The *Botrytis cinerea* endopolygalacturonase gene family

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You want to live a life time each and every day

You've struggled before, I swear to do it again
You've told it before, until I'm weakened and sore

Seek hallowed land

(Hallowed land, Paradise Lost-*d*raconian *t*imes)

Abbreviations

AOS	**a**ctive **o**xygen **s**pecies
Bcpg	***B**otrytis **c**inerea* endo**p**oly**g**alacturonase (gene)
BcPG	***B**otrytis **c**inerea* endo**p**oly**g**alacturonase (protein)
bp	**b**ase**p**airs
CWDE	**c**ell **w**all **d**egrading **e**nzyme
DP	**d**egree of **p**olymerisation
endoPeL	endo**p**ectat**e** **l**yase
endoPG	endo**p**oly**g**alacturonase
EST	**e**xpressed **s**equence **t**ag
exoPeL	exo**p**ectat**e** **l**yase
exoPG	exo**p**oly**g**alacturonase
GA	D-**g**alacturonic **a**cid
HPI	**h**ours **p**ost **i**noculation
kbp	**k**ilo**b**ase**p**airs
LRR	**l**eucine-**r**ich **r**epeat
nt	**n**ucleo**t**ides
OGA	**ol**igo**g**alacturonic **a**cid
PeL	**p**ectat**e** **l**yase
PG	**p**oly**g**alacturonase
PGA	**p**oly**g**alacturonic **a**cid
PGIP	**p**oly**g**alacturonase-**i**nhibiting **p**rotein
PME	**p**ectin **m**ethyl**e**sterase
PnL	**p**ect**in** **l**yase
PR	**p**athogenesis-**r**elated

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Chapter 1

Gray mould caused by *Botrytis cinerea*: An interaction on the cell wall?

Plants, plant pathogens and *Botrytis cinerea*

Plants are key components in the global food chain: they are the only higher organisms capable of converting solar energy into chemical energy. Like most animals, humans depend on higher plants for their survival which makes plant pathogens a threat for humans. A plant pathogen is an organism that harms a plant by disease. Disease is defined as any malfunctioning of host cells and tissues that results from continuous irritation by a pathogenic agent or environmental factor and leads to development of symptoms (Agrios, 1997). Different types of plant pathogens are distinguished: viroids, viruses, phytoplasmas, rickettsia, bacteria, protozoa, fungi and nematodes. Although damage inflicted by insects, birds or mammals may result in symptom development, these organisms are normally not regarded as plant pathogens. *Botryotinia fuckeliana* (de Bary) Whetz. is a plant pathogenic fungus, often referred to as *Botrytis cinerea* Pers.: Fr.. The name of the asexual stage or anamorph, *Botrytis cinerea,* is commonly preferred to the name of the sexual stage or teleomorph, *Botryotinia fuckeliana* (XIth International *Botrytis* Conference, 1996, Wageningen the Netherlands). Table 1 shows the classification of both stages. *B. cinerea* has a special position within the genus *Botrytis* since it has at least 235 known host plants (Jarvis, 1977). The host range of other *Botrytis* species is restricted to only a few species within a plant genus (Mansfield and Richardson, 1981), e.g. *Botrytis tulipae* causes petal blight on tulips but not on gladiolus, whereas *Botrytis gladiolorum* has the opposite host specificity.

Table 1: Asexual and Sexual Classification of Botrytis cinerea Pers.: Fr. Botryotinia fuckeliana (de Bary) Whetz.

	Asexual classification	Sexual classification
Kingdom	Fungi	Fungi
Division	Deuteromycota	Ascomycota
Class	Hyphomycetes	Discomycetes
Order	Moniliales	Helotiales
Family	Moniliaceae	Sclerotiniaceae
Genus	Botrytis	Botryotinia
Species	*Botrytis cinerea*	*Botryotinia fuckeliana*

B. cinerea and all other pathogenic species of the genus *Botrytis* mainly infect dicotyledons and non-graminaceous monocotyledons. They are necrotrophic pathogens that actively kill plant cells and subsequently live on killed tissue (Jarvis, 1977). They are renowned to cause soft rots and blights but can also cause leaf spot, damping-off (Hausbeck and Moorman, 1996) and stem cankers (Sweets *et al*., 1982; Hausbeck and Moorman, 1996; Kim and Cho, 1996,). They produce a white, woolly mycelium on decayed tissue that will turn grey during sporulation, which can occur already a few days after the start of the infection. The diseases caused by *Botrytis* species are therefore generally referred to as ‘gray mould’. Besides the production of macro-conidia, *Botrytis* species can produce sclerotia on the

surface of diseased hosts (Honda and Mizumara, 1991). These latter structures serve to overcome unfavourable conditions. Micro-conidia are produced but they have not been described to be able to cause disease. The broad host range of *B. cinerea* results in great economic losses not only during growth of the crop but also during storage and transport of the harvested product (Elad, 1988; Hammer *et al*., 1990; Berrie, 1994). Research on *Botrytis* species has mainly focused on disease prevention and disease development of *B. cinerea* in particular.

The destruction of the plant cell wall by *B. cinerea* is believed to be an important aspect of the infection process and is subject of this thesis. Although there is serious debate whether microbial **c**ell **w**all **d**egrading **e**nzyme**s** (CWDEs) in general have a major impact on pathogenesis, they are ubiquitously produced in many plant-pathogen interactions. This introduction describes our present understanding of the architecture of the plant cell wall, functions of cell wall components in plant resistance mechanisms and roles that microbial CWDEs play in infection processes. The pectic compound network of the plant cell wall as well as pectinases from *B. cinerea* are described in more detail in later sections since there are indications that pectinases are the most important CWDEs for *B. cinerea*. The introduction concludes with an outline of this thesis.

The plant cell wall is dynamic

Cell walls play an important role in the architecture of the plant: they provide the cell with mechanical strength and maintain its shape. The intercellular space including cell walls is referred to as the apoplast (Holmes, 1979), a continuous structure that stretches throughout the plant. The apoplast not only functions as a major transport structure but it also forms a barrier to harmful biotic and abiotic agents. The apoplast consists of a highly organised network in order to enable all these functions. It implies that the structure of the cell wall differs between plant species, between tissues within a species and even between different domains of the wall of a single cell (McCann and Roberts, 1991). The different questions addressed in plant cell wall research and the different methodologies that have been adopted have led to various models of the apoplast. The three most important models include the cellular model, the phase model and the network model. The latter two are strongly related. The description of the apoplast in this section (see also Figure 1) holds for dicotyledonous and non-graminaceous monocotyledonous plants. The major chemical compounds present in the plant cell walls are listed in Table 2A.

The cellular model represents a cell wall that is divided into three separate layers. Figure 1B shows a simplified representation of a cell wall with gradual transitions of chemical composition and a gradual transition of structural complexity. This model is based on the

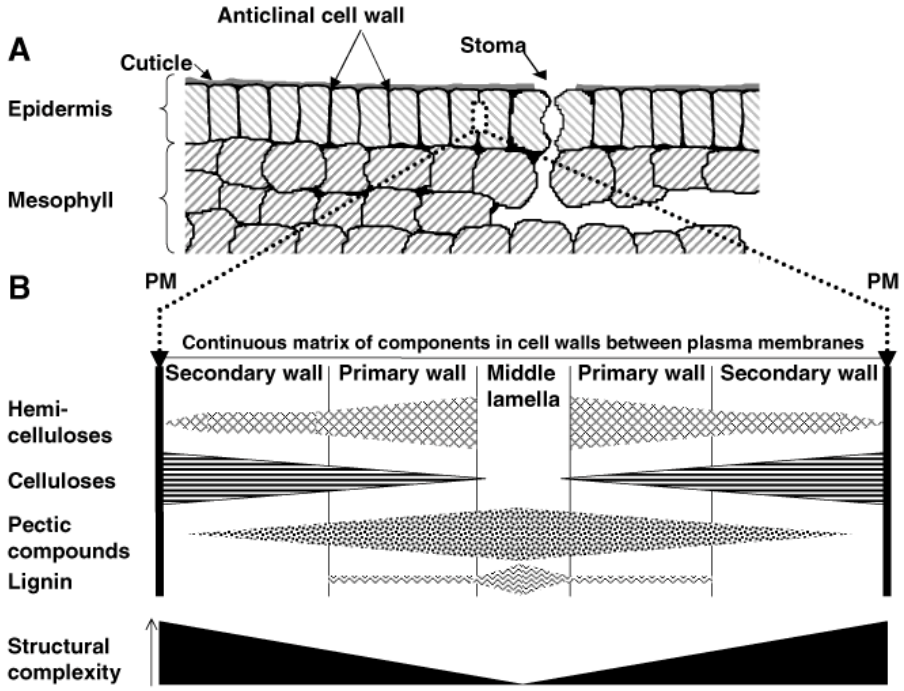

Figure 1: Organisation of the apoplast of dicotyledonous and non-graminaceous plants

1A: Schematic representation of the epidermis and underlying mesophyll

Cells can be seen as protoplasts surrounded by their cell walls. The cell walls and the intercellular space form the apoplast. The intercellular space can be in direct contact with the environment of the plant by the presented stoma but also by opened hydathodes and lenticels (not indicated).

1B: Schematic representation of the cell wall structure at intercellular level[1]

The cell wall between two cells is often described as a structure consisting of three separate layers, the secondary cell wall, the primary cell wall and middle lamella. A more accurate representation is that of continuous gradients of the structural components of which the apoplast is constructed, listed on the left. The structural complexity indicates the extent of linkages between networks. Complexity is high close to the plasma membrane (PM) and low at the interface of the cells, as indicated by the arrow.

1 After Bateman and Basham, 1976

development of the cell after division of the cell. Shortly after cell division, the cell elongates and forms a flexible extracellular layer, i.e. the primary wall. The subsequent apposition of the less flexible secondary wall is accompanied by arrest of cell elongation. The middle lamella (Fig. 1B) is regarded as the interface between two cells in which the cell wall material is often less organised.

The phase model originates from microscopical analyses and describes micro-fibrils that are embedded in a matrix, the so-called fibrillar and matrix phase (Vian *et al*., 1996). Chemical analyses revealed that the matrix phase consists of a diverse group of molecules (see Table 2A) whereas the fibrillar phase consists mainly of cellulose. Celluloses are unbranched β-1,4-glucans with a polymerisation degree of over 15,000. They form crystalline fibrils, i.e. bundles of 30 to 100 parallel cellulose molecules, mutually cross-linked by hydrogen bonds. The crystallisation degree gradually decreases towards the outside of the fibrils where increasing amounts of other sugar groups, like mannose and xylose, are present. In addition to the cross- links within one microfibril, cross-links are present between different

Table 2A: Cell wall components and the networks they can form[a]

Cellulose phase: cellulose, traces of xylose and mannose			
Cell wall component(s)	*Linked with*	*Linked by*	*Resulting structure*
Cellulose	Cellulose	Hydrogen bonds	Micro-fibrils
Micro-fibrils	Hemicelluloses	Hydrogen bonds	Cellulose-network

Matrix phase: pectic components, hemicelluloses, protein components, lignin			
Pectic components: pectins (galacturonans), arabinans, and galactans.			
Cell wall component(s)	*Linked with*	*Linked by*	*Resulting structure*
Pectic components	Pectic components	Ionic bonds (Ca^{2+})	Pectic compound network
Pectic components	Pectic components	Hydrogen bonds	Pectic compound network
Pectic components	Pectic components	Hydrophobic interactions	Pectic compound network
Homogalacturonan	Rhamnogalacturonan I	Covalent bonds	Pectic compound network
Hemicelluloses: xylan, glucans, mannans, callose[1] et cetera			
Cell wall component(s)	*Linked with*	*Linked by*	*Resulting structure*
Hemicelluloses	Hemicelluloses	Hydrogen bonds	Cellulose network
Xylan	Xyloglucan	Covalent bonds	Cellulose network
Protein components: structural and non structural proteins and glycoproteins			
Cell wall component(s)	*Linked with*	*Linked by*	*Resulting structure*
Extensin	Extensin	Covalent bonds	Extensin network
Arabinogalactan proteins	Polyphenols	Covalent bonds	Not defined
Arabinogalactan proteins	Pectic components	Non-covalent bonds	Not defined
Enzymes	Not applicable	Not applicable	Not defined
Cell membrane proteins	Not applicable	Not applicable	Not defined
Lignins			
Cell wall component(s)	*Linked with*	*Linked by*	*Resulting structure*
Lignin[2]	Lignin[2]	Covalent bonds	Lignin network

[a] Listed are the main components of plant cell walls. [1] In most species only upon induction by plant pathogens. [2] Present in most cell walls, major constituent in woody plants and in non-woody plants upon induction by plant pathogens.

Table 2B: Proposed connections between independent networks

Component	***Component***	***Linked by***	***Linked networks***
Cellulose	Extensin	Entanglement	Cellulose network and extensin networks
Cellulose	Pectin	Hydrogen bonds	Cellulose network and pectic compound network
Pectins	Xylans	Covalent bonds	Cellulose network and pectic compound network
Rhamnogalacturonan I	Xyloglucan	Covalent bonds	Cellulose netwrok and pectic compound network
Lignin	Pectin	Covalent bonds	Pectic compound network and lignin network
Pectins	Proteins	Covalent bonds	Not defined

microfibrils (Reis *et al*., 1994). Further chemical analysis of components extracted from cell walls, revealed the existence of additional bonds between the different chemical compounds (Table 2A). This resulted in a model of four networks: three networks within the matrix phase; the pectic compound network, the lignin network and the extensin network, and finally the fourth network between the fibrillar phase and the hemicellulose fraction of the matrix phase (see also Table 2A).

The pectic compound network is mainly composed of galacturonans bound by Ca^{2+} ions but also covalent bonds have been reported. The egg-box model (Fig. 2) represents a structure that might be formed by non-methylated **p**oly**g**alacturonic **a**cid (PGA) and Ca^{2+} ions. Since strong differences in the degree of methylation of PGA are found between different cell wall domains (from almost 0 up to ~80% (McCann and Roberts, 1996) it can be envisaged

that such a structure appears only occasionally. Hydrogen bonds and hydrophobic interactions predominate at low Ca^{2+} concentrations (Jarvis, 1984). In lignin and extensin networks covalent bonds are predominant. Both molecules polymerise in highly irregular shaped polymers that can span several cell layers. The fourth network consists of hemicellulose molecules, interconnected and connected to cellulose microfibrils by hydrogen bonds (Bauer *et al*., 1973; York *et al*., 1985). In addition to hydrogen bonds, covalent bonds between particular hemicelluloses have been reported (Carpita and Gibeaut, 1993).

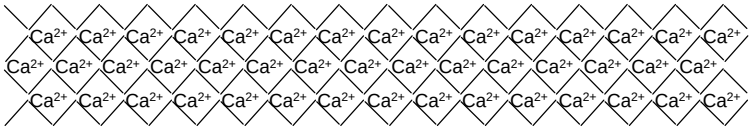

Figure 2: The egg-box model[1]

Ca^{2+} ions bind stretches of (homo)-galacturonans resulting in a structure similar to that of an egg-box.
1 After Morris et al., 1982

Although each of the cell wall networks is formed independently, cross-links between the networks exist (see Table 2B). The pectic compound network is directly connected to the microfibril-hemicellulose network by covalent bonds, whereas ferulic acid may connect the lignin network with pectins (Ishii, 1997; Williamson *et al*., 1998). Polymerisation of extensin entangles microfibrils (Lamport and Epstein, 1993). Hydrophobic and hydrophilic interactions between cell wall components strongly affect the final structure and thereby the organisation of the cell wall at both the cellular and the multicellular level.

The microfibril-hemicellulose network is believed to be the most important network in providing the cell with mechanical strength. Hence, it is the main network in the secondary wall. The pectic compound network strongly affects the overall architecture of the apoplast (McCann *et al*., 1992): it determines the pore size of the wall which affects the transport properties of the apoplast. A high content of the hydrophobic lignin will result in a cell wall with lower water content. This affects the transport capacity of the apoplast and results in more hydrogen bonds between cell wall components and thus to a more rigid cell wall. Therefore, the wall structure in fruits, which consist of up to 40% of pectic components, is very different from the wall structure in cereals, which contain only up to 1% of pectic components. The relative ratios, in which the four different cell wall networks occur in a certain compartment of the cell wall or apoplast, can therefore strongly influence the nature of that compartment. These cell wall dynamics convey flexibility to the plant in order to support specific functions of cells and tissues, respectively (McCann and Roberts, 1991). Strong differences in cell wall structure can occur within a single species. Xylem cells have perforations at their longitudinal ends in order to conduct water. On the radial side they often have high amounts of lignin in order to direct water transport. Another example is the endodermis that surrounds the central vascular cylinder in roots. The endodermis has a cell wall with a zone that contains high amounts of the hydrophobic suberin, the Casparian strip. This, again, prevents radial water

transport allowing the tissue to carry out its specific function. A different situation holds for the epidermal cell wall that has an extra protective layer referred to as the cuticle. The cuticle consists of cutin, a polymer mainly composed of fatty acids. It functions as the main barrier against evaporation and against attack by pathogens. However, the cuticle does not cover natural openings in the epidermis.

