show this expression pattern *pcpg*_556668 and *pcpg*_554601 that show a different pattern with the have highest expression level after 16h.

The *pcpg*_537485, *pcpg*_557807, *pcpg*_537480, *pcpg*_554601, *pcpg*_556668, are up-regulated in the germinated cyst and in the mycelium but the levels of expression in sporangia is very low. The second group *pcpg*_21386, *pcpg*_130325, *pcpg*_130406 we have the opposite situation high levels of transcript during sporangia but low in germinating cysts and mycelia. *pcpg*_130325 shows an equal expression in all the three stages, *pcpg*_511643 is up-regulated only in the mycelium, *pcpg*_556654 high levels in the germinating cysts and *pcpg*_130352 have is expressed highly in all the stages (figure 4.16).

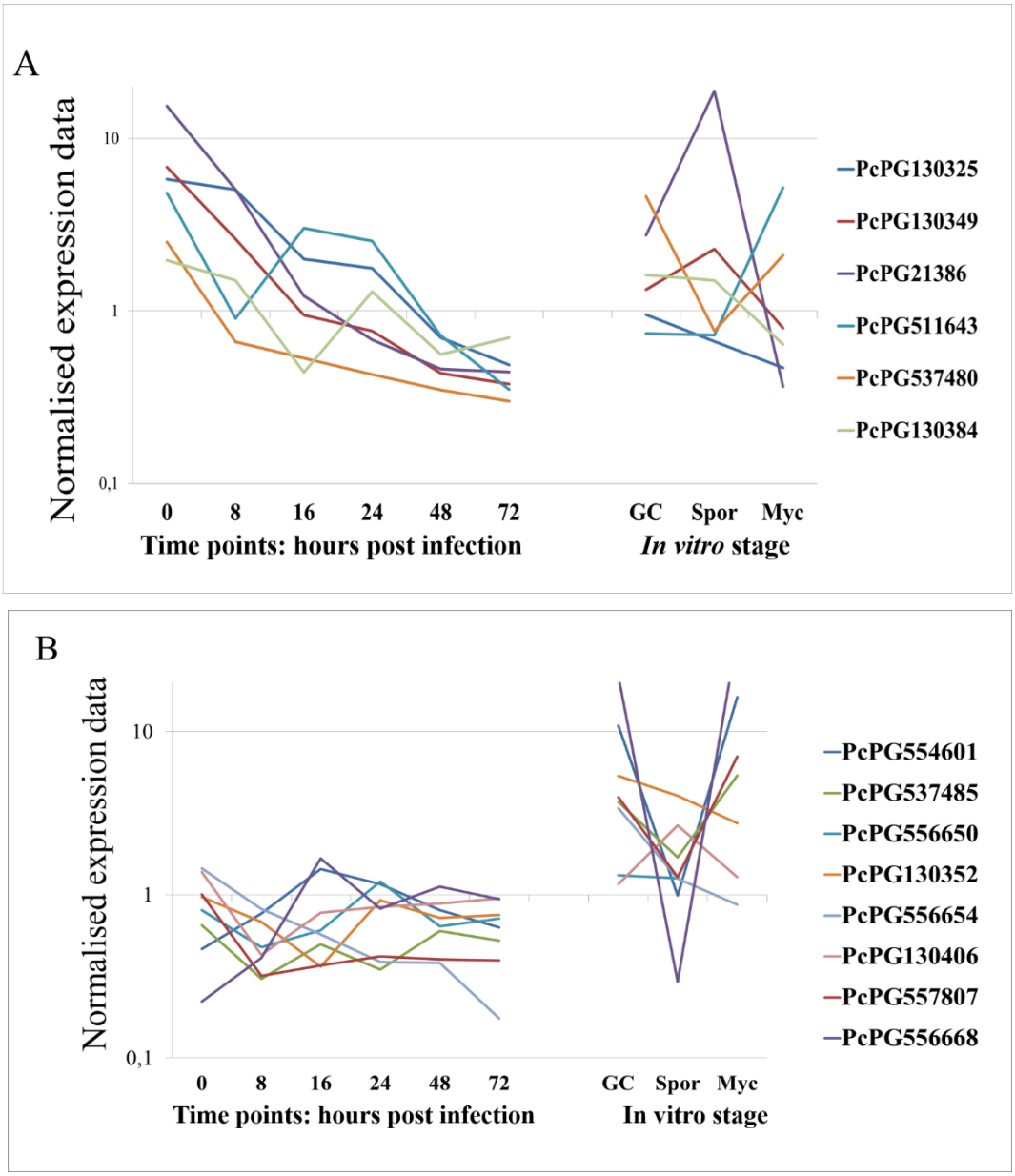


Figure 4. 16: PG gene expressions upon infection, expression profiles for PGs genes from *P.capsici* according to the microarray data. Samples taken during the infectious stages 0, 8, 16, 24, 48, 72h, and during in vitro stage sporangia/zoospores (Spor) at 0h post inoculation, germinating cysts (GC) 16h, and mycelia (Myc) were grown in 1 ml pea broth, and harvested after48 h. The PGs are divided into a two groups, A) the PGs were are upregulated, compared to the mean expression, at the very early point of the infection and B) PGs were have lower expression compared to the mean, during infection.

4.3.3 PGs cause necrosis on N. benthamiana leaf tissue

To test whether *P. capsici* PGs were active and able to induce cell death, seven candidates PGs were expressed in *N benthamiana.* Plants were grown in a glasshouse under 16 hours light and set at 26°C by day and 22°C by night. Microarray data were used for choose the candidates with different characteristics and expression patterns for further investigation. The candidates PGs are: *Pc*PG_21386, *Pc*PG_511643, *Pc*PG_556650,

*Pc*PG_130325, *Pc*PG_556668, *Pc*PG_537480, *Pc*PG_130384. The primers for the amplification of the PGs (Table 4.4) were designed to contain a *CACC* sequence at 5'to allow GATEWAY directional TOPO cloning in pENTR-D-TOPO (§3.7.2). PCR were carried out following these steps: initial denaturing of five minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 57°C for 45 seconds and 72°C for two minutes and then a final extension at 72° C for fifteen minutes using 10ng of cDNA from *P. capsici* as template. pENTR/D_TOPO-PG constructs were sequence verified and used for recombination into the binary vector pK7WG2, using Gateway LR reactions (§3.7.3). Constructs were sequence verified before transformation into *A. tumefaciens* strain AGL1 (§3.8.2). *N benthamiana* leaves were infiltrated on the left with the empty vector pK7WG2 and in the right with the pK7WG2_PG. After 2-3-4-5-6 days phenotypic effects were scored on a range from 1 (no symptoms visible) to 6 (severely necrotic, black tissue). In this analysis, two controls were used: the CRN_83152 and the Empty Vector pK7WG2. CRN_83152 cause necrosis and were used as yardstick for the measure of the cell death (Stam *et al.*, 2013b).

Name	Primer
*Pc*PG21386Fw	5'*CACC*ATGAAGCTTCTCTCCACTGC3'
*Pc*PG21386Rv	5'CACAGCAAGACTGTTAGGC3'
*Pc*PG511643Fw	5'*CACC*ATGAAGCTCTTCTCCATTCTA3'
*Pc*PG511643Rv	5'GCAGTTGATGCCACTGGG3'
*Pc*PG556650Fw	5'*CACC*ATGAAGCTCCTCTCCGCTG3'
*Pc*PG556650Rv	5'TTGGTGTATTTGGCGTTGG3'
*Pc*PG130325Fw	5'*CACC*ATGAAGGTTTTAGCCCCCGTT3'
*Pc*PG130325Rv	5'GCAACTGACCCCGCTGG3'
*Pc*PG556668Fw	5'*CACC*ATGAAGCTCTTCTCCACTGTC3'
*Pc*PG556668Rv	5'GCACTTGACGGTGCTGG3'
*Pc*PG537480Fw	5'*CACC*ATGAAGCTTCTCTCCGCTG3'
*Pc*PG537480Rv	5'CACGTTAAGACTGTTGGGC3'
*Pc*PG130384Fw	5'*CACC*ATGAAGCTCTTCACGTCTACG3'
*Pc*PG130384Rv	5'GCACTGCACGTTGCTGG3'

Table 4. 4: Primer used for the amplification of the PGs from the cDNA.

To assess whether there are differences in cell death inducing activity each pK7WG2_PG construct was expressed in *N. benthamiana* and scored for cell death across different time point. Assessment of cell death occurring from 1– 6 days showed significant differences in the timing and level of cell death between the PGs from day 2 to day 7. Expression of pK7WG2_556668 show the same pattern as the control CRN83_152, both lead to a fast cell death response, reaching maximum levels (6) within 3 days of agro-

infiltration. *Pc*PG_21386 and *Pc*PG_511643 have a strong necrotic phenotype five days post infiltration whereas *Pc*PG_556650 and *Pc*PG_537480 only induced marginal levels of cell death in the course of this experiment as the empty vector control. Compared to pK7WG2_556668 and *Pc*PG_537480, *Pc*PG_130384 *Pc*PG_130325 manifest an intermediate phenotype in these assays. The possibility of variation between leaves was excluded by expressing all the PGs and the EV on the same leaf (Figure 17_A) and using 12 leaves for each construct in 4 different experiments (Figure 17_B).