Contributions of cell wall components to resist pathogen invasion

The infection by *B. cinerea* is a process that can be described in three subsequent phases. During these different phases of infection, *B. cinerea* encounters different combinations of defence mechanisms. The apoplast is a ubiquitous physical barrier for *B. cinerea* but it also contains pre-formed components that can inhibit fungal growth and serve as chemical barrier. In addition, the plant can respond upon pathogen invasion by producing various components like phenols that contribute to both the physical and the chemical barrier. The objective of all these defence measures is to either kill the fungus or to create a fortified wall structure that surrounds and therewith restricts the fungus. This containment, however, is not always effective against *B. cinerea.* This section briefly describes infection strategies that are employed by *B. cinerea* and possible plant defences that the fungus may encounter.

Upon arrival of a conidium at a plant epidermis, the conidium can form a germ tube in order to penetrate the epidermis, as is schematically shown in Figure 3. It has been argued that, at least on a number of its hosts, *B. cinerea* is able to penetrate only through wounds, stomata or other natural openings (Poole and McLeod, 1994; Staples and Mayer, 1995). However, analyses at the ultrastructural level on several hosts indicated that *B. cinerea* is able to actively breach the cuticle. Appressorium-like structures (Rijkenberg *et al*., 1980) and a cutinase have been suggested to be involved in this process (Salinas, 1992; van der Vlugt-Bergmans *et al*., 1997a). A targeted mutant in a gene encoding a secreted cutinase was, however, not notably affected in its ability to infect tomato leaves or gerbera petals (van Kan *et al*., 1997). Other, so far unidentified, cutinases or lipases are suggested to be involved (van Kan *et al.,* 1997; Comménil *et al*., 1998; Gindro and Pezet, 1999). Once the cuticle is breached the fungus encounters the apoplast. When a stoma or other natural opening serves as entrance, the fungus encounters the intercellular space where only limiting amounts of nutrients are available.

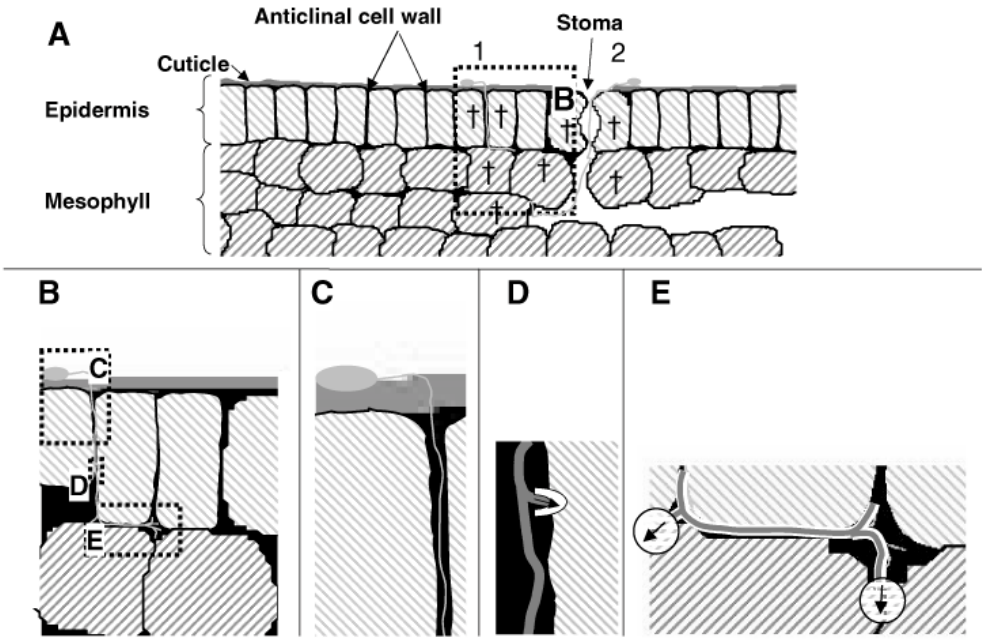

Figure 3: Cartoon envisaging possible strategies of penetration of epidermis
3A: *After Figure 1A: Penetration may occur by active breaching of the cuticle (1) or through a natural opening (2). Plant cell death (†) that occurs around the infection site may be caused by loss of cell wall coherence, toxins or by fungal growth into the protoplast.*
3B: Section of 3A *Fungal growth through the anticlinal cell wall of the epidermis into protoplasts and mesophyll.*
3C: Section of 3B *Close up depicts intercellular growth.*
3D: Section of 3B *Eventually a hypha may grow through the secondary cell wall into the direction of the protoplast, a process that is believed to be hindered by pathogen induced callose deposition (indicated by white arch).*
3E: Section of 3B *Intercellular growth through the pectin-rich middle lamella gives access to the underlying mesophyll tissue.*

A hyphal tip will eventually encounter a primary cell wall, independent whether penetration occurs anticlinal or through a stoma (indicated in Figure 3). From a primary wall, the hyphal tip can grow either into the middle lamella (see Figure 3C), which provides access to underlying tissue (see Figure 3E). Optionally, a hypha can grow into the secondary cell wall and subsequently reach the plasma membrane (see Figure 3D). Cell wall compartments that are chemically and structurally different will be encountered, depending on the direction in which the tip grows.

Plants respond to pathogen recognition with an oxidative cross-linking of phenolic cell wall components by the activation of pre-formed enzymes present in the apoplast (Levine *et al*., 1994). These enzymes catalyse the formation of cross-links between lignins and other phenolic components. This cross-linking will reduce the pore size in the middle lamella hindering further intercellular fungal growth as well as water transport. Deposition of callose in the secondary wall is another fortification process, which inhibits fungal growth into the protoplast (see Figure 3D). Normally, callose deposition starts within hours upon recognition of the pathogen. A more drastic response that can be observed following pathogen attack, is plant cell death. Plant cell death is rapidly observed following inoculation with *B. cinerea* but it

is not known whether it is induced by the pathogen, or whether it is the result of plant apoptosis or programmed cell death (Chapter 2). Slower defence responses, such as the formation of pathogenesis related (PR) proteins (Chapter 2; van Loon and van Strien, 1999) and phytoalexins (Hashim *et al*., 1997; Quidde *et al*., 1998) can be triggered upon pathogen recognition and/or cell death. Among the PR proteins are enzymes such as chitinases that have the potential to hydrolyse the fungal cell wall. Phytoalexins act as fungitoxins. Both these types of anti-fungal components are generally produced slower than the occurrence of oxidative cross-linking and callose deposition.

The plant appears to contain the fungus within the inoculation site and thereby arrests further outgrowth. This occurs in many interactions and for many fungi the infection stops here: the result is a restricted lesion, which is referred to as ghost spot in infections by *B. cinerea*. Although plant defence responses cause serious problems to *B. cinerea,* often this fungus overcomes these responses. As *B. cinerea* is a necrotroph and feeds on dead plant material it can be envisaged that plant cell death is not as effective against this fungus as it is against a biotroph. Under certain circumstances *B. cinerea* is able to grow through the thick layer of dead cells and fortified cell walls after a seemingly latent period. By then the fungus grows so vigorously that defence responses from the plant seem to be ineffective. The lesion will expand rapidly resulting in infestation of the complete organ or whole plant.

Contributions of cell wall degrading enzymes to plant infection

The previous sections describe the contributions of cell wall components to the defence system of the plant. Although cell walls form only a fraction of this organised defence system, it is conceivable that loss of cell wall coherence reduces the effectiveness of the containment of the pathogen. Many plant pathogens are believed to decrease cell wall coherence by cell wall degrading enzymes (CWDEs). Many different microbial enzymes that catalyse the degradation of one of the cell wall components have been described. In addition, some species produce different isozymes with specific activities. The occurrence of CWDEs in many plant-pathogen interactions prompted many investigators to study the function of these extracellular enzymes in infection processes (reviewed in Walton, 1994; Hugouvieux-Cotte-Pattat *et al*., 1996; Annis and Goodwin, 1997; Py *et al*., 1998). A wide variety of enzymes has been identified. Most research has been focused on the function of pectinases in plant-bacterium interactions (see Table 3 for examples of pectinases and their abbreviations). Pectate lyases (PeL) isolated from *Erwinia chrysanthemi* and *Erwinia carotovora* cause cell lysis in potato tubers (Mount *et al*., 1970; Basham and Bateman, 1975), indicating that these

Table 3: Pectinases secreted by B. cinerea classified according to the Enzyme Commission

Name	*E.C. number*	*Abbreviation*	*Substrate*[1]	*Action*	*End-product*[2]
Pectin lyase	4.2.2.10	PnL	Pectin	β-elimination	OGA-CH_3
Pectin methylesterase	3.1.1.11	PME	Pectin	Hydrolysis	PGA
exoPectate lyase	4.2.29	exoPeL	PGA	β-elimination	GA
endoPectate lyase	4.2.2.2	endoPeL	PGA	β-elimination	OGA
endoPolygalacturonase	3.2.1.15	endoPG	PGA	Hydrolysis	OGA
exoPolygalacturonase	3.2.1.67	exoPG	PGA	Hydrolysis	GA

[1]Pectin indicates methylated polygalacturonic acid, PGA indicates non-methylated polygalacturonic acid.
[2]OGA indicates oligogalacturonic acid, GA monogalacturonic acid.

CWDEs are important in infection. Similar studies have been performed for other types of bacterial CWDEs (reviewed in Hugouvieux-Cotte-Pattat *et al*., 1996). However, the simultaneous presence of various isozymes and/or different enzymes that can degrade the same substrate made it difficult to ascribe a certain effect *in planta* to a particular enzyme. Molecular genetic approaches were initiated to elucidate the role of individual CWDEs in pathogenesis. Introduction of a gene encoding a PeL from *E. chrysanthemi* in *Escherrichia coli* conferred the latter with the ability to cause significant tissue maceration on potato tubers (Payne *et al*., 1987). Although the latter study indicated that a CWDE may inflict damage during pathogenesis, there was no proof that such an enzyme is actually crucial for maceration *in planta*.

The first proof that CWDEs can be involved in pathogenesis was reported for the bacterium *E. chrysanthemi* (Roeder and Collmer, 1985). A directed mutation in *pelB*, encoding a pectate lyase resulted in a strain with reduced macerating capability. Targeted mutations in other PeL genes as well as genes encoding pectin lyase (PnL), endo**p**oly**g**alacturonase (endoPG) and pectin methylesterase (PME) were constructed in *E. chrysanthemi* (reviewed by Barras *et al*., 1994). Although not one single gene is required for virulence, most of the gene products can independently modulate the maceration of potato tuber. Targeted mutation of pectate lyase genes *pelA*, *pelD*, *pelE* and *pem* encoding a PME in *E. chrysanthemi* 3937, resulted in reduced systemic invasion of *Saintpaulia ionantha* (Boccara *et al*., 1988; Boccara and Chatain, 1989). However, this effect was not observed for mutants in *pelB* and *pelC*, indicating that contribution to maceration by a CWDE *in planta* does not necessarily contribute to the virulence of the pathogen. Regulatory aspects as well as substrate specificities might explain this phenomenon. Similar studies were performed with *E. carotovora* (reviewed by Barras *et al*., 1994), *Ralstonia solanacearum* (formerly referred to as *Pseudomonas solanacearum*) (Schell *et al*., 1988) and *Agrobacterium tumefaciens* biovar 3 (Rodriguez-Palenzuela *et al*., 1991). Targeted mutants of *R. solanacearum* and *Xanthomonas campestris* pv. c*ampestris* deficient in cellulases, enzymes that are generally produced late in the infection process only, also showed a reduced virulence (Gough *et al*., 1988; Roberts *et al*., 1988).

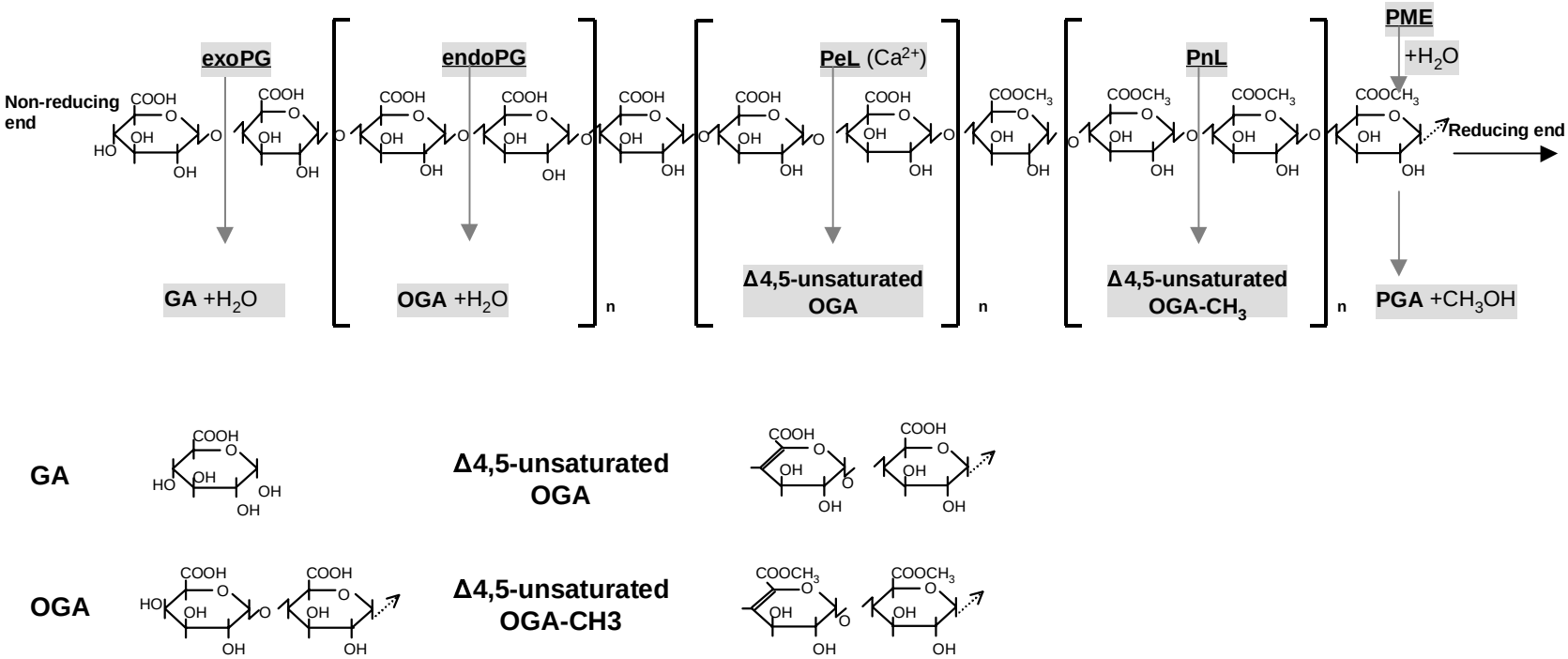

Figure 4: Cartoon of pectinase activities on galacturonan-molecule with methylated and non-methylated stretches. *Polygalacturonases (exoPG and endoPG) hydrolyse polygalacturonic acid (PGA) at the α,1-4 glycosidic bond resulting in monogalacturonic acid (GA) and oligogalacturonic acid (OGA) respectively. Pectate and pectin lyase (PeL and PnL) perform a β-elimination, the latter on methylated galacturonan, resulting in OGA with a Δ4,5 unsaturated bond at the non-reducing end. Pectin methylesterase (PME) demethylates pectin resulting in PGA. See text for details.*

In analogy to CWDEs from bacteria, CWDEs isolated from fungi were shown to degrade cell wall components *in planta* (Basham and Bateman, 1975, Bauer *et al*., 1977). In addition, a mutant of *Cochliobolus heterostrophus* exhibiting a defect in the secretion of CWDEs showed reduced virulence (Lyngholm *et al*., 1995). However, this defect results in distorted secretion of all CWDEs and probably additional, unknown factors. Therefore, no conclusions on the function of CWDEs for fungi could be drawn. The first targeted mutation of a CWDE gene in a fungus was reported for an endoPG from *Cochliobolus carbonum* (Scott-Craig *et al*., 1990). The mutant displayed no reduction in virulence on its natural host, maize, which could possibly be explained by the low amount of pectic components present in the apoplast (Carpita and Gibeaut, 1993). However, subsequent targeted mutations in glucanases in *C. carbonum*, did also not result in reduced virulence (Schaefer *et al*., 1994; Görlach *et al*., 1998). Even multiple targeted mutations did not result in reduced virulence (Apel-Birkhold and Walton, 1996; Scott-Craig *et al*., 1998). Targeted mutations in genes encoding CWDEs have also been made in other plant pathogenic fungi. Also in these cases no reduced virulence was observed (Bowen *et al*., 1995; Gao *et al*., 1996).

Residual enzyme activities that were reported for all fungal mutants might compensate a particular mutation that could possibly explain the lack of effect of targeted mutations in fungal CWDEs. Furthermore, reports on targeted mutations in fungal CWDEs are not supported by detailed expression studies. The complexity of the cell wall might be a complicating factor as well: CWDEs are active on a specific covalent bond in a specific cell wall component only. The activity of one CWDE might only decrease cell wall coherence to a certain extent and the effect of such an enzyme on the virulence of the pathogen would therefore be difficult to assess. It should also be emphasised that not all networks found in the apoplast are equally vulnerable to CWDE attack. Besides biochemical aspects of cell wall polymer degradation, aspects of structural integrity and accessibility of cell wall polymers are involved. The pectic compound network of the middle lamella allows transport of hydrophilic solvents enabling good access of CWDEs to this structure. Cellulose fibrils are dense structures and therefore not easily accessible for CWDEs and more resistant to enzymatic attack. In addition, the effect of the degradation of 1 molecule out 30-100 parallel molecules of which a cellulose fibril is made, is most probably small.

Apparently there are still many explanations for the lack of proof that fungal CWDEs are virulence factors. Nevertheless, the limited results with targeted mutations in CWDEs of fungi questions whether fungal CWDEs have a similar function in pathogenesis as described for CWDEs in certain bacterial pathogens. Since bacteria are monocellular organisms, it can be envisaged that CWDEs are more important for bacteria than for multicellular fungi. Fungal growth is accompanied by the development of a small turgor (Money, 1995 and references therein) that might help to breach through the middle lamella. A dense structural barrier might be sufficient to stop the spread of bacteria but not that of fungi. Provision of nutrients rather than growth facilitation could be a major function of fungal CWDEs. Fungi that strongly benefit

from plant cell walls for their nutrient supply, like *B. cinerea,* might therefore depend more strongly on their CWDEs than other fungi.

Degradation of the pectic compound network by pectinases secreted by *B. cinerea*

B. cinerea encounters many cell wall components during the infection process and accordingly secretes a great number of CWDEs. This thesis focuses on pectinases since these CWDEs are probably the most important CWDEs for *B. cinerea*. This is supported by the following observations.

(1) Most hosts of *B. cinerea* have an extensive pectic compound network. This forms a fragile part of the apoplast, as was mentioned before. The preference of *B. cinerea* for the pectic compound network is further supported by the observation that *B. cinerea* preferably penetrates at anticlinal positions (Fig. 3) (Mansfield and Richardson, 1981).

(2) An endoPG is generally considered as the first detectable CWDE *in planta,* although not many data on CWDE activities based on *in planta* studies exist. However, at least one endoPG isoform is constitutively expressed resulting in a high activity in liquid cultures (Leone and van den Heuvel, 1987; Johnston and Williamson, 1992a; 1992b; van der Cruyssen *et al*., 1994).

(3) Penetration of the cuticle is often followed by a swelling of the primary wall, indicating a loss of coherence of the pectic matrix (Mansfield and Richardson, 1981).

As was mentioned before, the components of the pectic compound network comprise various polysaccharide structures with a high content of galacturonides as well as rhamnoses, galactoses and arabinoses. Three main types of polygalacturonans can be distinguished: homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II, respectively. Homogalacturonans are made up of α-1,4-linked chains of D-**g**alacturonic **a**cid (GA). Rhamnogalacturonan I is a more complex pectic component consisting of a backbone of α-1,4-linked GA and α-1,2-linked rhamnose (Lau *et al*., 1985). Rhamnogalacturonan II is a minor component of the pectic compound network with a complex, yet not completely identified structure. All galacturonan residues can be methylated. acetylated or glycosylated. Homogalacturonan with a low degree of methylation is referred to as pectate whereas pectin is the name for homogalacturonan with a high degree of methylation. The term xylogalacturonan is used to describe galacturonan rich in xylose side groups. Besides these three types of galacturonides other polysaccharides can be found in the pectic compound

network. Arabinan is a highly branched molecule that has a α-1,5-linked arabinose backbone. Side chains may be α-1,2- or α-1,3- linked. Galactan, found in the primary wall, is a β-1,4-linked galactose chain with some β-1,6-linked galactose residues. Arabinogalactan I consists of galactan with arabinan side chains. Since these non-galacturonan components are found mostly within the pectic compound network, these components are being referred to as "pectic components".

Pectinases are defined as enzymes that degrade pectins. All enzymes that degrade pectic components are referred to as pectic enzymes or pectin complex enzymes. During infection *B. cinerea* secretes one or multiple forms of a variety of pectinases and other pectic enzymes (see Table 3). PnL (Fig. 2) can only degrade the backbone of pectin. **P**oly**g**alacturonases PGs and PeL can only degrade the backbone of de-methylated pectin (Fig. 2), i.e. PGA or stretches of PGA enclosed in pectin or rhamnogalacturonan I. PME demethylates pectin to pectate (Fig. 4) which can subsequently be degraded by PGs and PeLs. PGs and lyases are discriminated on basis of the end products that are formed. PGs hydrolyse the 1,4-glycosidic bond eventually resulting in GA (Rombouts and Pilnik, 1980). Pectin and pectate lyases catalyse a β-elimination resulting in Δ4,5 unsaturated GA at the non-reducing end (Fig. 4). Pectin and pectate lyase can be distinguished by the strict Ca^{2+} requirement of pectate lyase (Rombouts and Pilnik, 1980).

The chemical diversity between the different components of which the pectic compound network has been built, indicates that, on the one hand, for total degradation of the pectic compound network a broad range of pectinases is required. On the other hand, it should be emphasised that different types of galacturonides can alternate within one single polysaccharide. One pectinase might therefore be able to cause severe decrease of coherence of the pectic compound network. The degree of this decrease will be affected by the substrate specificity of this enzyme. Much remains to be discovered about the substrate specificities of pectinases (Visser and Voragen, 1996), despite the extensive studies that have been performed on pectinases from various fungi. The presence of methyl groups in galacturonan results in steric hindrance that, apparently, prevents digestion of the backbone by an endoPG or PeL. However, presence of only one or a few methyl group in a stretch of ten sugar residues will have effects different from the presence of methyl groups on all these residues (see Figure 4). EndoPG or endoPeL digestion might therefore occur in stretches with a degree of esterification that fits with the enzyme's substrate specificity. Alternatively PnL digestion might occur in a stretch with only a moderate degree of esterification. Future research will generate more detailed information on substrate specificities.

Pectic fragments: friend or foe?

Sensing the environment is an important aspect in the survival of most organisms and it is clearly indispensable in interactions. Conidia from *B. cinerea* do not strictly need a specific signal in order to germinate, they usually germinate under humid conditions. However, germination is strongly stimulated by the presence of low concentrations of glucose and organic phosphate (Rijkenberg *et al*., 1980). These conditions are similar to those near wounds on plant surfaces, which are favourite infection sites of *B. cinerea*. Apparently the fungus senses conditions that are well suited for growth, resulting in stimulation of germination. In order to prevent disease, plants need to sense invasion by pathogenic agents. Since in most plant diseases the first encounter occurs at the epidermis or in the apoplast, a great number of signal molecules can be found within these structures during infection. It can be envisaged that during several stages of the infection process both *B. cinerea* and its host sense certain conditions and respond accordingly. The course of the infection might be strongly influenced by these recognition processes.

The induction of the cutinase gene, *CutA*, gene from *B. cinerea* exemplifies the function of signal molecules. A *CutA*- *uidA* promoter-reporter fusion transgene in *B. cinerea* clearly showed induction of GUS activity during germination on the epidermis of gerbera flowers as well as on tomato leaves (van Kan *et al*., 1997). The cuticle contains substrate for the cutinase. Apparently, *B. cinerea* senses cutin and starts secreting an enzyme that is able to degrade this particular component. *B. cinerea* also secretes a number of endoPGs of which at least one is expressed constitutively whereas other endoPGs are induced by GA (van der Cruyssen *et al*., 1994). Concerted action of endo- and exoPG results in accumulation of this GA and this may increase endoPG secretion. The fungus senses the pectate degradation-product GA in its vicinity and responds by producing more pectate degrading enzymes. Nothing is known about the nature of the sensing process and mechanisms of induction of gene expression.

From the perspective of the plant, the plant has to sense its invader in order to alarm its defence system, as described before. This is based on the recognition of chemical substances referred to as elicitors, which include very distinct chemical structures. Elicitors are often produced as a result of the interaction. Gene-for-gene interactions are based on the presence or absence of elicitors and their cognate receptors in the plant. This type of elicitor is often strain-specific as well as cultivar-specific. Also CWDEs have been shown to induce plant responses. β-1,4 xylanase from *Trichoderma viride*, a saprophytic fungus, induces ethylene biosynthesis and a hypersensitive response in tobacco. This occurs in a cultivar specific manner and the enzyme may be recognised through a high affinity-binding site which suggests that the enzyme, rather than the enzyme activity, is recognised by the plant (Hanania and Avni, 1997). However, in most cases cell wall polymer fragments generated by the enzyme activity, rather than the enzyme itself, act as elicitor (Davis *et al*., 1984). This type of elicitor is referred to as endogenous elicitor and it is believed to act in a non-specific

manner. Cell wall degradation by any pathogenic CWDE but also damage inflicted by insect feeding or mechanical wounding results in an increase of oligogalacturonides in tomato (Bergey *et al*., 1996). This increase induces a number of defensive actions that are active against many predators and pathogens. Galacturonides with a **d**egree of **p**olymerisation (DP) of ten to 13, believed to be the products of pectinase activity are the most effective elicitors in soybean (Nothnagel *et al*., 1983). In tomato oligogalacturonides with a DP of 4-6 have been shown to induce ethylene production (Simpson *et al*., 1998). Furthermore, pectin is a heterogeneous component and pectinase activity will therefore result in a mixture of products rather than in one specific molecule. In addition differences might exist in the mechanisms of perception of galacturonides by different plant species. **P**oly**g**alacturonase-**I**nhibiting **P**roteins (PGIPs) have been suggested to be involved in the oligogalacturonide-based recognition of certain pathogens by plants. PGIPs were initially described as plant proteins that bind to, and thereby inhibit the activity of endoPGs (Albersheim and Anderson, 1971). In *Phaseolus vulgaris* PGIPs are expressed constitutively but are also induced during infection by *Colletotrichum lindemuthianum* (Bergmann *et al*., 1994; Devoto *et al*., 1997). The inhibition of endoPG activity is believed to slow down pathogen ingress directly. Furthermore PGIP mediated inhibition of PGs might also result in the formation of hydrolysis products with a specific length, which could act as elicitors of defence responses (Cervone *et al*., 1989). Co-evolution, as is described for many gene-for-gene relationships (de Wit, 1997), of endoPGs from pathogens and PGIPs from plants can be envisaged. Binding of endoPGs by PGIPs is very specific. PGIPI from *P. vulgaris* inhibits the endoPG from *Fusarium moniliforme* but not endoPG PGII from *A. niger,* whereas PGIPII from the same plant inhibits both endoPGs (Cervone *et al*., 1998). Mutagenesis experiments have shown that the exchange of one amino acid in PGIPI converted its specificity to that of PGIPII (Leckie *et al*., 1999). Interestingly, PGIPs contain a **l**eucine-**r**ich **r**epeat (LRR) domain (de Lorenzo, 1997). LRR domains are found in many plant resistance gene products that comply with the gene-for-gene model (Toubaert *et al*., 1992; Jones *et al*., 1994). PGIPs have been tested for inhibitory activity against endoPGs from *B. cinerea*. *B. cinerea* endoPG preparations were inhibited by protein fractions from raspberry fruits (Johnston *et al*., 1994), pear fruits (Stotz *et al*., 1994) but not by a PGIP preparation isolated from leaves of *Phaseolus vulgaris* (Cervone, personal communication).

There are additional functions claimed for oligogalacturonides in plants. Oligogalacturonides with a polymerisation degree of 12-14 induce flower formation on tobacco explants (Marfa *et al*., 1991). Since endoPGs from plant origin are ubiquitous during growth and development, this might imply that pectic fragments have important regulatory functions for plants (Albersheim *et al*., 1983; Ryan, 1988). Infection of some plants by a number of pathogens that secrete pectinases can result in the formation of cankers but it is not known whether they are induced by pectic fragments. A rhamnogalacturonan fragment was released in the infection of lettuce by *B. cinerea,* prior to the onset of fungal outgrowth (Kamoen and van der Cruyssen, 1996). Detection of the rhamnogalacturonan coincided with

polygalacturonase activity and induction of plant cell death. However, it is not known whether the compound is intrinsically toxic or whether it induces HR.

Not only plant cell wall fragments generated by fungal CWDEs induce defence in plants. Since plants possess enzymes that are capable of hydrolysing fungal cell walls, these enzymes might release fungal cell wall fragments that induce plant defence responses (Vierheilig *et al*., 1994). A β-1,3-endoglucanase from soybean turned out to be an elicitor-releasing factor, triggering the production of a phytoalexin (Takeuchi *et al*., 1990). Cell wall fragments from pathogenic and non-pathogenic rust fungi from bean have been reported to induce plant cell wall modifications in bean (Ryerson and Heath, 1992).

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Outline of this thesis

The goal of the studies presented in this thesis is to provide a basis for the elucidation of the function(s) of pectinases in diseases caused by *B. cinerea*. Although biochemical analyses suggest particular physiological functions for an enzyme *in vitro*, *in planta* analysis often requires an approach using functional molecular genetics. This is particularly true for *B. cinerea*, since for this fungus many isozymes for pectinases have been described. Chapter two describes the development of a reproducible infection on tomato leaves as well as expression analyses of both fungal and plant genes during the infection. Chapter three describes the molecular cloning and the functional analysis of the first gene encoding an endoPG in *B. cinerea*, including gene expression during infection of detached tomato leaves and the replacement of part of the gene. Chapter four describes the cloning of the other members of the gene family and the expression of the complete gene family when grown on various carbon sources that are known as substrates. *In planta* expression analyses of infections of tomato and broad bean leaves as well as apple and courgette fruit are described in chapter five. Finally, chapter six discusses all studies. The results obtained with *B. cinerea* are compared with CWDEs from other plant pathogens as well as from saprophytes. Possible spin-offs in crop protection are discussed.

Chapter 2

Fungal and plant gene expression during synchronised infection of tomato leaves by *Botrytis cinerea*[1]

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Abstract

An inoculation procedure was developed to obtain efficient and synchronous infection on detached tomato leaves by *Botrytis cinerea*. In spray-inoculated leaves incubated at 20°C, the infection process consisted of three phases: the formation of primary necrotic lesions (until 20 **h**ours **p**ost **i**noculation (HPI)), a quiescent phase (20-72 HPI), and the expansion of a proportion of the primary lesions (from 72 HPI onwards), resulting in full tissue maceration. At 4°C, the infection progressed slowly but steadily without inducing necrotic responses in the host. The actin and β-tubulin genes of *B. cinerea* were cloned, characterised and used as probes on blots containing RNAs from leaves at various stages of the infection. The genes displayed a similar expression pattern throughout the infection and the hybridisation signal reflected the amount of fungal biomass. The actin mRNA accumulated to higher levels than the β-tubulin mRNA. Tomato PR protein mRNAs (chitinase, β-1,3-glucanase and PR-1) were induced during the infection, albeit with different kinetics and to different levels. At 20°C, β-1,3-glucanase and PR-1 mRNAs were induced more rapidly than chitinase mRNAs. At 4°C, mRNAs encoding extracellular β-1,3-glucanase and intracellular, as well as extracellular chitinase were hardly induced.

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Introduction

Botrytis cinerea is an ubiquitous plant pathogenic fungus with a very wide host range (Jarvis, 1977). Due to their destructive effects on many important crops, *Botrytis* diseases have attracted great efforts in different research disciplines over the last century, aimed at the development of effective control strategies. Histological studies performed on various host plants indicate that *B. cinerea* utilises different infection mechanisms. Conidial germ tubes can penetrate through wounds or natural openings but they are also capable of directly penetrating and killing intact, healthy plant tissues, which are subsequently invaded by mycelium spreading from previously colonised, dead plant tissues (Jarvis, 1977). Different types of disease symptoms have been described, such as softening and rot in grapes (McClellan and Hewitt, 1973) or tomato (Verhoeff, 1970), as well as localised necrotic spots on gerbera flowers (Salinas and Verhoeff, 1995) or French bean leaves (van den Heuvel, 1981). When localised necrotic spots are formed, only a proportion of these develop into spreading lesions, and this may depend on a number of factors (van den Heuvel, 1981). Presumably, the induction of necrosis and the accompanying defence response in the host effectively restrict fungal growth from primary lesions for a certain period of time. In this respect, the physiology of the host plays an important role in the interaction between *B. cinerea* and its hosts (Elad and Evensen, 1995). Salinas and Verhoeff (1995) reported that the incubation of gerbera flowers, inoculated with *B. cinerea*, at 4°C resulted in spreading lesions instead of the restricted lesions that are obtained upon incubation at room temperature. Stress imposed on the host at low temperature might abolish or reduce defence responses in favour of *B. cinerea*, which is able to grow in culture and colonise plant tissues at temperatures just above 0°C. A well characterised defence response that coincides with necrosis involves the local and systemic accumulation of *de novo* synthesised **p**athogenesis-**r**elated (PR) proteins. Some of these have been identified as chitinases and β-1,3 glucanases, proteins with antifungal and/or antibacterial activity (Linthorst, 1991). In tomato, both intracellular (type I) and extracellular (type II) chitinases and β-1,3 glucanases accumulate upon infection by *Cladosporium fulvum* (Joosten and de Wit, 1989; van Kan *et al*., 1992; Danhash *et al*., 1993). In addition, two extracellular PR-1 proteins accumulate (Joosten *et al*., 1990a; van Kan *et al*., 1992) which possess inhibiting activity towards the oomycete pathogen *Phytophthora infestans* (Niderman *et al*., 1995).