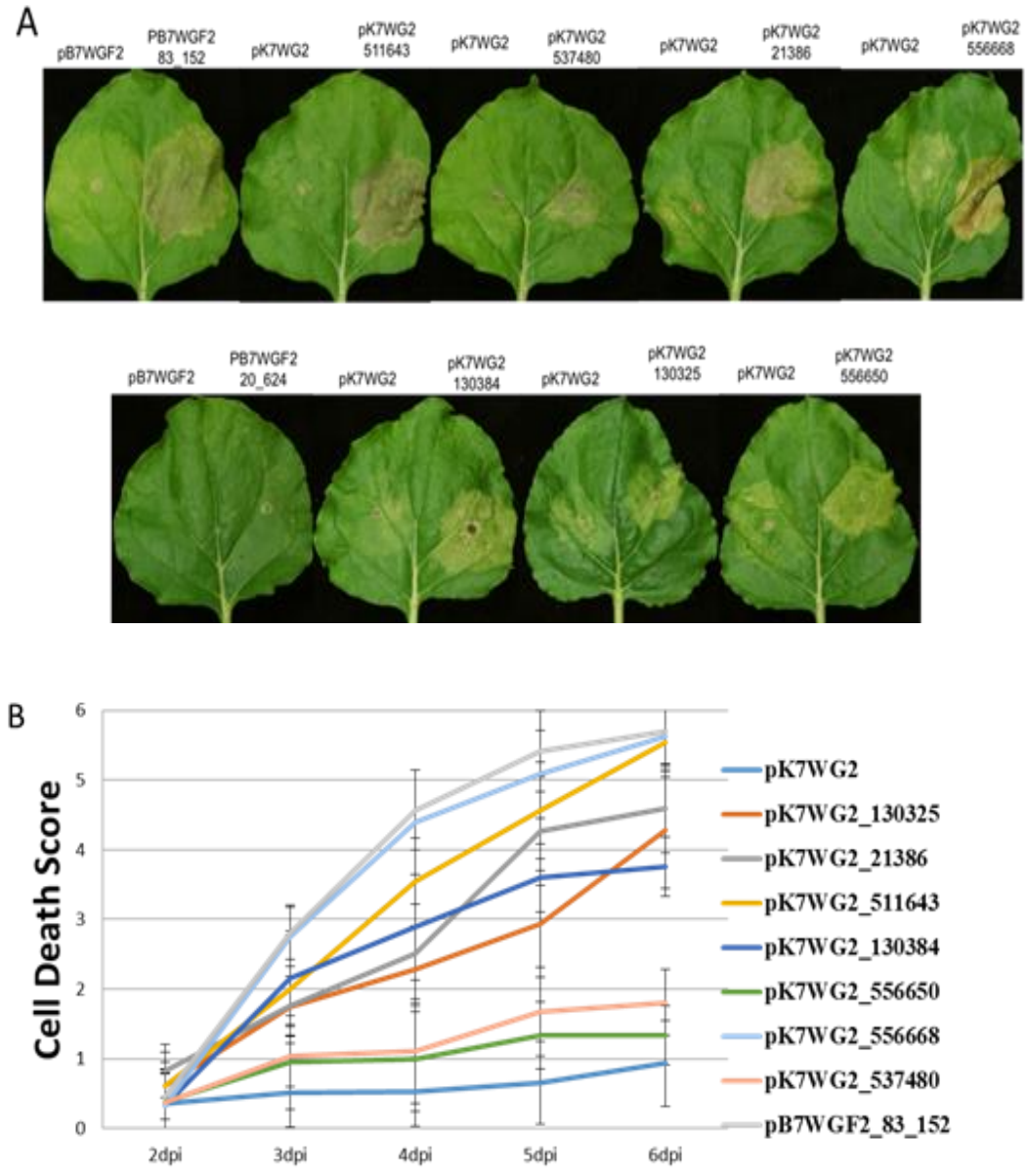


Figure 4. 17: Phenotypic and functional characterization of the candidates PGs. A) All the PGs causes necrosis after over expression in *N. benthamiana* with different profiles after five days post agroinfiltration. B) Progression of cell death in *N. benthamiana* leaves infiltrated with PGs. Cell death was scored every 24 h on a scale of 0–6. The graph shows average values ± standard deviation for one representative experiment.

4.3.4 Necrosis on N. benthamiana leaf tissue and fungal growth

To test whether PGs have an effect on the growth rate of *P. capsici* during infection, we performed a simple drop inoculation assay on leaves transiently expressing PGs proteins. The same candidates prepare as describe previously (§4.3.3) were transiently expressed in *N.benthamiana* plants. 3 days after infiltration, detached leaves were drop inoculated with 1 × 10^4/ mL zoospores incubate in a growth chamber at 24°C for 6 days. Lesion size was measure during three independent infection using 9 infection sites for construct. Three of the PGs had a direct positive effect on the growth rate of *P. capsici*, pK7WG2_556668 pK7WG2_511643 and pK7WG2_556650 (figure _C). Interestingly the pK7WG2_556668 pK7WG2_511643 showed a necrotic phenotype. This also indicates that the PGs are a diverse group of effectors with a wide array of functions, some of which directly affect *P. capsici* virulence (Figure 4.18).

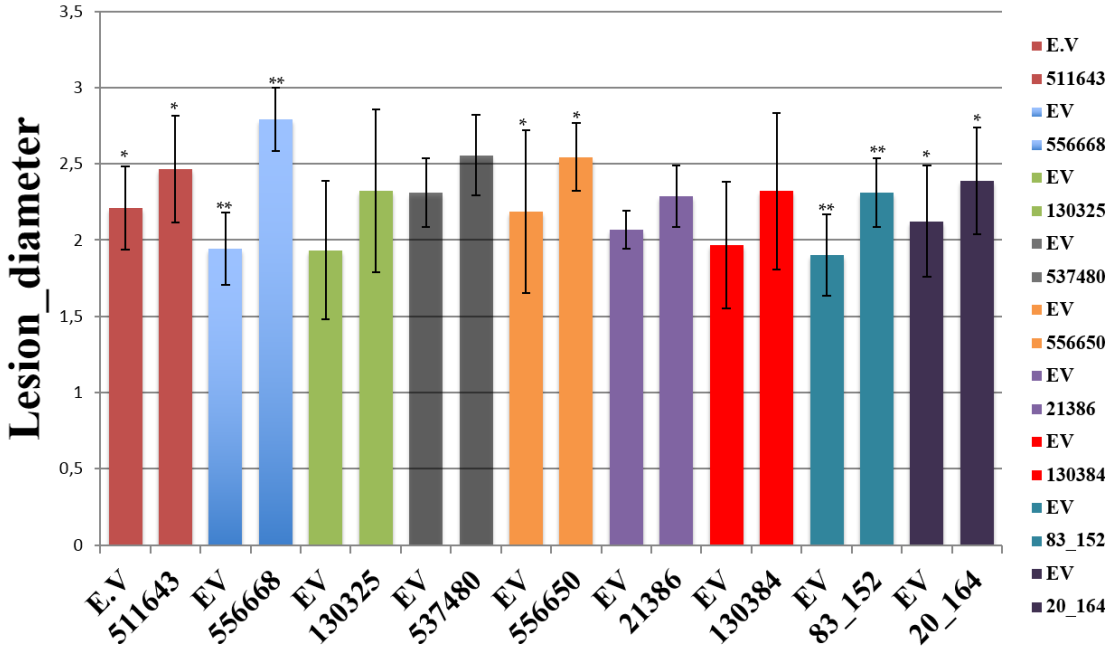


Figure 4. 18: Two PGs (PcPG_556668 and PcPG_556650) had significant differences respect to the Empty vector (EV), on the virulence of *P. capsici*. Lesion size for all other PGs was similar to that of the empty vector control. Error bars show standard deviations within the samples. *p<0,05 **p<0,01

4.4 Renewed method for knock out/down genes in *P. capsici*

4.4.1 Transformation of P.capsici with Cas9

Transformants of *P. capsici* were obtained using the protoplast method as described §3.9.3 and §3.9.4. The protoplast were treatment with 50µg of circular plasmid DNA of pTORCas9, and polyethylene glycol (PEG) CaCl2-Lipofectin protocol. As a result, I had obtained four transformants of *P.capsici* expressing Cas9. RT PCR carried out using 10 ng of cDNA from each transformant of *P.capsici* with the following protocol: initial denaturing of five minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 54°C for 45 seconds and 72°C for two minutes and then a final extension at 72° C for fifteen minutes. pTORCas9*8 shows higher level of transcript (Figure 4.19), grows very well in V8 agar and Pea broth with the addition of 50mg/ml of G418 and infect leaves of *N. benthamiana* as the WT LT1534 (Figure 4.20). pTORCas9*8 has been chosen for the following experiments of transformation

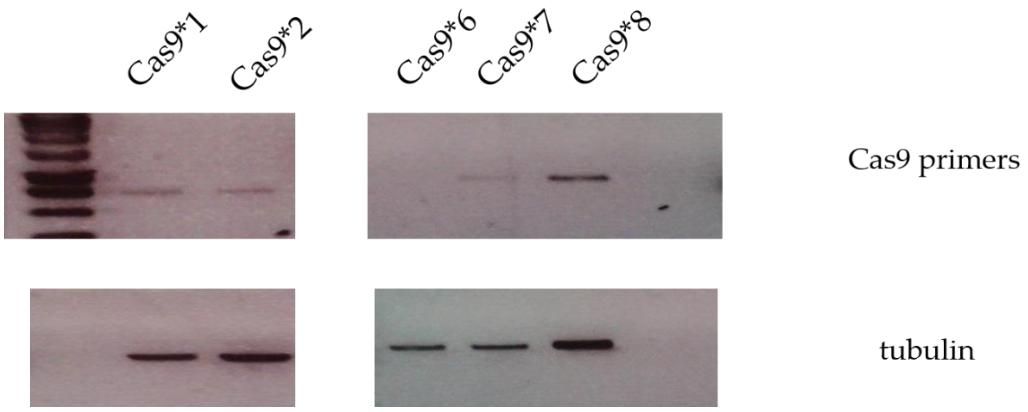


Figure 4. 19: RT-PCR for measured the relative mRNA level of the Cas9 in each transformants.