Our aim is to unravel the sequence of events during the infection of plants by *B. cinerea* at the gene expression level. In this context, molecular genetic approaches are undertaken to isolate and characterise fungal genes involved in pathogenicity. The rationale of this strategy is the assumption that fungal genes, that play an important role in pathogenicity, are expressed preferentially during growth of the fungus in the host. Therefore, we have initiated a detailed comparison of the mRNA expression pattern of the fungus *in planta* with its mRNA expression pattern during growth *in vitro* (Benito *et al*., 1996). To perform such an expression analysis, several tools need to be established. First, a standard

inoculation procedure under laboratory conditions is required, that reproducibly results in efficient and synchronous infection. To achieve this, high germination rates and a high rate of infection of host cells by germinated conidia are required. As the infection progresses, the fungal biomass within the host tissue increases. Consequently, the proportion of fungal RNA, present in the total RNA extracted from infected plant tissue ('interaction RNA'), increases with time. In order to estimate the infection progress and quantify fungal gene expression *in planta* in relation to the fungal biomass, genes with a constitutive expression pattern have been used as internal standards (Mahe *et al*., 1992; Pieterse *et al*., 1993; van den Ackerveken *et al*., 1993). For this purpose, we chose to test the usefulness of genes encoding actin and β-tubulin, highly conserved proteins which are ubiquitously expressed in all eukaryotic cells. They constitute microfilaments and microtubules, respectively, involved in the determination of the cell shape and in cellular and subcellular movements. Actin genes occur in a single copy in the genome of most fungi (Ng and Abelson, 1980; Fidel *et al*., 1988; Dudler, 1990), whereas β-tubulin is encoded by one gene in *Saccharomyces cerevisiae* (Neff *et al*., 1983) and *Neurospora crassa* (Orbach *et al*., 1986) and by two genes in *Aspergillus nidulans* (May *et al*., 1987) and *Colletotrichum gloeosporioides* (Buhr and Dickman, 1993; 1994). The *A. nidulans tubC* gene and the *C. gloeosporioides tub1* gene are differentially expressed during conidiation, whereas the *A. nidulans BenA* gene and the *C. gloeosporioides tub2* gene are considered as housekeeping genes (May *et al*., 1985; Buhr and Dickman, 1993; 1994).

In this paper we describe inoculation conditions used to obtain high efficiency and synchronicity of infection of *B. cinerea* on detached tomato leaves, both at low and room temperatures. Fungal colonisation was followed visually and microscopically over time and the amount of fungal biomass was quantified by RNA blot hybridisation, using *B. cinerea* actin and β-tubulin genes as probes. Furthermore, the expression levels of tomato PR protein mRNAs during the infection at different temperatures were monitored over time.

Results

Sugar and phosphate are required for successful infection

In laboratory inoculation systems, it has previously been reported that sugars and phosphate are essential components of the inoculum for infection to occur on intact plants (Harper *et al*., 1981; van den Heuvel, 1981). The effect of sugars and phosphate on the infection of detached tomato leaves by *B. cinerea* was tested in droplet inoculation experiments. Minimal inoculum doses of 500 conidia in 5-µl droplets, in solutions containing sucrose and potassium phosphate, were required to induce the formation of spreading lesions on tomato leaves. No necrotic lesions were obtained at all when either sucrose or potassium phosphate alone were present in the inoculum buffer. Sucrose, mannose and glucose stimulated infection in the presence of phosphate, while inositol did not. The minimal concentrations that yielded reproducible results were 10 mM potassium phosphate and 10 mM of either of the sugars.

Concentrations higher than 10 mM did not have a significant additional stimulatory effect. Therefore 10 mM was adopted as standard concentration, in order to limit nutritional effects of excessive supplements.

The first symptoms were observed at 18 **h**ours **p**ost **i**noculation (HPI) as necrotic spots of the size of the droplet applied. The lesions started to expand only from 72 HPI onwards. Microscopic observations of epidermal peelings prepared 20 HPI showed that the lesions consist of areas of collapsed epidermal cells. A high proportion of conidia (40%) had not germinated on the leaf surface and the germ tubes in the necrotic areas significantly varied in length. When conidial suspensions were prepared in Gamborg's B5 medium supplemented with sucrose and potassium phosphate (10 mM each) and preincubated at room temperature for 2-3 h prior to inoculation, equally high infection efficiencies were achieved. In this medium, and only upon the preincubation, conidia germinated within a narrow timespan (4-5 HPI) and the percentage of germinated conidia on the leaf surface at 20 HPI increased to 90%. Moreover, the size of the germ tubes was rather uniform (5-8 times the diameter of the conidium). This method of inoculum preparation was used as standard for further experiments.

Standard inoculation procedure

The amount of fungal biomass is the limiting factor for the analysis of fungal gene expression *in planta*, particularly during early stages of infection. A useful inoculation procedure should provide conditions determining the formation of as many lesions as possible. Therefore a foliar spraying inoculation method was used. Conidial suspensions were prepared as described above and sprayed onto detached tomato leaves. Upon incubation at 20°C the first symptoms are visible 18-20 HPI as small, brownish spots appearing all over the surface of the leaf. During the following 48 h neither the number nor the size of the lesions increased, but the lesions became darker and growth of the fungus in the lesions was arrested. Unlike the case in the single droplet inoculations, not all primary lesions expanded. At 72 HPI, a fraction of the lesions (1-10%) simultaneously started to spread. From these few spreading lesions the fungus was able to colonise the entire leaf. Viable mycelium could be recovered from non-spreading necrotic lesions transferred to water agar in all cases. At 120-144 HPI, total leaf necrosis was observed and the fungus sporulated vigorously on the surface of the macerated tissue. The three distinct phases in the infection process were observed in all subsequent experiments performed as described here. The percentage of lesions that eventually expanded varied from one experiment to the other but never exceeded 20% of the primary lesions. The appearance of primary necrotic lesions and the initiation of lesion expansion occurred at highly similar timepoints in every experiment.

The early stages of the infection were followed microscopically in epidermal peelings. At 16 HPI, before symptom appearance, penetration of epidermal cells by germ tubes was observed and death of the penetrated host cells was manifested by their brownish cell content. Microscopic observations of epidermal strips taken at 20 HPI showed that the dark

brown spots represent necrotic lesions in which a group of collapsed epidermal cells is in contact with *B. cinerea* germ tubes.

B. cinerea infection progresses differently at low temperature

Salinas and Verhoeff (1995) observed that low temperature incubation of inoculated gerbera flowers resulted in expanding, soft-rotting lesions instead of restricted, necrotic lesions. We set out to investigate whether similar differences could be observed in inoculated tomato leaves incubated at low temperature. Since tomato is rather sensitive to cold stress, it was expected that the plant tissue would serve as a rather inert, albeit living, substrate. Individual droplets of standard conidial suspensions were inoculated onto detached tomato leaves, which were incubated at 4°C in darkness for up to 144 HPI. The conidial germination rate on agar plates at 4°C was >75%. At 20 HPI small water-soaked translucent lesions of the size of the inoculum droplet were observed. No host defence responses, such as the accumulation of dark-coloured phenolic compounds, were observed. From each primary lesion, the fungus grew slowly but steadily into the neighbouring tissue. At 120 HPI at 4°C the lesions had reached a diameter of approximately 7 mm. Each lesion on a particular leaf grew at equal rate. Differences in lesion growth were observed on different leaves and among experiments. Especially the apical leaflet of composite tomato leaves was relatively resistant to infection.

Foliar spray inoculation and subsequent incubation at 4°C yielded similar symptoms as the single droplet inoculations although the number of lesions was much higher. Unlike the primary lesions developing after spray inoculation at 20°C, all primary lesions obtained at 4°C expanded into a soft-rotting lesion and each lesion on a particular leaf grew with approximately equal speed. Due to the large number of primary lesions that expanded, the leaf material degenerated severely around 144 HPI.

The structure of fungal genes used for quantitative estimations of infection progress

Assuming that the proportion of fungal RNA in the total RNA extracted from infected tissues ('interaction RNA') reflects the proportion of fungal biomass at any given moment during an interaction, its determination allows an estimation of fungal ingress and, therefore, provides the basis for normalisation of fungal gene expression *in planta.* The proportion of fungal RNA in the interaction RNA can be determined by quantifying the hybridisation intensity of constitutively expressed fungal genes and comparing it with the hybridisation signal of RNA from *B. cinerea* cultured *in vitro*.

The *B. cinerea tubA* gene encoding β-tubulin and the *actA* gene encoding actin were isolated by heterologous hybridisation as described in Materials and Methods. Figure 1 shows the sequence of the *B. cinerea tubA* gene and its flanking sequences. Partial sequences of the coding region of the *tubA* gene have been published previously (Yarden and Katan, 1993; Luck and Gillings, 1995). The general structure of the gene is identical to that of the *N. crassa tub2* gene and the *C. gloeosporioides tub2* gene. It comprises a 1341 nt coding region,

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-943                    gatatccttgctctttgtcgattgacctgatcccaattgcaaaggattgacagccatgaagaa
-880    ctcttcatatgactgggcgtcatcgggttgattgataagtttagaagcctcatactcgacggccttgaattccttcttgc
-800    tttgtttagcagcaatctttctttctgctttctccaacttctttttatcaaccctcgactctacttttctaacatttgcg
-720    gattccaaatcaacacctccaagagatacacccaaagtagacgacatattcctctgagccccaacttggatggcttggtc
-640    tagtcgtttggcagcggaagagagctgcttgttttgcccactgctatcaaatttttgaacccatttctccactatttgat
-560    tgatctttgactgattctctggactcggatcaccagaagctgatagtaagacaggagatagcaacggctgcttctgataa
-480    tggtgatggaccattttggtctgcctcgacatatgcatttgatgcatgggtgaggtacccaactgaatattccgatagaa
-400    ccggatcaatgttgggtaagaaagctttgatttcgttctccatgatgattaatatgatacagaaagtaaaatatcaattc
-320    gtaccgccaacaaagggaccctctacacgaatcgactactttgatgcgaattgcctcacaatccgcaagtttattttcac
-240    gtgatctgcagttttccgctgtccgacttactaagcccgttgaggaaattaatccccactagcgttttatttgtttaccg
-160    attactcctgcctagcggtgaacaacaaactccactatccacaaggacatctcagcaatccttaacctcttaatacttct
 -80    ctatcctcaacctcgacttctcaatacccaaactcccatatctcaatacctcaacacaagatcctaaatcaaccttcaaa

   1    ATGCGTGAGATTgtatgtatttctctctcttcatttacgatttctacgccttcttgcaagacgcgtcgactttacccctg
   1     M  R  E  I
  81    aaaagcaccccactatatatttttaaaagtaacatatcgctgaccaagtaacttttcaatctacagGTCCATCTTCAAA
   5                                                                    V  H  L  Q
 161    CCGGCCAATGTgtaagtaaacccatccaaatatattctatgagctttgctgacaatctgctcagGGTAACCAAATTGGTG
  10    T  G  Q  C                                                        G  N  Q  I  G
 241    CTGCTTTCTGgtacgagatctcggatctgcgaaacgtcttgcttcgcgacaacctcagattgcaactaaccatatcacag
  19    A  A  F  W
 321    GCAAACTATCTCTGGCGAGCACGGTCTTGACGGTTCCGGTGTgtaagtaaaatcacaaattcttctcgtacttgaaacgt
  22      Q  T  I  S  G  E  H  G  L  D  G  S  G  V
 400    tactgatattgtttacagCTATAATGGTACATCCGATCTCCAACTTGAGCGTATGAACGTCTACTTCAACGAGgtatata
  36                        Y  N  G  T  S  D  L  Q  L  E  R  M  N  V  Y  F  N  E
 481    tacacaatttcgactctgctgaaaaccgtccgctaacccctataagGCTTCTGGCAACAAGTATGTTCCCCGTGCCGTCC
  54                                                 A  S  G  N  K  Y  V  P  R  A  V
 561    TCGTCGATTTGGAGCCAGGTACCATGGATGCTGTCCGTGCCGGTCCTTTCGGTCAACTCTTCCGTCCCGACAACTTCGTT
  66    L  V  D  L  E  P  G  T  M  D  A  V  R  A  G  P  F  G  Q  L  F  R  P  D  N  F  V
 641    TTCGGTCAATCTGGTGCTGGTAACAACTGGGCTAAGGGTCATTACACTGAGGGTGCTGAGCTTGTCGACCAAGTTCTTGA
  92     F  G  Q  S  G  A  G  N  N  W  A  K  G  H  Y  T  E  G  A  E  L  V  D  Q  V  L  D
 721    TGTTGTCCGTCGTGAAGCTGAAGGCTGTGACTGCCTTCAAGGATTCCAAATTACCCACTCTCTCGGTGGTGGAACTGGTG
 119      V  V  R  R  E  A  E  G  C  D  C  L  Q  G  F  Q  I  T  H  S  L  G  G  G  T  G
 801    CCGGTATGGGTACGCTTTTGATCTCCAAGATCCGCGAGGAGTTCCCAGATCGTATGATGGCTACCTTCTCCGTCGTCCCA
 146    A  G  M  G  T  L  L  I  S  K  I  R  E  E  F  P  D  R  M  M  A  T  F  S  V  V  P
 881    TCGCCAAAGGTTTCCGATACCGTTGTCGAGCCATATAACGCAACTCTCTCTGTCCATCAATTGGTTGAGAACTCTGACGA
 172     S  P  K  V  S  D  T  V  V  E  P  Y  N  A  T  L  S  V  H  Q  L  V  E  N  S  D  E
 961    GACCTTCTGTATCGATAACGAGGCTCTTTACGATATTTGCATGAGAACCTTGAAGCTCAGCAACCCATCTTACGGAGATC
 199      T  F  C  I  D  N  E  A  L  Y  D  I  C  M  R  T  L  K  L  S  N  P  S  Y  G  D
1041    TTAACCACTTGGTTTCCGCCGTCATGTCCGGTGTTACCACCTGTCTCCGTTTCCCTGGTCAACTTAACTCAGATCTCCGA
 226    L  N  H  L  V  S  A  V  M  S  G  V  T  T  C  L  R  F  P  G  Q  L  N  S  D  L  R
1121    AAGTTGGCTGTTAACATGGTTCCATTCCCCCGTCTCCATTTCTTCATGGTTGGATTTGCTCCTTTGACCAGTCGTGGCGC
 252     K  L  A  V  N  M  V  P  F  P  R  L  H  F  F  M  V  G  F  A  P  L  T  S  R  G  A
1201    ACACTCTTTCCGTGCTGTCACCGTTCCAGAGTTGACTCAACAAATGTACGACCCTAAGAACATGATGGCCGCTTCCGATT
 279      H  S  F  R  A  V  T  V  P  E  L  T  Q  Q  M  Y  D  P  K  N  M  M  A  A  S  D
1281    TCCGTAACGGTCGTTACTTGACATGCTCTGCCATTTTgtaagtttgccctgtaatcaatctgccaaaatcttgtagaaac
 306    F  R  N  G  R  Y  L  T  C  S  A  I  L
1361    taactttctgtagCCGTGGTAAGGTTTCCATGAAGGAGGTTGAGGACCAAATGCGCAACGTCCAAAACAAGAACTCATCC
 318                 R  G  K  V  S  M  K  E  V  E  D  Q  M  R  N  V  Q  N  K  N  S  S
1441    TACTTCGTTGAGTGGATCCCTAACAACGTCCAAACCGCCCTTTGTTCCATTCCTCCCCGTGGTCTCAAGATGTCCTCCAC
 340     Y  F  V  E  W  I  P  N  N  V  Q  T  A  L  C  S  I  P  P  R  G  L  K  M  S  S  T
1521    CTTCGTTGGTAACTCGACATCCATCCAAGAACTTTTCAAGCGTGTCGGTGATCAATTCACTGCTATGTTCAGAAGAAAGG
 367      F  V  G  N  S  T  S  I  Q  E  L  F  K  R  V  G  D  Q  F  T  A  M  F  R  R  K
1601    CTTTCTTGCATTGGTACACTGGTGAAGGTATGGACGAGATGGAGTTCACTGAGGCTGAGTCCAACATGAACGATTTGGTT
 394    A  F  L  H  W  Y  T  G  E  G  M  D  E  M  E  F  T  E  A  E  S  N  M  N  D  L  V
1681    TCCGAGTATCAACAATACCAGGATGCCTCGATCTCTGAGGGAGAGGAGGAGTACGAAGAGGAGGTCCCAATTGAGGGCGA
 420     S  E  Y  Q  Q  Y  Q  D  A  S  I  S  E  G  E  E  E  Y  E  E  E  V  P  I  E  G  E
1761    GGAATAGatatcgttgagaatcgtttcatcggtctcaagtcccgtggatgttatgaaactcctggtctcacatgtctccg
 447      E  •

1841    ctccgcccacgttgatctcgaaggtttggttatggaccgtgaagtccgtgctcttcgtgaccaattcgtcacctacaact
1921    actctaagatcctttacaatggtctttacttctctcctgagcgtgagttcatcgaggaatctatcgttgcttcccagaag
2001    aatgtcaatggacaagtcagatgccgtgtgtacaagggtaccttcagtgtcttgggtcgttcctcagagaccgagaagtt
2081    gtacgatgcaagcgagagttcaatggacgaaattggttcattcgctcctgcggatactactggtttcatcagcgttcaat
2161    ctatcagattgaagaagtatggtgaggctaaggcagctgctggtgaaagactatagatggatcttgacaccataacggct
2241    tgaatttagtctgagtcttggattggacctgagacattcgggaacttgcaacatcgccacaaaatgcagatgagacaccg
2321    attgcctccggtcctcggagtgcagatgggaatgggaatatgtaaactaaaatgctacacaaaagtcgcatatgaatgaa
2401    aacagaccagcttgttattgccagtgccgcttcaattcatgatgatttatgtgtttgtttccaagaaaagacttaatgat
2481    gattactaacag
```

Figure 1: Sequence of the* B. cinerea *β-tubulin (*tubA*) gene

The nt sequence is numbered from the ATG initiation codon. Non-coding sequences are indicated in lower case letter. The sequence of the deduced encoded protein is shown below the nt sequence of exons 1 to 7 (in capitals) and is numbered from the first methionine residue. GenBank Accesion number Z69263.