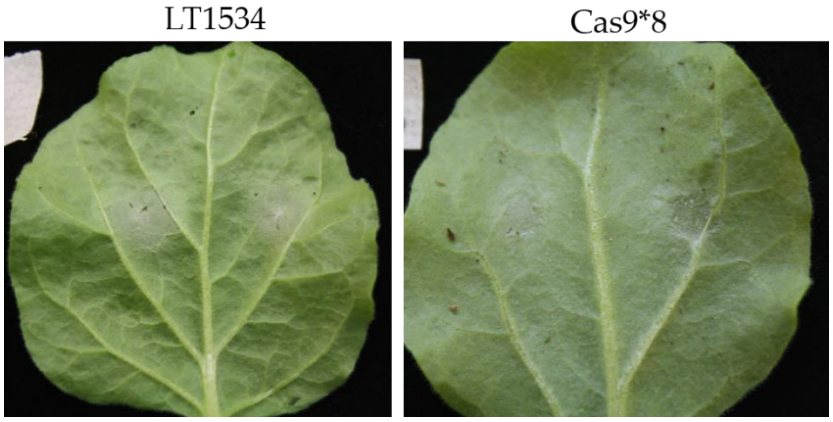


Figure 4. 20: infection of *N.benthamiana* leaves with *P.capsici* LT1534 and *P.capsici* expressing Cas9 (pTORCas9*8). No differences are presents in the infection.

4.4.2 Selection of Target sequence and vector construction for the CRISPR/Cas9 system and transformation in P.capsici

To test the efficacy of CRISPR/Cas9 in *Phytophthora capsici* I choose to target a gene that, when function is disrupted, would result in distinctive characteristic. A CRISPR/Cas9 construct was designed to target the haustorial plasma membrane gene *hmp1*, which has already been studied and silenced in *P. infestans* leading to the loss of pathogenicity and indicated involvement of this gene in the penetration and early infection processes (Avrova *et al.* 2008). A homologous sequence to *Pihmp*1 has been identified in the genome of *P. capsici*, that was shown to exhibit biotrophy specific gene expression (Jupe *et al.*, 2013). In addition, I choose a target region of the *pchmp1* sequence that contained the required binding region or Protospacer Adjacent Motif (PAM) for Cas9 cleavage, which is a 3'GGN located immediately after the 20 bp target DNA. Between the PAM sequence and the 20bp target region there is the restriction enzyme *Pst*I for easy identification of the transformans. The transformant with silenced *hmp*1 function will keep a disrupted target sequence if CRISPR-Cas9 has cleaved and the *Pst*I restriction enzyme will not cut the DNA (figure 4. 21). The desired single targeting gRNA was generated by *in vitro* transcription from a T7 promoter after linearizing the plasmid with *Bam*HI (§ 3.9.2).

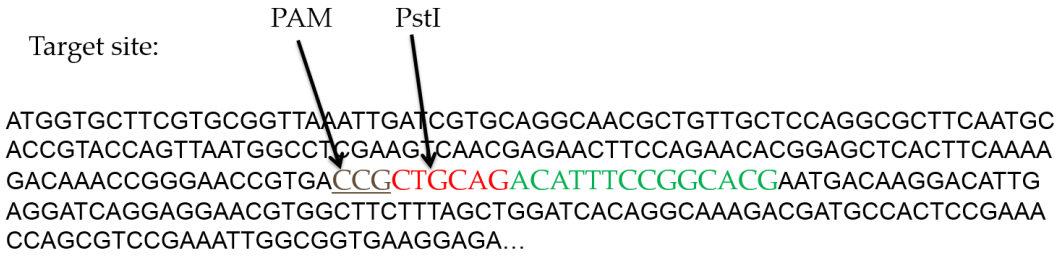


Figure 4. 21: Target site of CRISPR/Cas9-mediated target mutagenesis in the *P.capsici hmp1* gene. The PAM sequence (CCG) is shown in grey, in red is show the restrinction enzyme *PstI* and the 20 bp target sequence is shown in green.

4.4.3 Detection of on-target mutation in Cas9-gRNA transformants

After the co_transformation of the pTORCas9*8 with the gRNA (Figure 4.22) I analyzed the new transformants for the detection of the expected cleavage site that lies within the recognition sequence PAM and restriction enzyme *PstI*. Thus, cleaved amplified polymorphic sequences were used to detect mutations in the target region. 82 transformants were generated from the co_transformation of pTORCas9 with the gRNA. DNA was extracted from the independent transformants directly from the Mycelium. PCR products of 1.4 Kb length of the *hmp1* gene with mutations in the *PstI* recognition site should not be

cleaved into 150 and 1250bp fragments by digestion with *PstI* but should present only one band of 1.4Kb. This analysis revealed that no mutations were induced in all the transgenic lines (Figure 4.23) because the *PstI* manage to digest the fragment of DNA.

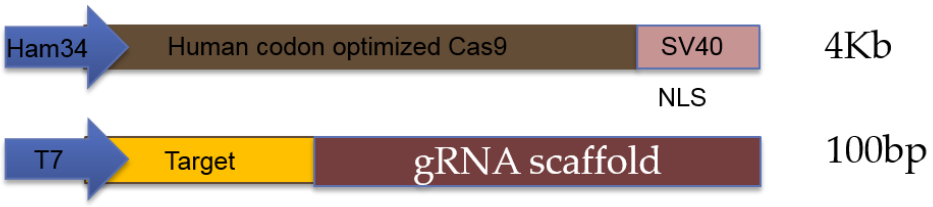


Figure 4. 22: plasmid used for the transformation of P.capsici pTORCas9 (4Kb) and gRNA (100bp)

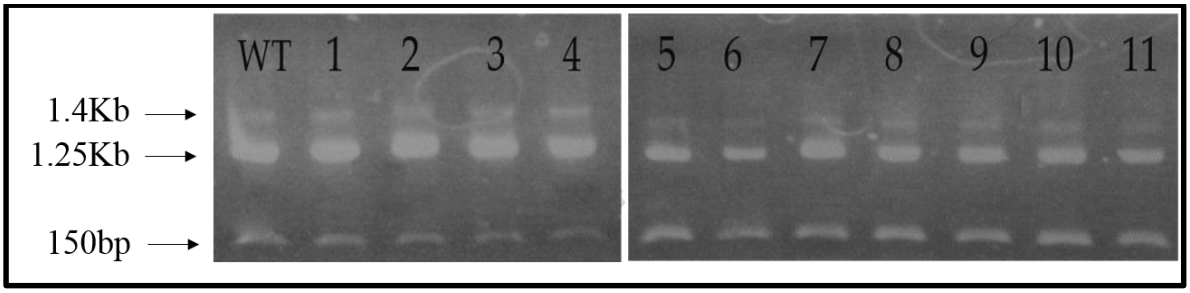


Figure 4. 23: DNA extracted from independent Cas9 transformants was subjected to PCR and subsequent *PstI* restriction enzyme digestion. In line 1 is the WT LT1534 2-11, transformants of *P.capsici* expressing Cas9 and treatments with gRNA. If they were, mutations at that cleavage site, on the gel had to have the presence of only one band at 1.4Kb.

The transformation experiments of *P. capsici* were repeated several times, changing the materials of transformation, enzymes for obtaining more and stable protoplasts and, different strain of *P. capsici*, not only LT1534 but also LT6535 and OP97. By this way has been obtained a better protocol for an improved efficiency of *P. capsici* transformation. Although the CRISPR-Cas9 system is very powerful and used in many organisms, in *Phytophthora* was not a success.

4.5 Silencing of PGs with Hairpin construct in *P.capsici*.

Although the failure of the CRISPR_Cas9 system in *Phytophthora* was important to try with other system to silencing the selected PGs. I use the conventional method of intron spliced hairpin construct using the pSTORA plasmid (§3.7.5) for silencing the PGs from *P. capsici*. The expression data and the experiments of the cell death help me to identify the candidates to be silenced. I choose two candidates: *pcpg*_21386 and *pcpg*_556668. This candidate presents different characteristics and different expression pattern during infection. *pcpg*_21386 is up-regulated during the very early stage of infection and after 8 hours going down instead of *pcpg*_556668 that has the higher expression at 16 hours after infection. Also in the *in vitro* stage (e.g. espressed in *P. capsici* and not *in planta*) this two genes are completely different having high levels of expression for *pcpg*_21386 during only the sporangia phase but low for germinating cyst and mycelia. The opposite situation shows the *pcpg*_556668 that has high levels during germinating cyst and mycelium but low level of expression during sporangia stage.

Construction of the pSTORA plasmid I take the whole gene, the sense plasmids of *pcpg*_21386 and *pcpg*_556668 were generate by amplifying the PGs genes from *P. capsici* cDNA using specific primers (Table 4.5). Primers contains the sequence for *Fse*I and *Sbf*I restriction enzymes. PCR reaction was carried out using 10 ng of cDNA from each *P. capsici* with the following protocol: initial denaturing of five minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for two minutes and then a final extension at 72° C for fifteen minutes. PCR fragments were purified and cloned in pSTORA. The Antisense plasmids of *pcpg*_21386 and *pcpg*_556668 were generate by amplifying the PGs genes from *P. capsici* cDNA using specific primers (Table 4.6). Primers contains the sequence for *Asc*I *and Sac*II restriction enzymes. PCR reaction was carried out using 10 ng of cDNA from each *P.capsici* with the following protocol: initial denaturing of five minutes at 95°C followed by 35 cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for two minutes and then a final extension at 72° C for fifteen minutes. PCR fragments were purified and cloned in pSTORA. The sense and the antisense sequences were separated by a 71-bp intron from Ste20-like gene of *P. infestans*. After the construction of the plasmids, transformants of *P. capsici* were obtained using protoplast. The protoplast were treatment with 50µg of circular plasmid DNA of pSTORA*Pc*PG, and polyethylene glycol (PEG - CaCl2-Lipofectin protocol).

Name	Sense Primer
*Pc*PG21386_pSTORA_FseI_F	5'TAGGCCGGCCATGAAGCTTCTCTCCACTGC3'
*Pc*PG21386_pSTORA_Sbfi_R	5'CACCTGCAGGTTACACAGCAAGACTGTTAGG3'
*Pc*PG556668_pSTORA_FseI_F	5'TAGGCCGGCCATGAAGCTCTTCTCCACTGTC 3'
*Pc*PG556668_pSTORA_Sbfi_R	5'CACCTGCAGGTTAGCACTTGACGGTGCTG3'

Table 4. 5: Primer used for the construction of the sense plasmid.

Name	Antisense Primer
*Pc*PG21386_pSTORA_AscI_F	5'AGGCGCGCCTTACACAGCAAGACTGTTAGG3'
*Pc*PG21386_pSTORA_SacII_R	5'ACCGCGGATGAAGCTTCTCTCCACTGC3'
*Pc*PG556668_pSTORA_AscI_F	5'AGGCGCGCCTTAGCACTTGACGGTGCTG3'
*Pc*PG556668_pSTORA_SacII_R	5'ACCGCGGATGAAGCTCTTCTCCACTGTC3'

Table 4. 6: Primer used for the construction of the antisense plasmid.

4.5.1 Analysis of transformants for silencing of pcpg_556668 and pcpg_21386

Two transformants for the silenced line *pcpg*_556668 and three for the line *pcpg*_21386 were obtained. All the transformants show a regular phenotype and, sporulate as the WT LT1534. To investigate if the expression profiles of the PGs from *P. capsici* silenced strain was different compared to WT, Real Time qPCR *In vitro* stage was carried on. Samples were prepared as described in Jupe *et al.*, 2013. Spor (taken at 0 hpi) and GC (taken at 16 hpi) were sampled from the same inoculum/sporangial suspension, differing only in harvesting times. The mycelia were grown in 1 mL pea broth (§3.4.2) and harvested after48 h. All samples were placed in the controlled incubator with the same settings and conditions as the leaf samples, and harvested after centrifugation for 2 minutes at 1,200 × g. After the supernatant was removed, the pellets were collected and frozen in liquid nitrogen (Jupe *et al.*, 2013).

Extraction of RNA and generation of cDNA was performed during *in vitro* stage. Transcript specific primers were designed using Primer Express (Applied BioSystems) with default settings. qPCRPcPG21386Fw 5'gtgtgaccctcgacctgag3' andqPCRPcPG21386Rv 5'acgtggtagtgccgagaaac 3' for the *pcpg*_21386 gene. For the quantification of the transcript from *pcpg*_556668 gene, qPCRPcPG556668Fw 5'taacggatcgcccatgat3' qPCRPcPG556668Rv 5'tacgtgccggagagggta 3' were used. As a control for the normalization of the quantification, primer for the amplification of the constitutive expressed gene in *P.capsici* Tubulin were used.

The Figure 4.24 shows the real time qPCR results. Into the panel A are shown the silenced two lines for *pcpg*_556668. Only *pcpg*_556668*1 transformant shows decreased

transcript level in all the three stage germinating cyst (GC), sporangia (SP) and mycelium (MYC) when compared with the mock LT1534. In panel B are shown three transformant lines for *pcpg*_21386. Only *Pc*PG21386*3 shows lower level of the transcript in all *In vitro* stage GC, SP and Myc when compared with the LT1534 Empty Vector.

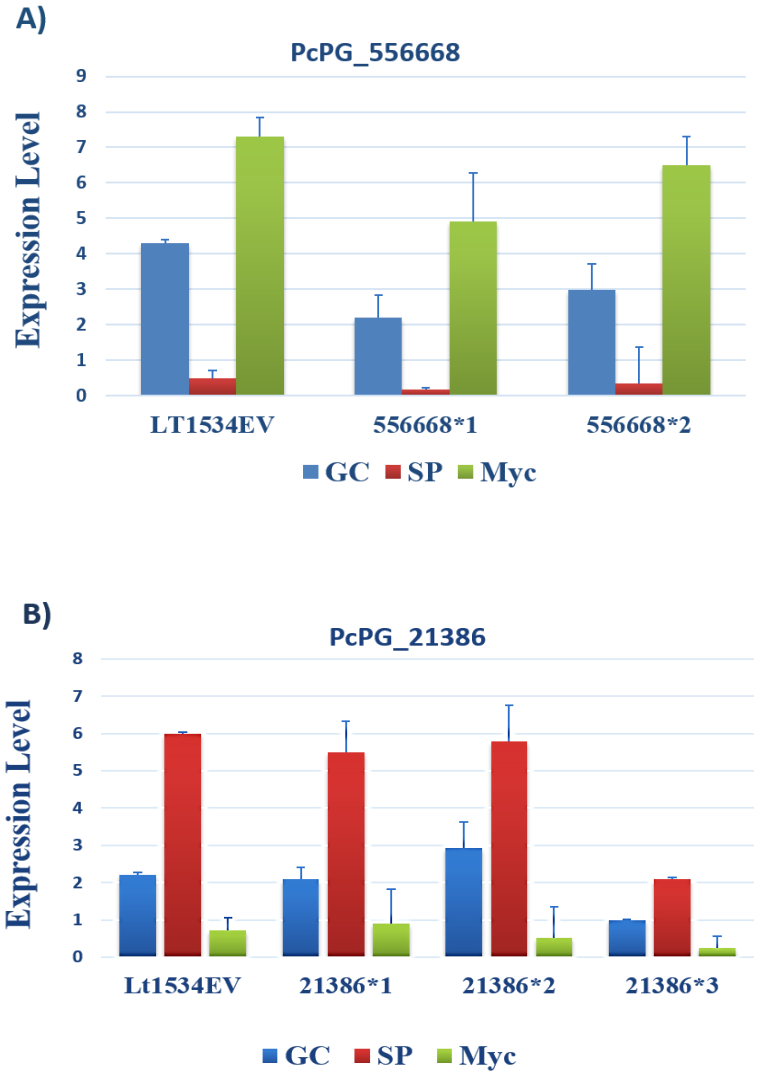


Figure 4. 24: Transcript quantification of *P. capsici* transformants in vitro stage GC: germinating cyst, SP: sporangia, Myc: mycelium. A) transcript level in WT LT1534 *pcpg*_556668*1 and *pcpg*_556668*2. The expression level between the WT and the line *pcpg*_556668 *2 are very similar. Only for the line *pcpg*_556668*1 the level of the transcript are lower when compared with the WT B)Also for the *pcpg*_21386 silenced gene, only the line *pcpg*_21386*3 show lower level of transcript when compared with the WT. Each value is reported as the percent of the maximum transcript value. Each transcript was normalized with the P.capsici b-tubulin gene as housekeeping of the oomycete genes.

4.5.2 Infection assay with the silenced lines PG of P.capsici

Gene silencing, was used to determine the role of the polygalacturonase during *P.capsici* infection on tomato. To verify if the role of these two PGs are important in pathogenesis, infection were performed on tomato *S. lycopersicum* 'Moneymaker' compared the wild type strain with the silenced lines. *P. capsici* wild-type strain LT1534 and the silenced lines *pcpg*_556668*1 and *pcpg*_21386*3 were grown in petri dishes on V8 agar medium in a dark climate chamber at 25°C for 4 days and under standard light at 22°C for 3 days. To induce zoospore release, plates were flooded with ice-cold distilled water, and spores were harvested from sporulating mycelia by dislodging the sporangia with a sterile glass spreader. Sporangial suspensions were collected and incubated at room temperature under bright light conditions. 25 detached tomato leaves were drop inoculate with 1×10^5/ml zoospores from each strain and incubate in a growth chamber at 24°C for 6 days. The infections made to compare the silenced lines with wt LT1534 did not show any difference, nor in the first days of infection and either after 6 days (Figure 4.25).

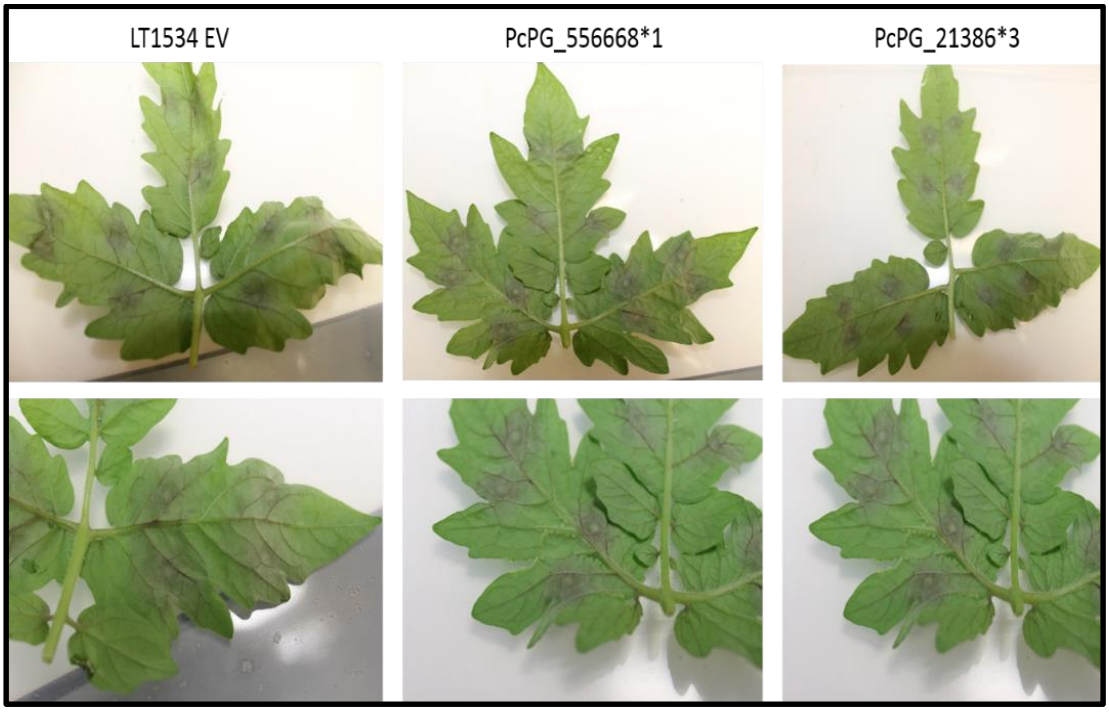


Figure 4. 25: Symptoms appearing on tomato WT after inoculation with P. capsici LT1534 EV and the silenced lines PcPG556668*1 and PcPG21386*3 2 days after inoculation 25 detached leaves of tomato *S. lycopersicum* 'Moneymaker', were drop inoculate with 1×10^5/ml zoospores from each strain. The lesions diameter of the infection not show any difference.