```
-225                tttcccatttactacgaccacgatcaccaattcttttgacaaaacaacaacacaccttcttctct
-160    ttcaaccatcttcttttttatcaaccacgaattaacaacttcgtgaatcttactttaatacaaaactccatcattctcga
 -80    gatcttaattatcacgagaattaactcaccgccatattccacaactcttttatttgacttaacgtcttaatttatcaacc

   1    ATGGAAGgtaagcttcaatccgacatgtatatggcgtgcccattccttgcgcagacgtctgcaacatcatgagcatctgc
   1     M  E  E
  81    taatatcatctcgcaatcaacagAAGAAGTCGCAGCCCTCGTCATTGACAATGGgtaagctcccagcatcccacaaaatg
   4                          E  V  A  A  L  V  I  D  N  G
 161    gtcgtcagcctcgaaagacagaggggtagctattggaaggaatcaacaaccgaccgagaaactccgaatcttctacagag
 241    gccaagacttcataattaagaagagtgagactgatgggttctgtagTTCTGGAATGTGTAAGGCCGGTTTCGCCGGTGAC
  14                                                  S  G  M  C  K  A  G  F  A  G  D
 321    GATGCTCCAAGAGCTGTTTTCCgtaagtagatcatccttacagacaactgttgcaatccaccaccttgatttctatcata
  25     D  A  P  R  A  V  F  P
 401    atcaatcatcgacaaccaccttgatgataatgaaatatctctaacgtgcaatacagCTTCCATTGTCGGTCGTCCCCGTC
  33                                                        S  I  V  G  R  P  R  H
 481    ATCATGGgtaagaattccatttccacgacaccccgccatttcccccacaactcgtgtcactcgaacaggaactgataagg
  41       H  G
 561    gattttatagTATTATGATTGGTATGGGTCAAAAGGACTCATATGTTGGAGATGAAGCGCAATCCAAGCGTGGTATTCTT
  43                I  M  I  G  M  G  Q  K  D  S  Y  V  G  D  E  A  Q  S  K  R  G  I  L
 641    ACCCTTAGATACCCAATCGAGCACGGTGTTGTCACCAACTGGGATGATATGGAGAAGATCTGGCATCACACTTTCTACAA
  66     T  L  R  Y  P  I  E  H  G  V  V  T  N  W  D  D  M  E  K  I  W  H  H  T  F  Y  N
 721    CGAACTTCGTGTAGCACCAGAGGAGCACCCAGTCTTACTTACTGAGGCCCCAATCAACCCAAAGTCCAACAGAGAAAAGA
  93      E  L  R  V  A  P  E  E  H  P  V  L  L  T  E  A  P  I  N  P  K  S  N  R  E  K
 801    TGACACAAATTGTCTTTGAGACCTTCAACGCCCCTGCATTCTACGTCTCTATTCAAGCCGTCCTCTCCCTTTACGCTTCC
 120    M  T  Q  I  V  F  E  T  F  N  A  P  A  F  Y  V  S  I  Q  A  V  L  S  L  Y  A  S
 881    GGTCGTACCACCGGTATCGTTCTCGATTCCGGTGACGGAGTTACTCACGTTGTTCCAATTTACGAAGGTTTCTCTCTTCC
 146     G  R  T  T  G  I  V  L  D  S  G  D  G  V  T  H  V  V  P  I  Y  E  G  F  S  L  P
 961    TCACGCCATTGCTCGTGTTGACATGGCTGGTCGTGATTTGACTGATTACCTCATGAAGATCTTGGCTGAGCGTGGTTACA
 173      H  A  I  A  R  V  D  M  A  G  R  D  L  T  D  Y  L  M  K  I  L  A  E  R  G  Y
1041    CTTTCTCCACCACTGCCGAGCGTGAAATCGTCCGTGATATCAAGGAGAAGCTCTGTTATGTTGCTCTTGATTTCGAGCAA
 200    T  F  S  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V  A  L  D  F  E  Q
1121    GAAATCCAAACCGCCAGTCAATCCTCCAGCTTGGAGAAGTCATACGAACTTCCTGATGGACAAGTTATTACCATCGGTAA
 226     E  I  Q  T  A  S  Q  S  S  S  L  E  K  S  Y  E  L  P  D  G  Q  V  I  T  I  G  N
1201    CGAGCGTTTCCGTGCTCCAGAAGCTTTGTTCCAACCATCTGTCTTGGGTCTTGAGAGCGGTGGTATCCACGTCACTACTT
 253      E  R  F  R  A  P  E  A  L  F  Q  P  S  V  L  G  L  E  S  G  G  I  H  V  T  T
1281    TCAACTCCATCATGAAGTGTGATGTTGATGTCCGTAAGGATTTGTATGGTAACATTGTTATGgtaagatttcccatctgc
 280    F  N  S  I  M  K  C  D  V  D  V  R  K  D  L  Y  G  N  I  V  M
1361    gaagtttacaggacgatatgctaacattttgacacagTCTGGTGGAACCACTATGTACCCAGGTATCTCCGATCGTATGC
 300                                          S  G  G  T  T  M  Y  P  G  I  S  D  R  M
1441    AAAAGGAAATCACTGCTCTTGCACCATCGTCGATGAAGGTCAAGATCATTGCACCACCCGAGAGAAAATACTCCGTCTGG
 315    Q  K  E  I  T  A  L  A  P  S  S  M  K  V  K  I  I  A  P  P  E  R  K  Y  S  V  W
1521    ATTGGTGGTTCTATTTTGGCATCTTTGTCTACTTTCCAACAGATGTGGATCTCAAAGCAAGAGTACGACGAGTCCGGACC
 341     I  G  G  S  I  L  A  S  L  S  T  F  Q  Q  M  W  I  S  K  Q  E  Y  D  E  S  G  P
1601    TTCCATTGTCCACCGCAAGTGTTTCTAAggtatgtaattatcaatggttcctcatatgacgggcctgtgctaatcatcgg
 368     S  I  V  H  R  K  C  F  •