5. DISCUSSION

5.1 General considerations

The research described in this thesis was focused on a group of apoplastic effectors, the polygalacturonases, encoded by *P.nicotianae* and *P.capsici.* Both species cause multibillion-dollar losses in crop production every year and are considered among the most dangerous plant pathogens.

P.capsici is a highly dynamic and destructive invasive pathogen that attacks solanaceous (pepper, tomato), legume (lima and snap beans) and most cucurbit hosts. (Erwin & Ribiero 1996; Hausbeck & Lamour, 2004). During epidemics, farmers fight damage and crop losses by *P. capsici* with chemical treatments. The absence of resistant crop cultivars, the high level of genotypic diversity within *P. capsici* isolates, as well as the threat of sexual recombination in the fields, makes *P. capsici* a worst-case scenario for crop production (Kamoun *et al.*, 2014).

P. nicotianae is a worldwide distributed pathogen (Erwin & Ribeiro, 1996). Primarily known to cause tobacco black shank and gummosis; it is also responsible for severe foliar and fruit diseases as well as root and crown rots on herbaceous and perennial plant species in more than 250 genera, including solanaceous crops, and horticultural and fruit trees (Cline *et al.,* 2008). *P. nicotianae* produces both asexual zoospores and thick-walled sexual oospores. Oospores constitute a potential source of genetic variation, and with resting chlamydospores contribute to survival in unfavorable conditions in soil or within infected plant tissues (Kamoun *et al.*, 2014; Meng *et al.,* 2014).

P. capsici and *P. nicotianae* are hemibiotrophics pathogens, the first 36 hours of the infection are biotrophics and, progressively it progresses to a necrotrophic phase (Jupe *et al.,* 2013). Infection starts when a hypha emanating from a germinated oospore, a directly germinating sporangium or an encysted zoospore penetrates the plant cuticle and gains access to host cells. Appressoria are observed, in some cases, at the infection site. Successful invasion is then followed by growth and the colonization of host tissues, which ultimately results in tissue collapse and sporulation (Figure 5.1) (Lamour *et al.,* 2012).

This particular lifestyle of the hemibiotrophics *Phytophthora spp* organisms can maybe explain why the PGs are expressed differently one from another and present distinct characteristics.

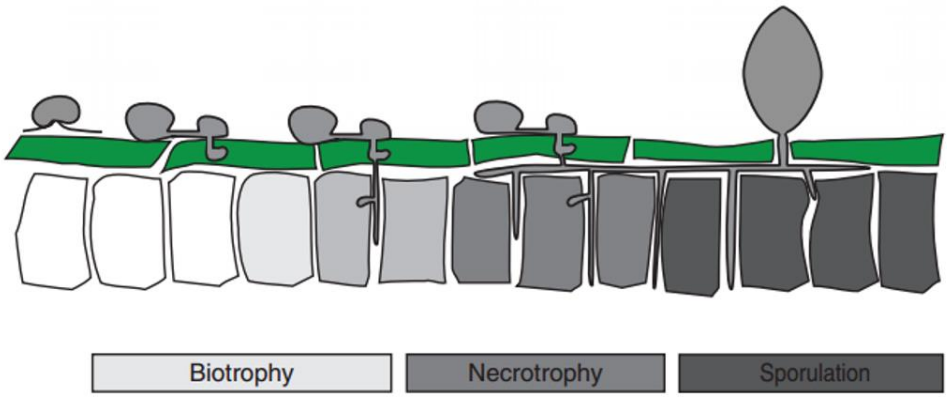


Figure 5. 1: Schematic representation of the infection process of the hemibiotrophic oomycetes *P.capsici*. Figure adapted from Lamour *et al.,* 2012

5.2 Identification of new PGs

In this thesis, *in silico* analysis was used for the identification of PGs sequences from different species of oomycetes such as *Phytophthora*, *Pythium* and *Aphanomycetes*. By this way, I have analyzed a broad part of oomycetes with different lifestyle and mode of infection. *Phytophtora* species including *P.nicotianae* are known to contain large multigene families encoding PGs and, some of them are studied for their role during pathogenesis process (Götesson *et al.,* 2002; Li *et al.,* 2012; Sun *et al.,* 2009; Torto, Rauser, & Kamoun, 2002; Wu *et al.,* 2008)

From previous described pipeline (§ 4.1), were identified the presence of these large families on *P. capsici* composed by 19 members and 10 new PGs *P. nicotianae,* that have never been studied before. Reduced was the number of the PGs identified for *Pythium* and *H. arabidopsidis,* between 3-5, however it is in agreement with the comparative analyses of oomycete pathogens, which have been shown variation in genome size, genome content, and evolution of host-pathogen interactions (Adhikari *et al.,* 2013). Several gene families that facilitate the infection process are expanded in *Phytophthora* species and significantly reduced in *P. ultimum* and *H. arabidopsidis* (Adhikari *et al.,* 2013). For example, analysis of the CAZymes in *Pythium* species showed a highly reduced set of these enzymes when compared to *Phytophthora* species (Zerillo *et al.,* 2013).

Among oomycetes, the genus *Aphanomycetes* belongs to the Saprolegniales and comprises species that are destructive on plants, crustaceans and animals such as *A. euteiches, A. astaci* and *A. stellatus* respectively. From the pipeline only for the *A. euteiches* were identified a large family of PGs composed of 18 genes, being the only plant-pathogen (Gaulin *et al.,* 2008).

The differential ability of oomycete pathogens to produce different hydrolytic enzymes acting on different complex carbohydrate molecules could determine their infection

strategy, host range, and most likely contribute to the different virulence mechanisms between oomycete pathogens (Bowler e*t al.*, 2008)

5.3 Tracking evolutionary of the Oomycetes

After the identification of 95 candidates PGs, a phylogenetic analysis was performed to investigate the relationship between the identified PGs from the different oomycetes species. The analysis has highlighted the phylogenetic distance between the *Phytophthora* and the others oomycetes such *Pythium* and *Aphanomycetes* (§4.1.2; Figure 4.4), they show PG sequences in separate clades over the phylogenetic tree. The ability to infect plants has evolved at least twice in the oomycete lineage (Figure 5.2). According to the literature, the acquisition of plant pathogenicity probably occurred early in the ancient monophyletic group *Pythiales*, which is comprised of the majority of plant-pathogenic genera of oomycetes including *Phytophthora*. More recently, a group of plant pathogens emerged within the genus *Aphanomycetes*. These species are distantly related to the *Pythiales* and closely related to animal pathogens. *Aphanomyces* species appear to have emerged as plant pathogens more recently and independently (Kamoun, 2001).

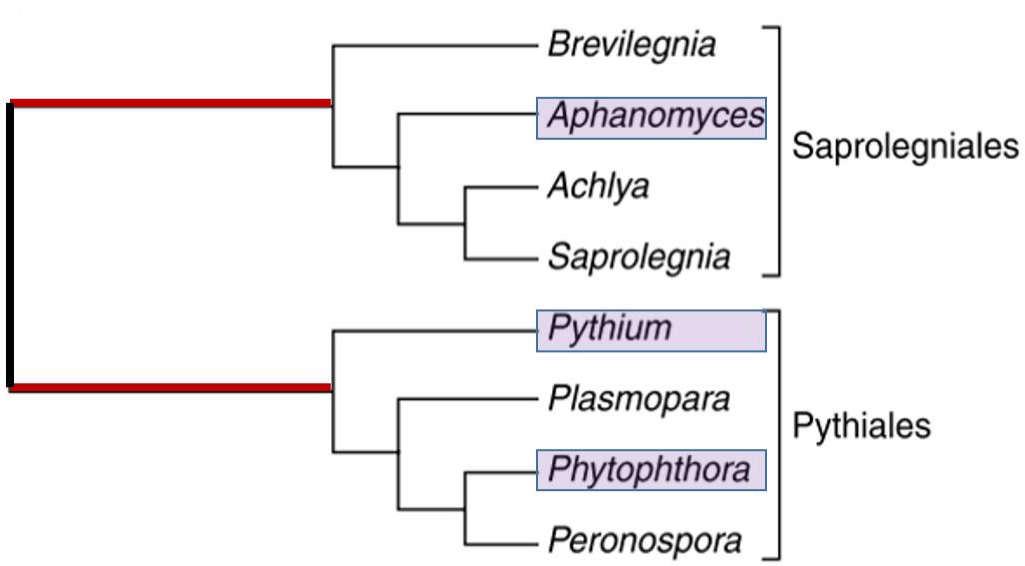


Figure 5. 2: Phylogenetic tree illustrating the evolutionary relationships among the major genera of oomycetes. Lineages are indicated with a red line. Note that plant pathogenesis has probably emerged at least twice during the evolution of oomycetes. First, in an ancient lineage that diverged into the *Pythiales* (including *Pythium*, *Phytophthora* and the downy mildews). Second, in a lineage that includes *Aphanomyces* species. Adapted from Kamoun, 2001.

In addition, the phylogenetic tree revealed the presence of gene duplication across the genomes of *Phytophthora* species. This fact is extended in *P. infestans* (duplication highlighted with • in Figure 4.4) and, it is also present in the other species of *Phytophthora* analyzed in this study as well as in *Phytophthora cinammomi* where 19 PG genes were identified with high levels of duplication events from Götesson *et al.,* 2002. In the phylogenetic tree, some PG genes of *P. infestans* are in pairs to genes from *P. capsici* and *P. nicotianae*, or there some PG genes from *P. capsici* are in pairs to PG genes from *P.nicotianae*, this suggests that they may have evolved prior to their speciation and are thus orthologs (Hardham & Cahill 2010). The existence of pairs of almost identical genes in the *Phytophthora* PG multigene family is likely to be due to recent duplication events. The presence of recently duplicated genes and, genes that have acquired mutations leading to loss of function indicate that the gene family is undergoing a process of birth-and-death evolution (Nei & Rooney, 2005). Gene duplication is a fundamental process in the evolution of species and is believed to play a leading role in the creation of sequence variability and novel gene functions (Jianzhi Zhang, 2003). Genes, across genomes, that are related by sequence or function similarity are called homologs and are grouped into a gene family. Initial analyses of the evolution of several pathogenic oomycetes led to the identification of large gene families. However, the individual contributions and the exact sequence of different evolutionary processes such as gene gains, duplications, and losses that caused the enormous increase in gene families sizes are still unknown (Seidl *et al.,* 2012).