1681    tattacagcgtatcacggtcgatttgtgtttacatcacgaacttcacgaatttgatccaaaagaagattcgagcaaaaca
1761    acaactcccagtgttttgtttcgccgcgaagtgttgttccgaaaattc
```

Figure 2: Sequence of the* B. cinerea *actin* (actA) *gene

The nt sequence is numbered from the ATG initiation codon. Non-coding sequences are indicated in lower case letter. The sequence of the deduced encoded protein is shown below the nt sequence of exons 1 to 6 (in capitals) and is numbered from the first methionine residue. GenBank Accesion number AJ000335.

consisting of seven exons separated by six introns located at identical positions in the three genes. Southern blot analysis using two different restriction enzymes, under moderate stringency hybridisation conditions, indicated that the *B. cinerea tubA* gene occurs in a single copy in the genome of SAS56 and nine other *B. cinerea* isolates (not shown). The protein shows more than 85% identity to other fungal β-tubulins. The *B. cinerea* β-tubulin resembles more closely the proteins encoded by the *A. nidulans BenA* gene (May *et al*., 1987) and the *C. gloeosporioides tub2* gene (Buhr and Dickman, 1994) than those encoded by the *A. nidulans tubC* (May *et al*., 1987) and the *C. gloeosporioides tub1* (Buhr and Dickman, 1993) genes.

The sequence of the *B. cinerea actA* gene is shown in Figure 2. The gene consists of six exons separated by five introns, and contains a coding region of 1125 nt encoding a protein of 375 amino acids, which shows 99% identity to the *A. nidulans* γ-actin (Fidel *et al*., 1988). The positions of the five introns of the *B. cinerea actA* gene exactly coincide with the positions of the five introns in the coding region of the *A. nidulans* γ-actin gene. There is a sixth intron located in the 3' untranslated region of the *A. nidulans* γ-actin gene (Fidel *et al*., 1988), which is probably also present in the 3' untranslated region of the *B. cinerea actA* gene since intron processing sequences are found at equivalent positions. As the *B. cinerea tub*A gene, the *actA* gene occurs in a single copy in the genome of strain SAS56, as demonstrated by Southern blot analysis (data not shown).

Estimation of fungal infection progress by RNA blot hybridisation

To test whether probes derived from these genes discriminate between plant and fungal β-tubulins and actins, and whether expression of the two fungal genes is detectable at early time points during the interaction, total RNA was extracted from mycelium of *B. cinerea* grown *in vitro*, from healthy tomato leaves and from *B. cinerea*- infected tomato leaves harvested 16 HPI. The upper panel of Figure 3 shows an ethidium bromide-stained gel containing 20 μg of each RNA sample and demonstrates that approximately equal amounts of total RNA were loaded in each lane. The gel was blotted and hybridised successively with a *tubA* probe and an *actA* probe. Equivalent exposures of this blot with both probes showed that there is no cross-hybridisation with β-tubulin or actin mRNAs from healthy tomato leaves under the conditions used (Fig. 3, panels *tubA* and *actA*). Single mRNA species of the predicted sizes (1.7 kb for *tubA* and 1.5 kb for *actA*) were detected in the RNA sample extracted from *B. cinerea* mycelium grown *in vitro*. The hybridisation intensity with the *actA* probe was about 4-5 times higher than with the *tubA* probe. The high hybridisation intensity of the *actA* mRNA allows easy detection at early stages of the interaction. Longer exposure times were required to detect expression of the *tubA* gene *in planta* at these time points (not shown). The same blot was probed with an 18S rDNA gene from radish (Grellet *et al*., 1989). This probe hybridised to the same extent with fungal and tomato 18S rRNA (Fig. 3, lower panel, lanes B.c. and L.e.). Therefore the hybridisation intensity of 18S rRNA in interaction samples (e.g. lane I.B.c. 16h) reflects the total sum of plant and fungal RNA loaded in each lane. In consequence, differences in the intensity of this band in different lanes was used to correct for differences in loading between lanes (see below).

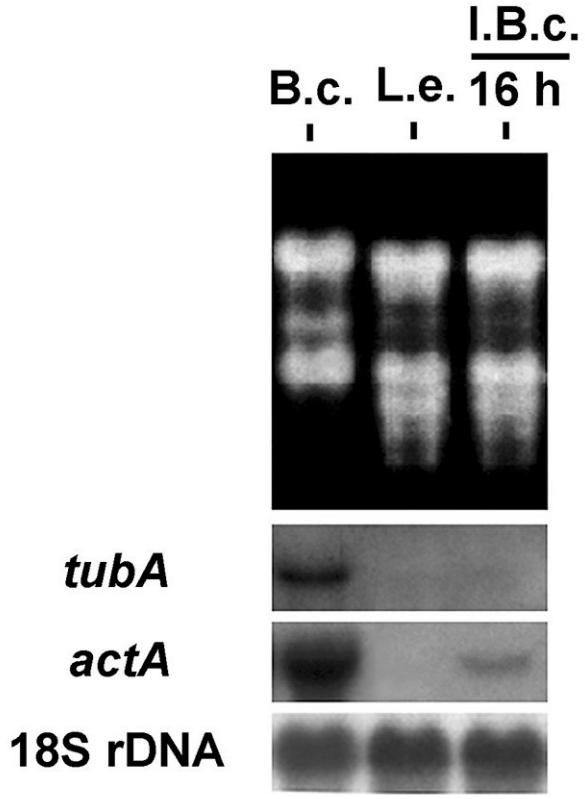

Figure 3: Detection of the* B. cinerea *β-tubulin and actin mRNAs at early stages of the infection process

Upper panel: ethidium-bromide stained RNA gel containing 20 µg of total RNA extracted from B. cinerea *grown in liquid culture (lane B.c.), from healthy tomato leaves (lane L.e.) and from tomato leaves inoculated with* B. cinerea *collected 16 hours post inoculation (lane I.B.c. 16h). Middle and lower panels: autoradiograms of the corresponding RNA blot hybridised with probes derived from the* B. cinerea tubA *gene (*tubA*), the* B. cinerea actA *gene (*actA*) and the radish 18S rDNA gene (18S rDNA). After hybridisation, the blots were washed and exposed for 16 h (*tubA *and* actA*) or 5 h 18S rDNA).*

The expression *in planta* of the *B. cinerea actA* gene was analysed in a time course experiment. Detached tomato leaves were inoculated according to standard procedures, incubated at 20°C or at 4°C, and harvested at different time points after inoculation. Total RNA was extracted and electrophoresed on a denaturing agarose gel in parallel with RNA extracted from mycelium of *B. cinerea* grown *in vitro* and from healthy tomato leaves. A blot containing equal amounts of total RNA from each sample was hybridised with an *actA* probe and an 18S rDNA probe (Fig. 4A, upper panel). The *B. cinerea actA* mRNA is detectable at 16 HPI, remains at a similar level until 72 HPI, and the intensity rapidly increases at 96 and 120 HPI. Hybridisation of the same blot with the radish 18S rDNA gene confirmed equal RNA loading.

The intensities of the hybridisation signals obtained with the *actA* probe were analysed densitometrically. The graph in the lower part of Figure 4A represents the ratios between the hybridisation intensity obtained at each time point and the intensity of the signal obtained with the RNA sample isolated from *B. cinerea* grown *in vitro*, corrected for equal loading by means of the 18S rDNA hybridisation intensity. For a constitutively expressed gene, these ratios provide an estimation of the proportion of fungal RNA in the interaction RNA at each time point. At 20°C, the ratio remains low and constant during early stages of the infection process, until 72 HPI. From this timepoint onwards there is a rapid increase of the ratio up to 120 HPI. The increase coincides with the infection phase in which colonisation and extensive maceration of the plant tissue occurs. At 4°C, the ratio increased more and extensive maceration of the plant tissue occurs. At 4°C, the ratio increased more

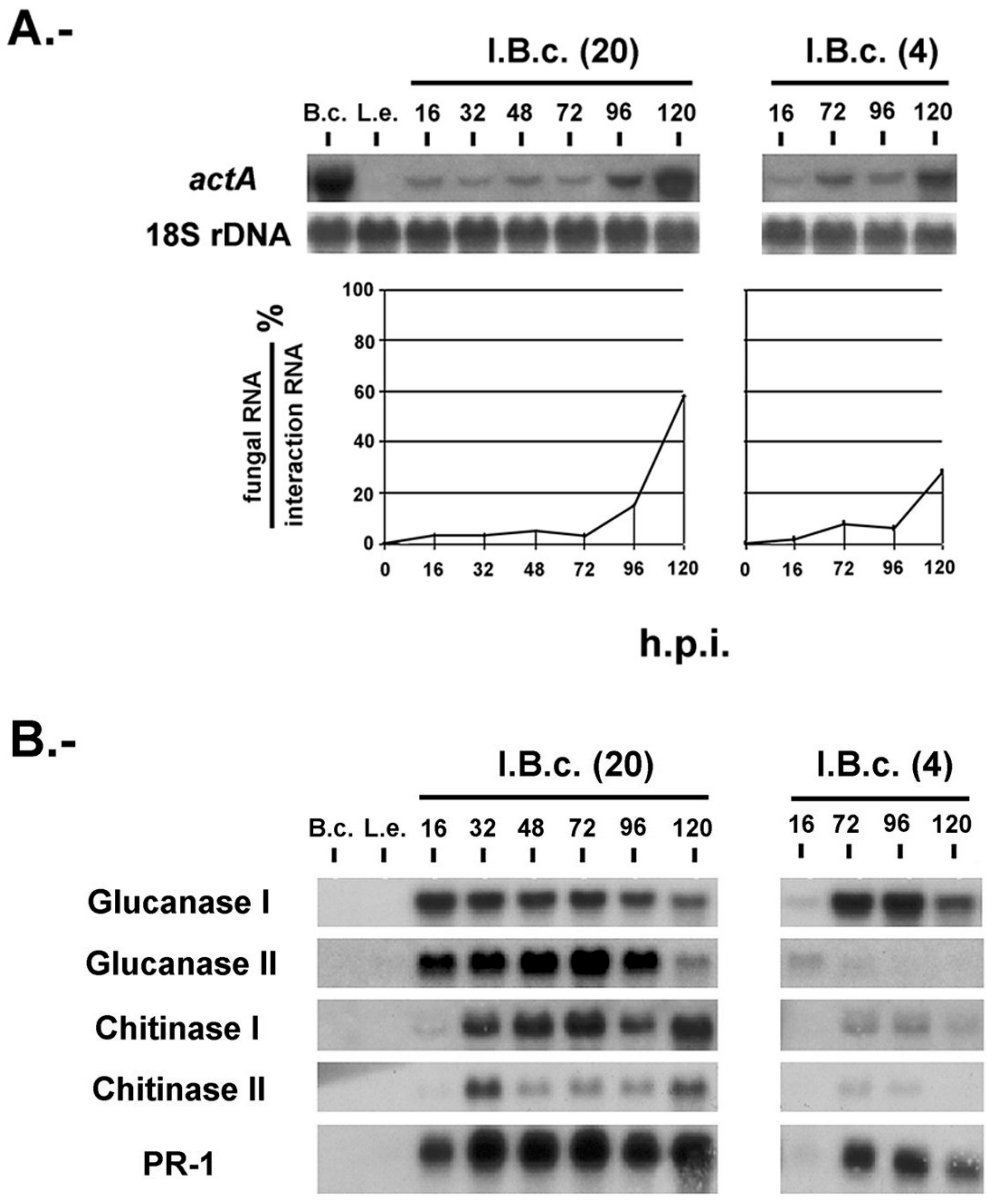

Figure 4: Fungal and plant gene expression during infection of tomato leaves

A: *Estimation of the infection progress of* B. cinerea *on tomato leaves. Autoradiograms of an RNA blot containing total RNA extracted from* B. cinerea *grown* in vitro *(B.c.), from healthy tomato leaves (L.e.) and from tomato leaves inoculated with* B. cinerea *collected at the indicated timepoints hours post inoculation (h.p.i.)after incubation at 20°C (I.B.c. (20)) or at 4°C (I.B.c. (4)). The blot was successively hybridised with a B. cinerea* act*A probe (actA) and a radish 18S rDNA probe (18S rDNA). The graphs below represent the proportion of fungal RNA in the interaction RNA at a given timepoint after inoculation, calculated as follows:* I_{actin} *(interaction)/*I_{actin} *(B.c.* in vitro*) multiplied by* I_{rDNA} *(B.c. in vitro)/*I_{rDNA} *(interaction) x 100%. This proportion was calculated for each timepoint and is displayed graphically in relation to the h.p.i..*

B:*. Analysis of PR protein mRNAs expression during the infection process. Autoradiograms of blots containing the same RNA samples as in panel A, hybridised with probes derived from the tomato intracellular type I β-1,3-glucanase clone GLUB (panel Glucanase I), the extracellular type II β-1,3-glucanase clone GLUA (panel Glucanase II), the intracellular type I chitinase clone CHI9 (panel Chitinase I), the extracellular type II chitinase clone CHI3 (panel Chitinase II) and the extracellular PR-1 clone P6 (panel PR-1). Exposure times were 16 h except for the 18S rDNA and PR-1 blots which were exposed 5 h.*

gradually during the infection process. The slight decrease between 72 HPI and 96 HPI in the experiment shown in Figure 4 was caused by sample-to-sample variation among inoculated leaves (not shown). At 120 HPI the ratio of fungal RNA / interaction RNA was about half of the value determined at the same timepoint at 20°C (see graph in Figure 4A). Hybridisation of the blot with the *tubA* probe yielded very similar results, but the exposure time for autoradiograms had to be increased as compared to the *actA* Hybridisations (not shown).

PR protein mRNAs are differentially induced by B. cinerea infection at different temperatures

Blots identical to the one shown in Figure 4A were hybridised with tomato cDNA probes encoding intracellular (type I) and extracellular (type II) chitinases and β-1,3 glucanases, as well as extracellular PR-1. The results of the Hybridisations are shown in Figure 4B. None of the five probes cross-hybridised to either of the others (not shown). None of the five PR protein mRNAs was detected in uninoculated tomato leaves. At 20°C all mRNAs were induced at 16 HPI, before any symptoms became apparent, but the hybridisation intensities were different. PR-1 mRNA showed a very high, nearly constant, level of expression until 120 HPI. Induction of both β-1,3 glucanase mRNAs was higher and faster than of the two chitinase mRNAs. The level of the type I β-1,3 glucanase mRNA was constantly high between 16 and 72 HPI and slowly declined later on. The level of the type II β-1,3 glucanase mRNA was comparable to the expression of the type I β -1,3-glucanase at 16 HPI but it increased slightly until 72 HPI and declined at later time points. Both chitinase mRNAs were transiently induced during the interaction but the kinetics of their accumulation were slightly different. For the type I chitinase, the mRNA level was maximal at 72 and 120 HPI while for the type II chitinase maximal mRNA levels were reached at 32 and 120 HPI.

At 4°C, the mRNA expression patterns were clearly different (Fig. 4B). Both β-1,3-glucanase mRNAs were weakly induced at 16 HPI, whereas induction of the two chitinases and the PR-1 mRNAs could not be observed. The type I β-1,3-glucanase mRNA accumulated strongly at later time points, from 72 HPI, whereas the type II β-1,3-glucanase mRNA declined. The type I and type II chitinase mRNAs were induced to relatively low levels at 4°C as compared to their induction at 20°C and the induction occurred rather late in the infection. As the type I β-1,3-glucanase mRNA, the PR-1 mRNA accumulated to high levels at 4 °C at later time points. When inoculated leaves were incubated at 4°C for 72 h and subsequently transferred to 20°C, the expression of all five PR proteins was strongly induced at 48 h upon transfer (not shown).

Discussion

In this paper we describe experimental conditions to obtain highly efficient and synchronised infections of detached tomato leaves with *B. cinerea*. Preincubation of conidia in an appropriate medium (Gamborg's B5 medium supplemented with sucrose and phosphate) prior to spray inoculation was important for obtaining uniform germ tube sizes and synchronous penetration of host tissue. In foliar spray inoculations incubated at 20°C, the infection process develops in three distinct phases. The first phase involves adhesion, germination and penetration into epidermal cells and the occurrence of host cell death, leading to the formation of primary lesions within a short time lapse, between 18 and 20 HPI. During the second phase, between 20 and 72 HPI, growth of the fungus is restricted and no new primary lesion is formed. In the third phase, starting 72 HPI, a proportion of the primary lesions is able to grow out into spreading lesions. The three-phase infection process in tomato leaves strongly resembles the observations made in French bean leaves under similar experimental conditions (van den Heuvel, 1981). However, no attempts were made to synchronise the infection in French bean leaves as we have described here. The similarities between the results obtained with bean and tomato leaves suggest that the synchronised infection method might be applicable to leaf tissue of a range of other host plants. On the contrary, at 4°C all primary lesions developed into spreading lesions, slowly but steadily, as was also observed in gerbera flowers by Salinas and Verhoeff (1995). The successful host tissue colonisation by *B. cinerea* at low temperatures might be a general phenomenon on a range of host plants, since *B. cinerea* is renowned for its destructive post-harvest effects, even under low temperature storage conditions. Post-harvest colonisation by *B. cinerea* may result from the inability of the host to activate effective defence mechanisms at low temperatures.

The strict requirement of sugars and phosphates in the inoculum to stimulate *B. cinerea* infection was described earlier by several groups (Harper *et al*., 1981; van den Heuvel, 1981; Edlich *et al*., 1989). Nutrients in the inoculum may mimic the situation during natural infections starting via wounded or dead tissue. It has been proposed that phosphates stimulate the production of pectolytic enzymes (van den Heuvel and Waterreus, 1985; Leone 1990) and that sugars provide energy for superficial growth and the formation of prepenetration structures (Akutsu *et al*., 1981; Clark and Lorbeer, 1976). Edlich *et al*. (1989), however, presented evidence that sugars were not required as nutrients, but rather as substrates for oxidation by fungal glucose and/or xylose oxidases. Such an oxidation generates H_2O_2, which can be converted to superoxide (O_2^-) and hydroxyl ($OH^\cdot$) radicals, **a**ctive **o**xygen **s**pecies (AOS) capable of destroying relatively inert material such as cutin and membrane lipids. H_2O_2 is also able to diffuse across cell membranes and exert toxic effects on neighbouring plant cells (Levine *et al*., 1994). Recently, von Tiedemann (1997) detected both H_2O_2 and $OH^\cdot$ radicals during the infection of bean leaf disks by *B. cinerea*. The aggressiveness of the fungal isolate was related to the amount of AOS produced (von

Tiedemann, 1997). Such experiments do, however, not discriminate between AOS generated directly by extracellular fungal enzymes or by host tissue in response to the perception of fungal constituents such as sterols (Granado *et al*., 1995), chitin fragments (Felix *et al*., 1993), phytotoxins (Rebordinos *et al*., 1996) or other, as yet unidentified molecules. The involvement of AOS in the infection by *B. cinerea* is in agreement with the observed protective effect of antioxidants (Elad, 1992). The production of AOS at the host-pathogen interface may be responsible for induction of tomato catalase mRNA from the moment of necrotic lesion appearance onwards (van der Vlugt-Bergmans *et al*., 1997b). The observation that, at the same time, the *B. cinerea* catalase mRNA is not induced *in planta* (van der Vlugt-Bergmans *et al*., 1997b), suggests that the oxidative stress imposed by AOS is not experienced equally severely by both organisms.

After penetration and primary lesion induction at 20°C, a relatively long quiescent period is observed during which no lesion expansion or fungal growth is observed. Quiescent infections have mainly been described for various fruits (reviewed by Williamson, 1994; Prusky, 1996). The successful restriction of fungal growth has been ascribed to substances present prior to infection (e.g. the saponin tomatine (Verhoeff and Liem, 1975) or polygalacturonase inhibiting proteins (Johnston *et al*., 1994)), or synthesised in response to pathogen invasion. It was demonstrated that the growth of *B. cinerea in planta* could be inhibited by the grapevine phytoalexin resveratrol, when overproduced in transgenic tobacco (Hain *et al*., 1993). In the lesions that developed on tomato leaves, the darkening of lesions during the quiescent phase (20-72 HPI) is indicative of the accumulation of *de novo* synthesised phenolic compounds, some of which presumably have fungistatic activity. The quiescent period may be required for the fungus to produce enzymes that either degrade growth inhibiting compounds (Pezet *et al*., 1991) or secrete these compounds from the mycelium by ABC transporter activity (de Waard, 1997). Plants also respond to fungal infection by producing PR proteins (Fig. 4B), several of which have hydrolytic activity towards fungal cell wall components (Linthorst, 1991). Although tomato PR proteins possess antifungal activity (Joosten *et al*., 1995) it is questionable whether they contribute to (temporary) fungal growth restriction, since *B. cinerea* is insensitive to chitinases from various plants (Broekaert *et al*., 1988).

During the quiescent phase, *B. cinerea* is not killed within the lesion. It could be argued that only 1-5% of the primary lesions develops into a spreading lesion because the plant defence mechanism successfully kills the invading fungus in the other lesions. However, viable mycelium could be recovered from all primary lesions, including the non-spreading lesions. Moreover, if the pathogen were killed within the necrotic lesions, hyphae would degenerate and fungal mRNAs would become exposed to nucleases, resulting in a decreased transcript level of actin and β-tubulin. Since the *actA* mRNA level remained constant until the end of the quiescent phase (Fig. 4A) without the development of new primary lesions, it can be concluded that the fungus is alive within the lesion and probably needs a signal to enter into the third, expansion phase. It remains to be determined whether

such a signal is released from host tissue or whether the fungus needs time to degrade and/or excrete growth inhibiting compounds before being able to resume hyphal growth (Pezet *et al*., 1991; de Waard, 1997).

The infection of tomato leaves by *B. cinerea* induced all PR protein mRNAs analysed. Remarkably, the mRNA induction kinetics are different from those induced by the biotrophic pathogen *Cladosporium fulvum*. Both in compatible and incompatible interactions with *C. fulvum*, all intracellular, type I PR protein mRNAs display very similar induction kinetics, as do the extracellular, type II PR protein mRNAs. Among the two types, there is a distinct timing of induction (van Kan *et al*., 1992; Danhash *et al*., 1993). In the tomato-*B. cinerea* interaction, the two β1,3-glucanase mRNAs are induced at the same rate and to equally high levels at 20°C but not at 4°C. The slow and transient induction of the type I and type II chitinase mRNAs at 20°C is particularly intriguing, since these mRNAs are the first to be induced during HR, triggered by injection of race-specific elicitors of *C. fulvum* into appropriate tomato genotypes (Wubben *et al*., 1996). At both temperatures the mRNA encoding the extracellular protein PR-1 has induction kinetics most similar to the intracellular type I β-1,3-glucanase mRNA. The extracellular, type II chitinase and β-1,3-glucanase mRNAs were hardly induced at 4°C and this coincides with the absence of host tissue necrosis. It will be very interesting to investigate why host cell death induced by the necrotrophic pathogen *B. cinerea* leads to clearly different PR protein induction kinetics than HR-associated host cell death induced by an avirulent race of a biotrophic pathogen, or its purified race-specific elicitors. Whether the generation of ethylene (Elad, 1990) or salicylic acid in response to the infection plays a role in the induction of the various PR protein mRNAs remains to be determined. Most tomato PR protein mRNAs investigated here are not induced by ethephon or only to low levels, and type I chitinase mRNA is not induced by salicylate (van Kan *et al*., 1995).