5.4 Sequence analysis reveal distinct characteristics

As mentioned in the Results paragraph §4.1.3, PGs have been studied in detail from a bacteria, plant, fungi and oomycetes (ten Have *et al.* 2001; Blackman, Cullerne & Hardham 2014; Götesson *et al.* 2002; Hong *et al.* 2013; Kalaitzis, Solomos & Tucker 1997) and all show four highly conserved motifs. Two "catalytic regions" (G/QDD and G/SHG) and the other two "substrate-binding regions" (NTD and RIK) (Palanivelu, 2006). Multiple sequence alignment was carried out, based on the HMM algorithm (Yoon, 2009) between the PGs from *Phytophthora* and three well known PGs from fungi, such as *Aspergillus niger*, *Collecotrichum lupini* and *Fusarium phyllophilum*. These conserved regions are all present in *P. capsici* and *P. nicotianae* PGs, except for the PcPG12536, which have only the RIK motif but lacks all the other regions. The areas around these motifs are very well conserved in all the *Phytophthora* PGs. By adding the PGs from fungi, the presence/absence of

aminoacids, which have been found to be important in the interaction with the inhibitor of polygalacturonase PGIP (see § 5.5).

The multiplicity of PG isoforms may reflect the complexity of the pectin molecule in plant cell walls and the need for enzymes capable of cleaving the homogalacturonan backbone in a variety of structural contexts (D'Ovidio *et al.*, 2004). The amino acid sequences analysis indicate that the *P. capsici*, and *P. nicotianae* PGs are divided into two groups with respect to potential glycosylation sites (Table 4.1). Glycosylation, thought to confer a higher stability (Yan & Liou, 2005) and increased protease resistance (Rudd *et al.*, 2001) has been observed in many fungal PGs (Götesson *et al.*, 2002; ten Have *et al.*, 2001). Differential glycosylation of PGs is also present in *B. cinerea* (Wubben *et al.*, 1999), but the division of the *Phytophthora spp* PGs into lightly or potentially heavily N-glycosylated isoforms is striking. Deglycosylation of *Pp*PG1 (*P. nicotianae*) protein, resulted in a complete loss of the PG activity as well as in *A. niger* (Sakamoto *et al.*, 2002; Yan & Liou, 2005). In a recent paper the activity of the PG form *P. capsici* strain SD33 (*Pc*ipg_5) was lower when compared to the WT (Li *et al.*, 2012). Another structural feature that may determine the functional diversification of these enzymes is the isoelectric point. The predicted pIs of the *Phytophthora* PGs vary between 4 and 9.87. The pI may influence enzyme specificity for substrates with different charge. A basic pI may confer affinity for negatively charged polygalacturonate and decrease protein mobility within the cell wall, leading to highly localized wall degradation, as observed upon secretion of the extremely basic polygalacturonate lyase by the biotroph *Uromyces vicaefabae* (Götesson *et al.*, 2002 ;Mendgen *et al.*, 1996).

The presence of N-terminal extensions such as the polyglutamine regions (Figure 4.4) of *Pc*PG_537480, *Pc*PG_21386, *Pc*PG_556654 from *P. capsici* and four from *P. nicotianae Pn*PG_2, *Pn*PG_3, *Pn*PG_15164, *Pn*PG_15165, may play a role on functional diversification of these enzymes. It has been suggested to influence substrate specificity and to play a role in their interaction with particular regions of the pectin polymer (Götesson *et al.*, 2002; Pařenicová *et al.*, 2000). In humans, expanded runs of consecutive trinucleotide CAG repeats encoding polyglutamine (polyQ) stretches are observed in the genes of a large number of patients with different genetic diseases such as Huntington's and several Ataxias. Protein aggregation, which is a key feature of most of these diseases, is thought to be triggered by these expanded polyQ sequences in disease-related proteins (Schaefer *et al.*, 2012).

5.5 Interaction PG-PGIP

Plants produce distinct PGIP isoforms that display different specificity of recognition for different PGs. For example, in *Phaseolus vulgaris* four genes encode PGIPs, and their recognition abilities against PGs from *Aspergillus niger*, *Fusarium phyllophilum*, *Stenocarpella maydis* and *Colletotrichum lupini* have been characterized. Among them, only *Pv*PGIP2 inhibits all tested fungal PGs, while *Pv*PGIP1, *Pv*PGIP3 and *Pv*PGIP4 are unable to inhibit PG from *F. phyllophilum* (*Fp*PG). *Pv*PGIP3 is also unable to recognize a PG from *A. niger* (D'Ovidio *et al.*, 2004). In Arabidopsis, two inhibitors, encoded by the tandemly repeated genes *At*PGIP1 and *At*PGIP2, also exhibit different inhibitory capability against fungal PGs (Ferrari *et al.*, 2003). The isoform 2 of *Phaseolus vulgaris* (*Pv*PGIP2) is the best characterized inhibitor and has the strongest inhibitory activity against most of the tested PG from various pathogens. *Pv*PGIP2 inhibits different PGs with different inhibition mechanisms (Manuel Benedetti *et al.*, 2013). Genes encoding PGIPs are under selection pressure for diversification and a number of hot spots for the interaction with PGs have been identified in the LRR concave surface of the inhibitor. Furthermore, it has been shown that a few PGIP residues, sometimes only one, are critical for a stable PG-PGIP interaction. (Casasoli e*t al.*, 2009).

A structure obtained by SAXS analysis supports the idea that the interaction between *Fp*PG and *Pv*PGIP2 is the result of a specific and unique set of contact points (Benedetti *et al.*, 2013). The structure, for example, indicates that residues S^{120} and N^{121} of *Fp*PG are contact points in the complex. These two residues belong to the loop SNSN, which is under selective pressure for diversification and is located in proximity of the active site cleft; this loop is typical of *Fp*PG while it is generally absent or mutated in other PGs. For instance, the loop is absent in *A.niger* PGII and this may consequently cause the non-competitive mechanism of inhibition of this enzyme by *Pv*PGIP2. Mutation of H^{110}, the inhibitor residue that faces the enzyme N^{121} in the SAXS structure, resulted in a marked loss of inhibition, suggesting the importance of the SNSN loop in *Fp*PG recognition. From the multiple alignment shown in the Figure 4.2 is clear that none of the motifs or the aminoacids are presents on the *Phytophthora* PGs sequences considered, with the exception of two amino acids, Lysine (K^{360}) and the Serine(S^{362}), present in certain sequence of *P.capsici* and *P.nicotianae* PGs. Inhibition capability of *Pv*PGIP2 against the purified and *in vitro* induced polygalacturonase from *P. nicotianae* (§4.2.1) and partially purified PGs from *P.capsici* was measured and none of the analyzed PG from *P. nicotianae* and from *P.capsici* (data no show) exhibits any capability to interact with *Pv*PGIP2.

5.6 P. capsici-PGIP plants

Transgenic plants from various species with increased levels of polygalacturonase-inhibiting proteins (PGIPs) are known to have better protection against pathogens. The importance of PGIPs in resistance against the necrotrophic fungus *B. cinerea* is well established: transgenic tomato and grapevine plants expressing a pear PGIP or transgenic tobacco and *Arabidopsis* plants expressing, respectively, bean or *Arabidopsis* PGIPs are more resistant to *Botrytis* infection in greenhouse experiments (Ferrari *et al.,* 2003; Manfredini *et al.,* 2005; Powell *et al.,* 2000). Conversely, Arabidopsis plants with pgip silencing show increased susceptibility towards *Botrytis* infection (Ferrari *et al.,* 2006). Borras-hidalgo *et al.,* 2012 have shown that the expression of the *Pv*PGIP2 in tobacco confers to transgenic plants a strong resistance, against an important fungal pathogen (*Rhizoctonia solani*) and two dangerous oomycetes (*Phytophthora parasitica var. nicotianae* and *Peronospora hyoscyami f. sp. tabacina*). Clearly, PGIP expression levels affect pathogen infection and host-pathogen interaction. In this work, I tested whether *Pv*PGIP2 protects tomato, *Solanum lycopersicum overexpressing Pv*PGIP2 against *P. capsici*. The results from this analysis have shown that, the observed protection conferred is not complete (Figure 4.14_4.15). Only during the early stage of the infection (2dpi- 3dpi), the differences in the sizes of the lesions were significant between the WT and the transgenic plants after inoculation with the zoospores from *P. capsici*. These differences became less significant after 6 dpi (Figure 4.6). One explanation for this result is that the *P. capsici* present a large family of PGs and each of this isoform shows distinct characteristics and not all can be inhibited from the *Pv*PGIP2. This is consistent with the observation that when the polygalacturonase isoform 1 from *B.cinerea* (*Bc*PG1) is deleted, the virulence of the fungus is significantly decreased but not completely abolished (ten Have *et al.,* 1998), indicating that multiple enzymatic activities contribute to symptoms development. Results are also consistent with the soybean PGIP that inhibits only one of the two PGs secreted by *Sclerotinia sclerotiorum* at conditions mimicking those during infection (Favaron *et al.,* 2004). It is conceivable that plant protection can be improved when all PG activities expressed in planta by a fungus are fully inhibited. This may be obtained by transforming crop plants with PGIPs that efficiently inhibit multiple PGs or with a set of different pgip genes with complementary and high-affinity recognition abilities that can be different expressed during infection (Manfredini *et al.,* 2005).