As a measure for the progress of the infection, we used the hybridisation intensity of the *B. cinerea* actin and β-tubulin mRNAs. Several parameters have been used for estimating the fungal biomass of a pathogenic fungus growing within its host: ergosterol (Padgett and Posey, 1994), chitin (Ekblad and Nasholm, 1996), the activity of reporter genes such as β-glucuronidase (GUS) (Couteaudier *et al*., 1993; Oliver *et al*., 1993), and the accumulation of constitutively expressed fungal mRNAs (Mahe *et al*., 1992; Pieterse *et al*., 1993; van den Ackerveken *et al*., 1993). Quantification of ergosterol and chitin in infected host tissue by chemical detection methods provides the advantage that it discriminates between fungus and plant. It does, however, not discriminate between dead and living fungal hyphae. Moreover, the induction of chitinase activity in infected host tissue (Fig. 4B) might lead to partial chitin hydrolysis and consequently, to underestimation of the fungal biomass. The quantification of GUS reporter gene activity as a parameter for *B. cinerea* biomass has been investigated. Histochemical staining for GUS activity was successfully used in early stages of the infection of gerbera flowers and tomato fruits by *B. cinerea* (van Kan *et al*., 1997), but it proved very irreproducible in necrotic tomato leaf tissue (van 't Klooster and van Kan, unpubl.) for several reasons: first, it is difficult to infiltrate necrotic tissue with aqueous solutions containing the

substrate for GUS; second, the structure of necrotic leaf tissue was disintegrated to a larger extent than the infected tomato fruit or gerbera flower tissue (van Kan *et al*.,1997); third, the activity of GUS is irreversibly inhibited by the presence of oxidising agents released during and after the collapse of host cells (Pineiro *et al*., 1994). Therefore we decided to determine the progress of fungal infection by measuring the proportion of fungal actin and β-tubulin mRNAs in a constant amount of total 'interaction RNA'. This proportion (Fig. 4A) correlated well with the development of symptoms as they were visually observed over time. In previous work, the expression of the *tubA* gene was used as internal standard to estimate fungal infection progress (Benito *et al*., 1996; van der Vlugt-Bergmans *et al*., 1997a; 1997b). Here we show that the quantification of the expression *in planta* of the *actA* gene is even more useful because of its higher hybridisation signals. The expression profile in time course experiments was highly similar with both probes suggesting that indeed both genes are constitutively expressed throughout the fungal growth phases considered in this study.

The proportion of fungal RNA in the interaction RNA might not be entirely accurate as an absolute measure for the fungal biomass in infected host tissue for two reasons. First, the amount of RNA per cell or mass unit might differ among plant and fungus (by differing water content). Second, the efficiency of RNA extraction might differ among plant cells and fungal hyphae. On the other hand, the quantification of actin mRNA in each RNA sample corrects for sample-to-sample variation. It provides an excellent marker for the relative amount of living, metabolically active mycelium in the plant tissue. Hence it can be used as internal standard in quantifying the mRNA level of other fungal genes, in order to assess whether a particular gene is induced at a particular stage of the infection process (Benito *et al*., 1996). Our future interest lies in the characterisation of changes in the gene expression pattern in *B. cinerea* during two stages of the infection process: at the penetration of the host cell (16 HPI) and at the onset of the formation of spreading lesions (60-70 HPI). We have developed tools for the analysis of fungal gene expression *in planta* by differential display RT-PCR (Benito *et al*., 1996), using the inoculation method described here. Furthermore, this bioassay can be used to determine the relative pathogenicity of *B. cinerea* gene disruption mutants in comparison with the wild type isolate (ten Have and van Kan, unpubl.).

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Materials and Methods

Inoculations. *Botrytis cinerea* strain SAS56 was cultured as described by van der Vlugt-Bergmans *et al*. (1997a). Conidia were harvested from sporulating plates by washing with 5 ml of sterile water, containing 0.05% Tween 80. The conidia were filtered through glass-wool, washed three times by centrifugation (8 min, 800 rpm, 114 x g) and resuspending in sterile water, and finally resuspended in inoculum buffer as specified in the text.

Tomato plants (cultivar Moneymaker genotype *Cf4)* were grown in soil in the greenhouse for approximately 7 weeks. Leaves were cut from the plant with a scalpel knife and inserted with their stems in a block of water-saturated floral foam. The block was placed in a petridish in a plastic box (30 x 45 cm) with a grid on the bottom, such that the leaf was spread out over the grid. Wet filter paper was placed beneath the grid to obtain high humidity. Leaflets were inoculated with conidial suspensions on the upper side either by pipetting individual droplets or by spraying using a DeVilbiss atomizer. The leaves were dried at room temperature for 30 minutes. The box was closed with a plastic transparent cover in order to obtain high humidity, and incubated either at 20°C with a 16 h photoperiod or at 4°C in the dark.

Initial experiments performed to determine the effect of inoculum composition on infection efficiency were carried out by applying twenty to forty 5 µl droplets (10^5 conidia ml^{-1}) of each suspension on one half of a leaflet. On the other half of the leaflet, sterile water was applied as control. Two leaflets were used per type of suspension in each inoculation. Each solution was tested in three independent experiments. The percentage of germination on the leaf surface was determined by counting 100 conidia in five different lesions at 16 or 20 HPI.

The standard inoculation procedure used for the gene expression studies was as follows: conidia were harvested, washed and resuspended at a density of 10^6 ml^{-1} in Gamborg's B5 medium (Duchefa BV, Haarlem, the Netherlands), supplemented with 10 mM sucrose and adjusted to 10 mM potassium phosphate (pH 6). The suspension was preincubated without shaking for 2-3 h at room temperature and inoculated by spraying droplets (±1-2 µl) onto detached tomato leaves until saturation. Leaves were dried and incubated as described above.

Light microscopy of inoculated tissues. Epidermis strips from inoculated tomato leaves were prepared at different time points, destained in acetic acid:ethanol:chloroform (1:6:3, v/v/v) for one hour, dipped into 0.2% cotton-blue in lacto-phenol for 3 min, washed in ethanol and examined using a Leitz microscope.

Isolation of the* B. cinerea tub*A and* act*A genes. A genomic λ-EMBL3 library of *B. cinerea* strain SAS56 was screened and positive phages were purified and characterised as described by van der Vlugt-Bergmans *et al*. (1997a). The probes used were derived from the *tub2* gene from *N. crassa* (Orbach *et al*., 1986) and the *actA* gene from *C. fulvum* (van den Ackerveken, unpubl.). DNA manipulations and cloning experiments were performed according to standard procedures (Sambrook *et al*., 1989).

RNA blot analysis. RNA isolation, electrophoresis and RNA blot hybridisation were performed as described by van der Vlugt-Bergmans *et al*. (1997a). RNA concentrations were determined spectrophotometrically and 20 µg of total RNA was loaded in each lane. For hybridisation, the following DNA fragments were radioactively labelled by random priming in the presence of (α-^{32}P) dATP: a 730 bp *Eco*RI-*Hin*dIII fragment containing most of the fifth exon of the *B. cinerea actA* gene, a 480 bp *Kpn*I-*Bgl*II fragment containing part of the sixth exon of the *B. cinerea tubA* gene, a 1.7 kbp *Eco*RI fragment of the radish 18S rDNA gene (Grellet *et al*., 1989) and the entire *Eco*RI-*Xho*I inserts of the cDNA clones GLUA, GLUB, P6 (van Kan *et al*., 1992), CHI3 and CHI9 (Danhash *et al*., 1993). The specific activity of the probes was routinely 7-9 x 10^5 dpm per ng of DNA. Hybridisations were carried out in 10 ml of hybridisation solution containing 1.5 -2 x 10^6 dpm per ml. The blots were washed at 65°C in 0.2 x SSC/ 0.1% SDS and exposed to Kodak X-OMAT AR films. The intensities (I) of the signals obtained on the autoradiograms, shown in Figure 3 and in Figure 4A, were quantified using an LKB Ultroscan XL densitometer. The proportion of fungal RNA in the interaction RNA at a given timepoint after inoculation was calculated as follows: I_{actin} (interaction)/I_{actin} (*B.c. in vitro*) multiplied by I_{rDNA} (*B.c. in vitro*)/I_{rDNA} (interaction) x 100%. This proportion was calculated for each timepoint and displayed in a graph in relation to the time after inoculation (Fig. 4A, lower part).

Chapter 3

The endopolygalacturonase gene *Bcpg*1 is required for full virulence of *Botrytis cinerea*[1]

Arjen ten Have, Wietse Mulder, Jaap Visser and Jan A.L. van Kan

Abstract

Botrytis cinerea, a fungus that causes diseases in over 200 plant species, secretes a number of endopolygalacturonases that have been suggested to be involved in pathogenesis. However, so far the corresponding genes have not been isolated from this fungus. We cloned *Bcpg*1, encoding endopolygalacturonase, using the *pga*II gene from *Aspergillus niger* as a heterologous probe.

The *Bcpg*1 gene is expressed to similar levels in liquid cultures of *B. cinerea* containing either 1% polygalacturonic acid or 1% sucrose, and it is expressed throughout the infection process in tomato leaves. The *Bcpg*1 gene was eliminated by partial gene replacement, and the resulting mutants were tested for virulence on tomato leaves and fruits, as well as on apple fruits. Although the mutants were still pathogenic and displayed similar primary infections when compared to control strains, a significant decrease in secondary infection, i.e. growth of the lesion beyond the inoculation spot, was observed on all three host tissues. These results indicate that the *Bcpg*1 gene is required for full virulence.

Data deposition footnote:
DNA sequence is in Genbank/EMBL/DNA Data bank of Japan under accession number U68715.

[1] **A slightly modified version of this chapter was published by Arjen ten Have, Wietse Mulder, Jaap Visser and Jan A.L. van Kan in Molecular Plant-Microbe Interactions 11:1009-1016 (1998).**

Introduction

The plant pathogen *Botrytis cinerea* Pers.: Fr. *Botryotinia fuckeliana* (de Bary) Whetz., the causal agent of gray mould, is renowned for its broad host range. Over 200 species can be infected (Jarvis, 1977) resulting in considerable economic losses. The fungus often invades senescent or damaged plant tissue. Since the fungus is able to cause disease in plants at temperatures down to 2°C, it causes many problems in post harvest chains. To establish successful infection on healthy, undamaged tissue, supplementation with nutrients is necessary (Rijkenberg *et al.,* 1980). Therefore, *B. cinerea* is regarded as a weak pathogen.

An important function for a cutinase was suggested by studies on the penetration of bean and tomato cuticles (Rijkenberg *et al.,* 1980). Reduction of lesion formation in gerbera flowers, as a result of treatment with antibodies raised against cutinase A, supported this hypothesis. (Salinas 1992). However, gene disruption studies demonstrated that cutinase A is not essential for the infection of both tomato fruits and gerbera flowers (van Kan *et al.,* 1997), suggesting that penetration of the cuticle occurs either by mechanical breaching or by other enzymes (Clark and Lorbeer 1976, van Kan *et al.,* 1997).

Many reports describe the secretion of **c**ell **w**all **d**egrading **e**nzyme**s** (CWDEs) by *B. cinerea* during early stages of infection. This is accompanied by a swelling of the epidermal cell wall, suggesting that the CWDEs are actively involved in penetration (Elad and Evensen 1995, Mansfield and Richardson 1981). However, it cannot be excluded that CWDEs are not required during penetration, but are rather involved in the degradation of the cell wall during subsequent host tissue invasion.

Among the *B. cinerea* CWDEs described, an endo**p**oly**g**alacturonase (endoPG, E.C. 3.2.1.15) with a basic pI is one of the first enzymes that is secreted (Leone and van den Heuvel 1987; Johnston and Williamson 1992a; 1992b; van der Cruyssen *et al.,* 1994). Activity of this enzyme, denoted as PG-A (van der Cruyssen *et al.,* 1994), PG2 (Leone and van den Heuvel 1987) or PG-I (Johnston and Williamson 1992a; 1992b), is found when the fungus is grown on **p**oly**g**alacturonic **a**cid (PGA) as well as on sucrose. In planta PG-A is predominantly detected in the outer zone of the infected area, whereas other endoPGs are only detected in the central zone of the infected area (Kamoen and van der Cruyssen 1996). Although much biochemical research has been performed, there is no clear understanding of the possible functions of endoPGs in the pathogenesis of *B. cinerea.* Molecular genetic studies in several other plant pathogenic fungi have not given support for a role in pathogenesis for these enzymes (reviewed by Annis and Goodwin 1997). However, it was recently reported that the elimination of an endoPG in the saprophytic fungus *Aspergillus flavus* resulted in a mutant with reduced virulence on cotton bolls (Shieh *et al.,* 1997). Here we describe the isolation, characterisation and elimination of *Bcpg*1, a member of an endoPG gene family from *B. cinerea*, and provide evidence that this gene plays a functional role during pathogenesis on three different host plant tissues.

Results

*Bcpg*1 is a member of a gene family encoding endoPGs

The *Bcpg*1 gene was isolated from a genomic λ-EMBL3 library (Kusters-van Someren *et al*., 1992) using the heterologous probe from *A. niger*. Twenty-two strongly hybridising and 50 weakly hybridising plaques were identified in a primary screening of 1.5×10^5 plaques (30 genome equivalents). Two of the 22 strongly hybridising plaques were identified as false positives in a secondary screening. DNA from purified recombinant phage 25, one of the intensely hybridising phages, was subjected to Southern analysis. Two overlapping hybridising fragments were isolated from this phage, cloned and used for sequence analysis. Figure 1A shows a restriction map of *B. cinerea* genomic DNA encompassing the region of DNA which hybridised to the heterologous probe. DNA sequence analysis revealed an open reading frame of 1149 bp. Putative core promoter elements, such as TATA and CAAT motifs, are present (Fig. 2), but the sequence lacks a typical polyadenylation signal. Using homology to polygalacturonases and the percentage of GC in third base of the codons, no intron was found (Ficket 1982). The encoded translation product (Fig. 2) contains a putative cleavage site for a signal peptide between Ala^{20} and Ala^{21} (Nielsen *et al.,* 1997). In analogy to PGII from *Aspergillus niger* Arg^{42} might serve as a monobasic cleavage site (Visser *et al.,* 1995). This would result in a mature protein, denominated BcPG1, consisting of 340 amino acids with a predicted molecular mass of 33.7 kDa and a calculated pI of 8.07. The protein contains an N-

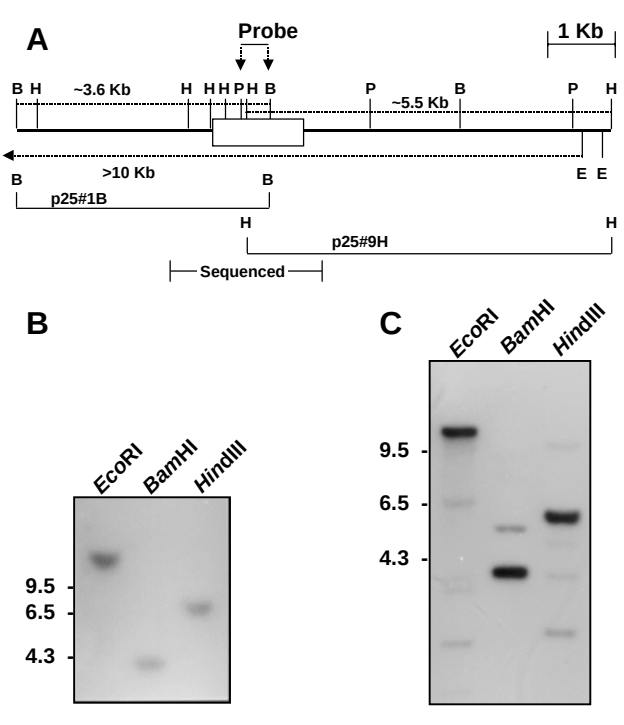

Figure 1: Analysis of* Bcpg*1 locus

A: *Restriction map of the cloned genomic DNA encompassing the* Bcpg*1 gene. Phage 25 contained two overlapping restriction fragments, 25#1B and 25#9H, which hybridised to the 700 bp* Sal*I* pga*II probe from* Aspergillus niger*. The sequenced region identified an open reading frame, indicated by the open box. The probe indicates the part of the gene which was used for Southern analysis, the dashed lines indicate the hybridising fragments. B:* Bam*HI, E:* Eco*RI, H:* Hind*III, P:* Pst*I (panel B).*

B: *Southern blot analysis of genomic DNA. Each lane contains 2 µg genomic DNA from strain SAS56 digested with restriction enzymes as indicated. The blot was hybridised with the probe indicated in panel A, and washed at high stringency conditions.*

C: *Low stringency hybridisation of blot shown in panel B. Molecular sizes were determined by comparison with comigrated* Hind*III digested λ-DNA, indicated on the left-hand side of the panel (Kb).*

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-862                    caaacggccgatccaacctttctttttactatcagattgaaattccaaaaatgtaatccct
-800   acgattctagatggcactcgcaaaaccatcagttagaa101tctcaggggttcttaaccacaccaatcatatccttctaa
-720   acttccatcaaatctctagtgcagccgtcgtttgttgacatttcatcaactcaaaacatccaacaacttcgccgcatcat
-640   tttttgggttagtcgttggttaattacactaattgaggcgttgtgctctggaattagtctagaagagctccaatttcatc
-560   accttgacaagtaattaaggaggaacggtacttcgaaagtacttcgaaattacttcggagtgatgttgtaatataccgga
-480   gggtctagtgccttttatgcttgtccaaagcttcttttgcttcgtttcatcttccaagcctttcgttccccccttgatgta
-400   tagcccattcaacaagaaagtggtgacattttgtactccacaaggcaatctctccttatttcaaagcttatgtttgatga
-320   tgttccaaggatcgctggggtcagaatgcggaccttgatcttacatggcagagcaaaagcgtaccatagcatcaaatata
-240   ctttggctcaccgcgggttaggtttaccgctgttaacatttcgcatcaacagccgacggcggattcaaccgggttccact
-160   tctttgatgcttttccgaatttcaatcatgtatttacatatataaggaagcttcgaatacctttttttcattcctcatct
 -80   catcatcacttacattcttttccatactgtctagtacattcactctttaaacttacattcttttaacactactagccaat

   1   ATGGTTCAACTTCTCTCAATGGCCTCCGGCCTCCTTGCGCTGAGCGCAATCGTCTCTGCTGCTCCAGCACCAGCACCAAC
   1    M  V  Q  L  L  S  M  A  S  G  L  L  A  L  S  A  I  V  S  A *A  P  A  P  A  P  T
  81   CGCAGCACCAAACCCAGCTGAGGCTCTCGCCGCTATCGAGCAACGTGGTACCGCCTGTACCTTCTCCGGATCTGGCGGTG
  28     A  A  P  N  P  A  E  A  L  A  A  I  E  Q  R  G  T  A  C  T  F  S  G  S  G  G
 161   CTGCAGCCGCTTCCAAGTCCAAGGCTTCCTGCGCCACCATCGTCCTCAGCGCCCTCTCCGTCCCATCCGGTACTACTCTT
  54   A  A  A  A  S  K  S  K  A  S  C  A  T  I  V  L  S  A  L  S  V  P  S  G  T  T  L
 241   GACTTGACTGGCCTCAAGTCCGGCACCCAGGTCATCTTCGAGGGTACCACCACCTTCGGTTACGAAGAATGGTCCGGTCC
  81    D  L  T  G  L  K  S  G  T  Q  V  I  F  E  G  T  T  T  F  G  Y  E  E  W  S  G  P
 321   TCTCTTCTCTGTCTCCGGAACTGACATCACTGTCAAGGGTGCCTCCGGCAGCAAGCTTGATGGTCAAGGAGCCAAGTACT
 108     L  F  S  V  S  G  T  D  I  T  V  K  G  A  S  G  S  K  L  D  G  Q  G  A  K  Y
 401   GGGATGGTAAGGGAACCAACGGTGGAAAGACCAAGCCAAAGTTCTTCTACGCCCACTCCTTGAAGGGTAAATCAACAATC
 134   W  D  G  K  G  T  N  G  G  K  T  K  P  K  F  F  Y  A  H  S  L  K  G  K  S  T  I
 481   TCTGGTATCAACATCTTGAACTCTCCAGTTCAAGTCTTCTCCATCAACGGTGCTTCCGGTCTTACTCTCTCCAACATCAA
.161`   S  G  I  N  I  L  N  S  P  V  Q  V  F  S  I  N  G  A  S  G  L  T  L  S  N  I  N
 561   CATTGACAACTCTGCTGGAGATGCTGGTTCCCTTGGCCACAACACCGATGCTTTCGATGTTGGTTCCTCCAGCGATATCA
 188     I  D  N  S  A  G  D  A  G  S  L  G  H  Ⓝ  T  Ⓓ  A  F  D  V  G  S  S  S  D  I
 641   CCATCTCCGGTGCCGTCGTTAAGAACCAAGATGACTGTCTCGCTATCAACTCCGGTACTGGTATTACCTTCACCGGTGGA
 214   T  I  S  G  A  V  V  K  N  Q  Ⓓ  Ⓓ  C  L  A  I  N  S  G  T  G  I  T  F  T  G  G
 721   ACCTGTTCCGGTGGTCACGGTCTCTCCATCGGATCTGTCGGTGGACGTTCCGACAACACTGTCTCCGATATCATCATCGA
 241    T  C  S  G  G  Ⓗ  Ⓖ  L  S  I  G  S  V  G  G  R  S  D  N  T  V  S  D  I  I  I  E
 801   ATCATCTACTGTCAAGAACTCTGCCAACGGTGTCCGTATCAAGACTGTCTCCGGTGCCACTGGATCCGTCTCCGGCGTTA
 268     S  S  T  V  K  N  S  A  N  G  V  Ⓡ  I  Ⓚ  T  V  S  G  A  T  G  S  V  S  G  V
 881   CCTACAAAGACATCACCCTCTCCGGCATTACATCCTACGGTGTTGTCGTTCAACAAGATTACAAGAACGGTTCCCCAACT
 294   T  Y  K  D  I  T  L  S  G  I  T  S  Y  G  V  V  V  Q  Q  D  Ⓨ  K  N  G  S  P  T
 961   GGTACCCCAACTTCAGGTGTCCCCATCACTGATGTCACTTTCTCCAACGTCAAGGGTACCGTCGCCTCCGGTGCCACCAA
 321    G  T  P  T  S  G  V  P  I  T  D  V  T  F  S  N  V  K  G  T  V  A  S  G  A  T  N
1041   TGTCTACGTTCTTTGCGCCAAGTGCTCTGGCTGGTCTTGGGATGTCAGTGTTAGCGGTGGTAAGACCTCCAGCAAGTGTG
 348     V  Y  V  L  C  A  K  C  S  G  W  S  W  D  V  S  V  S  G  G  K  T  S  S  K  C
1121   CTGGTCTCCCATCTGGTGTCAAGTGTTAA-1149
 374   A  G  L  P  S  G  V  K  C  •

1150   acatcttaacaccttgaccatcaaacccttgcagggtttggaacaattttatagaaggggatgggggtggtgttgttg
1230   gagcagtgctcagtgttcgcgttctttgtatatatagcttctattgtccacatatcgactgtttaaaccattgtaatcga
1310   attaatttgattaccttccaaaactttagcttccaatcatgctcttcagtgaactctccaggtctctgtgaaaccaatcg
1390   atttctcgccgtataagtcttaccctctgtatcagc-1425
```