The plant of tomato expressing *Pv*PGIP2 are more resistance to *P. capsici* but the inhibition assays showed a non-direct recognition between the PGs from *Phytophthora* and

*Pv*PGIP2 protein. Microarray analysis on transgenic tobacco plants over-expressing grapevine (*Vitis vinifera*) compared to the WT brought out that PGIP may directly influence defense responses in the plant possibly by strengthening the cell walls; whether by virtue of its structural features, which contains a LRR structure shared with many receptor involved in pathogen recognition or its integration in the cell wall. This evaluation of PGIP over-expressing plants performed under pathogen-free conditions exclude the classical PGIP-PG inhibition interaction (Alexandersson *et al.*, 2011).

5.7 Differential expression patterns

The reasons for large multigene families for CWDEs are yet to be fully clarified but are likely to include advantages conferred by differential expression of individual genes, under different environmental conditions (Hatsch e*t al.*, 2006; Tomassini *et al.*, 2009), production of isozymes specialised to cleave the diverse range of linkages present in cell wall polysaccharides (Hatsch *et al.*, 2006; Niture, 2008) and evolution of isoforms that can evade recognition by plant inhibitory proteins (Misas-Villamil & van der Hoorn, 2008). During my period at Dr, Huitema Lab. I had the possibility to investigate the expression level of the PGs *in vivo* in *P. capsici* during infection in tomato, through microarray data already available in the lab. From this analysis has led in evidence that the PGs from *P. capsici* exhibit differential expression pattern during infection (Figure 4.16). Some are not present in the microarray data, but these PGs may can be expressed in other conditions such as different hosts. Is obvious that the most of the PGs are expressed during the early phase of infection (biotrophic phase), between 0 and 24h, only few exception such as *pcpg*_556668, *pcpg*_554601 and *pcpg*_556650 which are up-regulated during the late stage of infection and during the transition to the necrotrophic phase. Also *in vitro* stage, the PGs have a differential expression pattern. The expression analysis of the polygalacturonase g through qRT-PCR in *P. nicotianae* revealed a differentially expression, since there are genes such *pppg1, pppg4, pppg6, and pppg7* up- regulated at 12 hours post inoculation (hpi) and peaking at 48 hpi which suggests their importance in the early phase of plant infection. The expression of other genes, however, was very low or even undetectable. The expression of *pppg2* and *pppg10* was detected in inoculated plants but was very low in germinated cysts, which indicates that their expression is induced in planta (Wu *et al.*, 2008). In *B. cinerea*, *Bc*pg1 through *Bc*pg6 are differentially expressed, depending on stage of infection and host (ten Have *et al.*, 2001). In addition, studies of *Sclerotinia sclerotiorum* revealed that different pg genes are expressed consecutively during pathogenesis: *pg1, pg2*, and *pg3* are expressed during invasion and colonization, *pg5* in the late stage of maceration and *pg6* and *pg7* are

constitutively expressed across the colonization process (Kasza *et al.,* 2004). Studies on the two polygalacturonase expressed from *Fusarium graminearum* showed that both PGs are involved in the colonization of different part of the plants (Tomassini *et al.*, 2009).

Expression of the PG genes is generally subjected to regulation by the carbon source available. The induction of the polygalacturonase isoform 1 from *P.nicotianae* was already demonstrate previously (Yan & Liou, 2005). For investigate also the expression of the others PGs, RT-PCR was performed on *P. nicotianae* RNA extracted from the mycelia growning on a minimal medium containing 2.5% glucose and 1% of pectin from apple. The results allowed me to demonstrate that all the PGs are repressed by glucose while 8 PGs are induced by pectin from apple (Figure 4.5). The result gives more value to the theory said above that wants the pathogen to secrete an arsenal of PGs that are expressed differentially and a lot depends on the environment surrounding. It has been demonstrated in several fungal systems that glucose represses PG accumulation and CREA may contribute to catabolite repression of pectinase gene in fungi (Di Pietro & Roncero 1996; Panozzo & Cornillot, 1998). Although *Phytophthora* is phylogenetically distinct from true fungi, sequence analysis revealed the presence of several CREA-binding site consensus upstream of the pppg1 transcription initiation site (Yan & Liou, 2005).

5.8 Biochemical characterization of the PGs revealed distinct characteristics

Five polygalacturonases gene from *P. nicotianae* (*Pn*PG2, *Pn*PG3, *Pn*PG5, *Pn*PG7 and *Pn*PG9) were cloned in *P. pastoris* and expressed. PGs were purified and characterized and tested for pH optimum, effect of temperature on enzyme activity. *Pn*PG5, *Pn*PG7 and *Pn*PG9 were further analyzed for finding thermal stability and kinetic parameters. The multiplicity of PGs, known to be important components of the offence arsenal, may give higher flexibility to a pathogen by allowing invasion in a variety of different conditions and hosts, as well as protecting the fungus from losses of pathogenicity functions. The pH optimum of the five enzymes were cover from 3.5 to 6.5 with the maximum activity of individual enzymes with each PG.

The results indicated that maximum quantity of PG activity was considerably influenced by temperature. The effect of the temperature on PG activity was analyzed a range of 28 – 55 °C. The optimum of the temperature for *Pn*PG2, *Pn*PG3 and *Pn*PG9 is 45°C. The optimum of the temperature of the PnPG5 is 50 °C but its activity is still high across the range of the tested temperatures. The optimum of the temperature of the PnPG7 is 40 °C, it is still high at 45°C but rapidly decrease at 50 °C. (Figure 4.10). This clearly showed that higher temperatures affected the activity of the enzymes.

The purified *Pn*PG5, *Pn*PG7 and *Pn*PG9 showed a typical Michaelis-Mentan profile, and the Km and Vmax values for the PGs were determined using both linear and non linear regression analysis by the Michaelis–Menten equation and Lineweaver-Burk plot, as reported in figure 4.11. Km and Vmax values for *Pn*PGs were analyzed indicating PnPG7 the polygalacturonase with higher affinity for polygalacturonic acid (0,17 mg/mL) than *Pn*PG5 and *Pn*PG9. PnPG5 shows a higher Vmax (22.55 U/mL) and K_M (6.8 mg/mL). In literature were reported hundreds of Km and Vmax values from different phytopathogenic microorganism but no informations are available on kinetic parameters about Oomycetes PGs.

In a previous paper, analysis of recombinant PG proteins from *P. nicotinanae* by gel activity showed the activity of two PGs pppg5 and pppg7 are much weaker compared to the others, which suggests differences in biochemical properties from the other proteins encoded by this pathogen (Wu *et al.*, 2008). These observations partially agrees with my results demonstrate that systemic expression of different PG genes from a pathogen may cause distinct effects in plants. In *B. cinerea*, *Bc*PGs also were found to differ in many aspects, including maximum activity, Km, optimal pH, and mode of action, and to have also differences in necrotizing activity (Kars et al., 2005).

Thermal stability represents the capability of an enzyme molecule to resist thermal unfolding, deamination, hydrolysis of the peptide bonds, interchange, and destruction of disulphide bonds and oxidation of the amino acids side chains in the absence of a substrate, whereas thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of a substrate. The stability of enzymes in this case is always judged by the residual activity (Georis *et al.*, 2000). To measure the kinetics of the thermal inactivation of *Pn*PGs, the enzyme were incubated at 30-60 °C. The *Pn*PG were found to be stable at 30 – 40 °C, except *Pn*PG7 where the half-life at 40 °C decrease to 28 min. When increasing the temperature further, the inactivation rate increased drastically. *Pn*PG5 shows a decrease in the half-life to 16 min at 50 °C and 3,4 min at 60 °C; *Pn*PG7 shows a decrease in the half-life to 10 min at 50 °C and 3,9 min at 60 °C; *Pn*PG9 shows a decrease in the half-life to 14 min at 45 °C and 2,9 min at 50 °C. Thus, purified *Pn*PGs cannot be considered thermostable.

5.9 Candidates PGs from P.capsici for further investigation

The composition of plant cell walls varies significantly among cell types and species (Vorwerk, Somerville, & Somerville, 2004). For pathogens to succeed in infection, a profile of PGs that function differently in plant tissues may be required to compensate for the complicated composition and structure of pectins in the cell walls (Wu *et al.*, 2008) this was

confirmed for *P.capsici* PGs from the microarray analysis. To further investigate this possibility, seven *Pc*PGs were transiently expressed in *N. benthamiana*, using the binary vector pK7WG2. The expressed proteins of the were secreted into the apoplast of the infiltrated plants. Effects caused by the *Pc*PG proteins were classified into two groups according to the different level of necrosis causing to *N. benthamiana* leaves (Figure 4.17). *Pc*PG_130325, *Pc*PG_21386, *Pc*PG_511643, *Pc*PG_130384, and *Pc*PG_556668 showed high necrosis effects on the leaves during the first 5days post inoculation. The second group with *Pc*PG_537480 and *Pc*PG_556650 not cause any significant effect. *In vitro* analysis showed four of the PGs from the first group: *Pc*PG_130325, *Pc*PG_21386, *Pc*PG_511643, and *Pc*PG_556668 to be secreted by *P.capsici* in proteomics analysis (E. Huitema, unpublished results). Only two PGs in our screen had a positive effect on *P. capsici* virulence. *Pc*PG_556668 and *Pc*PG_556650 enhances lesion growth rates in ectopic assays. However, this phenotype was unrelated to the cell death observed upon because these two PGs fall into different groups.

Why did these PGs function differently in planta? It is not easy to explain. No relation was found between the effects, the characteristics of the proteins, such as isoelectric point values, number of putative N-glycosylation sites, ectopic expression and microarray data (Table 5.1). Similar results were obtained for the PGs from *B. cinerea*. Infiltration of *Bc*PGs into plant tissue caused chlorosis or necrosis to different extents depending on the *Bc*PG and they have found that the PGs hydrolyze pectin each in a different way. The enzymes display differences in product progression profiles, which could lead to (temporary) accumulation of different types of oligomers (Kars *et al.*, 2005).

P.capsici PG	pI value	Glycosylation sites	Induction of necrosis	Secreted	Enhance infection	Microarray data
***Pc*PG_130325**	4.21	1	high	Yes	No	Biotrophic phase
***Pc*PG_21386**	9.04	1	high	Yes	No	Biotrophic phase
***Pc*PG_511643**	8.89	1	high	Yes	No	Biotrophic phase
***Pc*PG_130384**	5.6	9	high	No	No	Biotrophic phase
***Pc*PG_556668**	9.7	1	high	Yes	Yes	Early Necrotrophic phase
***Pc*PG_537480**	9.32	1	low	No	No	Biotrophic phase
***Pc*PG_556650**	6.29	0	low	No	Yes	Early Necrotrophic phase

Table 5. 1: Summary table shows the features found for the PGs analyzed from *P.capsici*.

5.10 Gene inactivation

Gene inactivation experiments have shown in several cases that pectinases are virulence factors for plant pathogenic fungi. Targeted replacement mutants have been constructed in a variety of presumed pathogenicity genes of several fungi, and have confirmed their involvement in pathogenesis. However, the elimination of CWDEs in plant-pathogenic fungi has thus far not indicated that these enzymes play any significant role in pathogenesis. The endoPG encoded by pecA in *Aspergillus flavus* contributes to invasion and spread of the pathogen in cotton bolls (Shieh *et al.,* 1997), and the endoPG gene *Bc*pg1 of *Botrytis cinerea* is required for full virulence, although mutants remain pathogenic(ten Have *et al.,* 1998). An endoPG and an exoPG did not result in reduced virulence *of Cochliobolus carbonum* on maize. Even the elimination in one strain of both an endoPG and an exoPG (Scott-craig *et al.,* 1998) had no effect on virulence. The endoPG (enpg-1) mutant of *Cryphonectria parasitica* (Gao et al. 1996) did not display a phenotype different from the corresponding wild type.

In this thesis, experiments of silencing of two PGs from P.capsici were carried out on two very interesting candidates: *pcpg*_21386 and *pcpg*_556668. These two PGs are very different with distinct characteristics and showed diverse expression patterns during infection from the microarray data (Figure 5.3). The *pcpg*_21386 is expressed during the very early point of the infection (0,8h) and then going down where the *pcpg*_556668 has the highest expression. Very interesting is the expression *in vitro* stage where the *pcpg*_21386 is expressed more in sporangia (Spor) and little in germinated cyst (GC) and mycelium (Myc). The opposite situation we have for *pcpg*_556668 with the highest expression levels in GC and Myc.

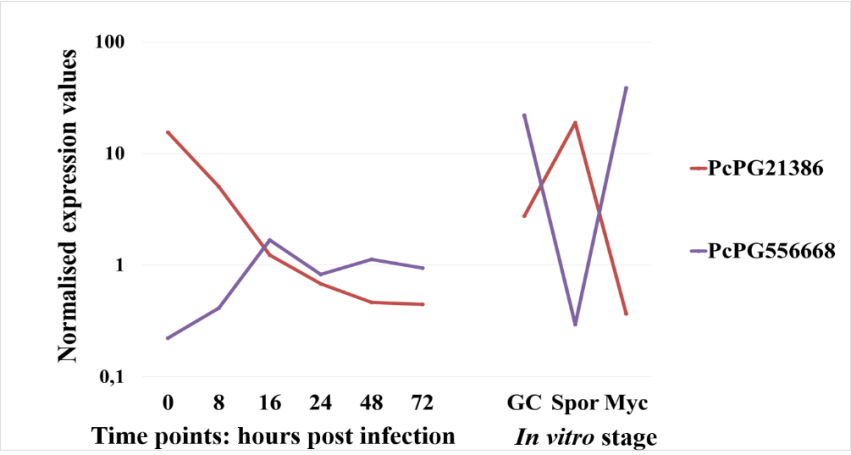


Figure 5. 3: *pcpg*_21386 (red line) and *pcpg*_556668 (purple line) display different patterns upon infection both during infection (0,8,16,24,48,72 hours post infection) and *in vitro* sporangia (Spor) germinated cyst (GC) and mycelium (Myc).

These two genes were cloned in to the silencing vector encoding hairpin RNA of pSTORA vector for the transformation of *P. capsici*. Two transformants for the silenced line *pcpg*_556668 and three for the line *pcpg*_21386 were obtained. All the transformants show a regular phenotype and, produce sporangia. Real time qPCR results shown one silenced lines for *pcpg*_556668 and one for *pcpg*_21386*3 with decreased transcript level in all the three stage germinating cyst (GC), sporangia (Spor) and mycelium (Myc) when compared with the *P. capsici* LT1534 transformed with the empty vector. Infection on leaves were carried out but no differences are presents between silenced lines and LT1534 Empty Vector.

The gene knockout approach is comparatively difficult for *Phytophthora spp* due to its diploid nature (Ah-Fong *et al.*, 2008). So far, homologous recombination has not been detected and integration of the introduced DNA into the genome is thought to be through heterologous recombination. High rates of tandemly integrated plasmids are often observed. The rates of transformation remain limited for some applications (Kamoun, 2000).

The functions of only a few of these genes have been tested. By expressing sense or antisense copies of their open reading frames in transformants of *P. infestans*, a Cdc14 phosphatase, α and β G-protein subunits, and a bZIP transcription factor were silenced and proved important in sporulation, zoospore behavior, or infection (Ah Fong & Judelson, 2003; Blanco & Judelson, 2005; Latijnhouwers & Govers, 2003; Latijnhouwers *et al.*, 2004). Other studies suggested that homology-based silencing in Phytophthora is transcriptional and not associated with DNA mutation (The feasibility to suppress gene expression was first illustrated in *P. infestans* where a transgenic strain expressing the β-glucuronidase (gus) reporter gene was further transformed with gus antisense constructs resulting in suppression of gus expression (Judelson *et al.*, 1993b). So far, this technology has been applied only once

to inactivate an endogenous gene: the *inf1* gene of *P. infestans*, which encodes a peptide elicitor from the elicitin protein family However, the presence of multiple *inf* genes in the pathogen did not allow complete suppression of elicitin production (West *et al.,* 2008; West *et al.,* 1999). Transient gene silencing of *Pihmp1* led to loss of pathogenicity and indicated involvement of this gene in the penetration and early infection processes of *P. infestans* (Whisson *et al.,* 2005). A glycoprotein that is involved in cell deposition and adhesion to cellulosic substrates from *P.nicotianae* has been silenced by sense and antisense method (Gaulin e*t al.,* 2002).

5.11 CRISPR/Cas9 system

During the last few years, there has been rapid development of genome editing strategies that make it possible to directly target regions of genes in a DNA sequence-specific manner. Two of these strategies, zinc-finger nucleases (ZFNs) (Carroll, 2011) and transcription activator- like nucleases (TALENs) (Joung & Sander, 2013), are based on protein-DNA interactions, whereas a third technology, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 endonuclease (Ran *et al.,* 2013), is an RNA-guided DNA endonuclease system. Each technology has advantages and disadvantages with regards to cost, ease of construction, efficiency of targeting, and specificity (Brooks *et al.,* 2014). Of the three genome- editing approaches, CRISPR/Cas9 has seen a meteoric rise during the past two years with applications to bacterial, animal, human, and most recently, plant systems (Pennisi, 2013). This is in part due to a greater number of advantages of CRISPR/Cas9, including the straightforward construct design and assembly, as compared to ZFNs and TALENs. CRISPR/Cas9 is a rapidly developing genome editing technology that has been successfully applied in many organisms, including model and crop plants. Cas9, an RNA-guided DNA endonuclease, can be targeted to specific genomic sequences by engineering a separately encoded guide RNA with which it forms a complex. As only a short RNA sequence must be synthesized to confer recognition of a new target, CRISPR/Cas9 is a relatively cheap and easy to implement technology that has proven to be extremely versatile (Belhaj *et al.,* 2015).

In this thesis, CRISPR/Cas9 system was used in *P.capsici* as a new system for gene editing. The first step was to improve the transformation efficiency of *P.capsici* that was very low. The expression of Cas9 was successful and I obtain transformants expressing Cas9 transcript. When the second transformation has occurred with gRNA and analysis of the target site in the sequence chosen it was been noticed that no mutation, cutting or other modifications had been obtained in the target site. Is not easy to explain why Cas9 not

worked in *Phytophthora,* but in my opinion there are some things to discuss. First, the chosen Cas9 to express in *P.capsici* was a human codon optimized e not optimized for the *Phytophthora spp.* Many experiments have been made for the visualization of the protein of Cas9 but with no success. The benefits of using codon optimized genes have been extensively demonstrated during the past decade both in industrial biotechnology in general (Elena *et al.,* 2013) and in many occasions for the expression of Cas9 in many organisms as discussed and published last year (Belhaj *et al.,* 2013; Endo, Mikami, & Toki, 2014; Jao *et al.,* 2013). The second problem is the diploid nature of *Phytophthora* which can cover the mutation and /or the disruption made by Cas9 (Belhaj *et al.,* 2015).

5.12 Conclusions

There are some main conclusion from the work presented in this thesis:

1. The research for new sequences that encoding PGs by oomycetes, confirmed the presence of multiple members in *Phytophthora spp.* Reduced was the number of the PGs identified for *Pythium* and *H. arabidopsidis.* These three species exhibit different lifestyles that could determine their ability to produce hydrolytic enzymes.

2. The phylogenetic analysis confirmed the distances between *Phytophthora, Pythium, and Aphanomycetes* species and revealed an extended gene duplication across the genomes of *Phytophthora* species for the Polygalacturonase.

3. Aminoacid sequences analysis and characterization of the PGs from *P.nicotianae* and *P.capsici* has shown a multiplicity of isoforms with distinct characteristics that may are necessary to the pathogens to colonize the different hosts.

4. It has not been possible to create mutants of *P. capsici* for investigate the role of the PGs in the pathogenesis of *Phytophthora*, using either CRISPR/Cas9 or hairpin vector. However, the use of renewed methods as CRISPR/Cas9 helped to improve the knowledge towards these species.

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