***Figure 2: Nucleotide sequence of the* Bcpg*1 gene from* B. cinerea**

Coding sequence is in uppercase and the deduced amino acid sequence is indicated underneath. Possible core promoter motifs are underlined. Amino acid residues which are strictly conserved among polygalacturonases are indicated in a circle. A cleavage site for a proposed signal sequence is indicated by an asterisk (*) whereas the putative monobasic protease cleavage site is double underlined. The potential N-glycosylation site is boxed.

glycosylation signal at position 316 and it contains all nine amino acids which are conserved among all known exo- and endopolygalacturonases (Benen *et al.,* 1996). BcPG1 shows 53% amino acid identity to PGII from *A. niger* and 84% to PG1 from *Sclerotinia sclerotiorum*, a closely related plant pathogenic fungus.

A DNA blot was hybridised with a part of the *Bcpg*1 coding sequence, indicated in Figure 1A. A single band was observed under stringent conditions for all three restriction enzyme digests, indicating that *Bcpg*1 is a single copy gene (Fig. 1B). At low stringency, up to five additional bands were detected. (Fig. 1C).

*Bcpg*1 is expressed during pathogenesis in tomato leaves

Following inoculation with a conidial suspension of strain B05.10, a haploid derivative of strain SAS56, the leaves were incubated under conditions that result either in leaf spot (20°C, 16 hour light period) or soft rot (4°C, in the dark). Watersoaked lesions appeared 16 **h**ours **p**ost **i**noculation (HPI), when leaves were incubated at 20°C. All lesions became brown and necrotic within 22 HPI. Of these initially formed lesions, in general 10-20% showed aggressive growth, i.e. rapid expansion beyond the inoculation spot. The remaining 80-90% of the initially formed lesions did not expand at all. The aggressive lesions fully colonised the leaf at 144 HPI. At 4°C, the lesions appeared at 18 HPI, and did not become necrotic. All lesions expanded, resulting in full leaf maceration at 192 HPI.

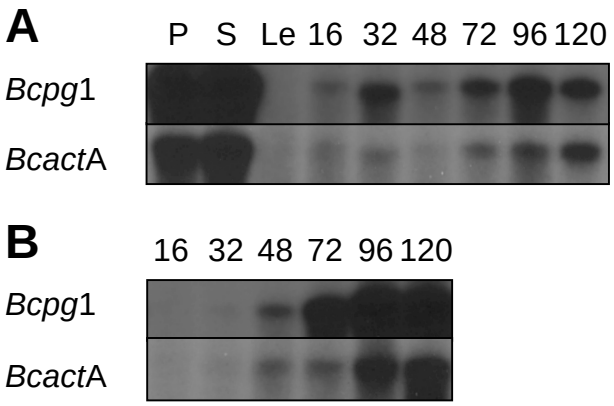

Figure 3: RNA blot analysis of time course infection of tomato leaves
A: *20°C incubation: P: 1% PGA, S: 1% sucrose (controls from liquid cultures), Le: uninoculated tomato leaf, and time course infection (16, 32, 48, 72, 96, 120 HPI).*
B: *4°C incubation (16, 32, 48, 72, 96, 120 HPI). The blot was consecutively hybridized with gene-specific fragments of* Bcpg*1 and* Bcact*A, the latter encoding actin. Each lane contains 20 µg of total RNA. Loading was checked by staining the blot with ethidiumbromide (not shown).*

Samples of infected plant material were taken for RNA extraction at six timepoints after inoculation, while RNA isolated from overnight grown liquid cultures of *B. cinerea* served as a control. Figure 3 shows the expression of *Bcpg*1 and the constitutively expressed actin gene (*Bcact*A), which is taken as a measure for the fungal biomass (Chapter 2). *Bcpg*1 transcript levels are similar when B05.10 is grown on either sucrose or PGA. In planta, *Bcpg*1 transcript can be detected at 16 HPI at 20°C (Fig. 3A), when typically 1-5% of the total RNA is of fungal origin (Chapter 2). The *Bcpg*1 transcript level increases during the course of the infection process in a similar way as the *Bcact*A transcript. The small decrease in the hybridisation level of both transcripts, at 48 HPI in the 20°C time course, is probably the result of a sample that was less well infected, when compared to the other samples. At 4°C, the *Bcpg*1 transcript is first detected by hybridisation at 32 HPI. The *Bcpg*1 transcript level is low during early infection and increases at later stages of the infection process, when compared to the *Bcact*A transcript level (Fig. 3B).

Construction and characterisation of *Bcpg*1 mutants

*Bcpg*1 elimination mutants were made by transformation-mediated gene replacement in haploid strain B05.10. A part of the coding sequence of *Bcpg*1 was exchanged by a cassette containing the *hph* gene, which confers resistance to hygromycin, as depicted in Figure 4A. Fifty independent hygromycin resistant colonies were isolated from five separate

transformation experiments. The transformants were further purified by two subsequent transfers to high selection plates and by two subsequent isolations of single germinating conidia, in order to obtain homokaryotic transformants. PCR detected the replacement of the locus in five transformants. Correct replacement was confirmed in transformants Δ1 and Δ39 by Southern analysis (Fig. 4C). No additional ectopic integrations were detected by hybridisation and no *Bcpg*1 mRNA was detectable on blots in mutants Δ1 and Δ39 when grown on PGA or sucrose (not shown). The mutants did not show significant polygalacturonase activity when grown on sucrose for 6 hours, whereas a clear activity was found for the wild type. When grown on agarose plates with sucrose as metabolite, the mutants show no significant difference in radial growth as compared to wild type. However, when grown on PGA as sole carbon source, there is a reduction in radial growth rate (data not shown) as compared to wild type strain B05.10.

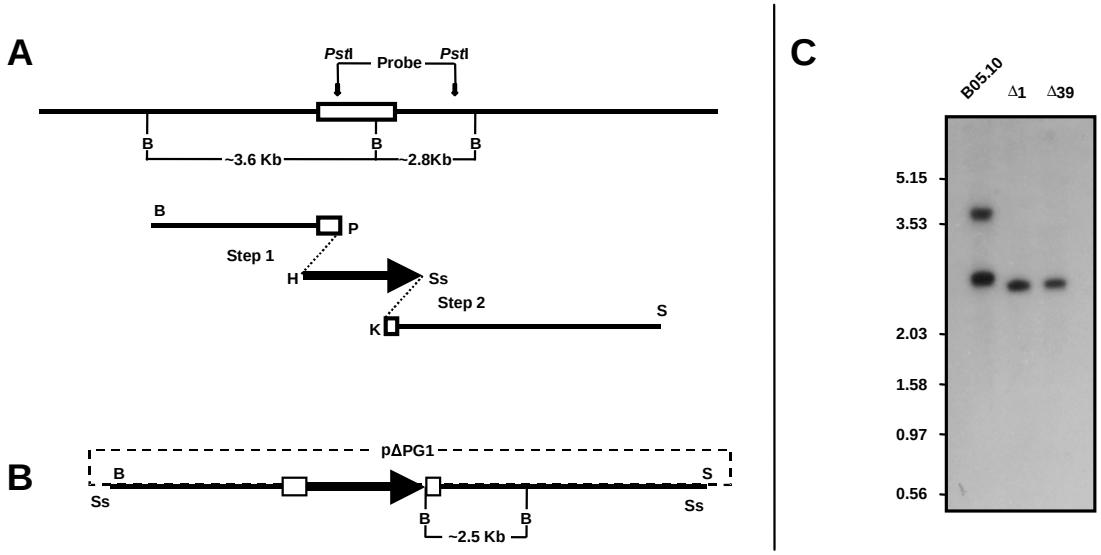

Figure 4:* Bcpg*1 gene replacement

A: *Schematic representation of subcloning of the replacement construct. All cloning steps were performed by blunt end ligation. The hph cassette, indicated by the arrow, was first ligated to the 5' terminus of the* Bcpg*1 gene (Step1). Subsequently the 3' terminus was cloned after the hph cassette (Step 2).*

B: *Replacement construct pΔPG1 in which approximately 700 bp of the coding sequence are exchanged with the hph cassette. The obtained construct was digested with* Sst*I in order to obtain linear replacement DNA without vector sequences.*

C: *Southern blot analysis of transformants Δ1 and Δ39 compared with recipient strain B05.10. 1 µg genomic DNA was isolated from recipient strain B05.10 and transformants and digested with* Bam*HI. The probe, indicated in panel A, hybridizes to a 3.6 Kb and a 2.8 Kb fragment of the recipient strain, indicated in panel A, and to one, 2.5 Kb fragment of the transformants, indicated in panel B. Molecular sizes were determined by comparison with comigrated* Hind*III*/Eco*RI digested λ-DNA, indicated on the left-hand side of the panel (Kb). The open box in panels A and B indicate* Bcpg*1 coding sequence whereas the solid lines indicate the flanking sequences. The dashed line in panel B indicates vector sequences. B,* Bam*HI, P,* Pst*I, H,* Hind*III, S,* Sal*I, K,* Kpn*I, Ss,* Sst*I.*

*Bcpg*1 mutants are less virulent on tomato leaves

The effect of *Bcpg*1 elimination on primary infection, i.e. the ability to penetrate the leaf epidermis, as well as on secondary infection, i.e. the subsequent invasion of surrounding tissue, was determined in droplet inoculation experiments on tomato leaves. If BcPG1 is

involved in the penetration of the epidermis, the obtained mutants might be expected to give either a lower frequency of primary infection or a delay in the process of primary infection. When BcPG1 is involved in the subsequent invasion of surrounding tissue it might be expected that the obtained mutants exhibit a reduced outgrowth of primary lesions. *B. cinerea* causes disease at both moderate (20°C) and low (4°C) temperatures. Therefore the efficacy of primary and secondary infection were tested at both temperatures.

Table 1: Reduction of number of expanding lesions on tomato leaves formed upon infection by BcPG1 deficient strains (Δ1 or Δ39) when compared to infection by wild type strain (B05.10) (20°C)[a]

		Number of expanding lesions		
Experiment	n	Δ1	Δ39	B05.10
1	40	0	-	10
2	20	1	-	6
3	20	1	-	2
4	60	-	0	6
5	60	9	-	24
6	60	-	15	30

[a] From each plant one detached leaf was taken of which 4 leaflets were inoculated with, from each strain indicated, five 1 µl droplets containing approximately 10^3 conidia. Upon inoculation leaves were incubated at 20°C with a 16h photoperiod. Lesions that expand beyond the inoculation spot before 120 HPI were scored as expanding lesions. The total number of inoculation droplets per strain is indicated by n. A dash (-) indicates not done. The germination frequencies of conidia were always between 70 and 80%.

No reduction or delay in occurrence of initial lesions by the mutants was observed at both 20°C and 4°C, when compared to the recipient strain. However, the mutants displayed a smaller number of aggressive lesions at 20°C when compared to the recipient strain B05.10 (see Table 1). Furthermore, the mutants show less aggressive growth at 4°C when compared to the recipient strain B05.10 (Fig. 5). Measurements of the lesion sizes at 96 and 144 HPI, indicated that the reduction in lesion growth rate over 48 hours was significant in all experiments ($P < 0.05$, $P < 0.01$ or $P < 0.001$ depending on the experiment, see Table 2). Similar results were obtained when mutant Δ39 was compared to a transformant with a single copy integration outside the *Bcpg*1 locus, T132 ($P < 0.001$, see Table 2).

Figure 5: Virulence assay of mutant Δ39 on tomato leaflet at 4°C
The left part of the leaflet was inoculated with conidia of Δ39 whereas the right part was inoculated with conidia of the recipient strain B05.10. Photo was taken at 144 HPI.

Table 2: Reduction in radial growth of lesions on tomato leaves formed upon infection by BcPG1 deficient strains (Δ1 or Δ39) when compared to infection by control strain (B05.10 or T132) (4°C)[a]

Experiment	n	Radial growth ± SD in mm				T test	Growth reduction
		Control B05.10	Control T132	Mutant Δ39	Mutant Δ1		
1	20	4.4 ± 1.1	-	3.4± 0.62	-	0.003	22%
2	20	3.6 ± 1.0	-	-	3.1 ± 0.62	0.048	14%
3	30	5.0 ± 1.1	-	2.3 ± 0.54	-	1.6 E-10	54%
4	30	-	3.6 ± 0.31	2.3 ± 0.30	-	0.0005	37%
5	28	-	5.7 ± 0.79	4.6 ± 0.69	-	0.021	20%

[a] Experiments 1-4: From each plant one detached leaf was taken of which 4 leaflets were inoculated with, from each strain indicated, five 1 µl droplets containing approximately 10^3 conidia. Upon inoculation the leaves were incubated at 4°C in the dark. Radial growth of the lesions was determined as the diameter increase after the lesions had established beyond the inoculation spot over a timespan of 48 hours (72-96 HPI). Experiment 5: Leaflets remained attached to the plants (2). Each leaflet was inoculated with, from each strain indicated, four or five 1 µl droplets containing approximately 10^3 conidia. The lesion size was determined once, at 144 HPI. SD means standard deviation. Statistical analysis was performed using a T test. Germination rate of conidia was always between 70 and 80%.

*Bcpg*1 mutants are less virulent on apple and tomato fruits

The mutants showed less aggressive growth on both apple and tomato fruits. Droplets of 5 µl conidial suspension were applied to wounded apple or tomato fruits. Apples developed brown, rotten lesions around the inoculation sites at 48 HPI, when incubated at 20°C. The growth rate of lesions, formed by the *Bcpg*1 elimination mutant between 48 HPI and 112 HPI, is significantly reduced ($P < 0.001$) when compared to the control, T132 (see Table 3). Similar results were obtained in a second experiment.

Table 3: Reduction of radial growth of lesions formed upon infection by BcPG1 deficient strain (Δ39) on tomato (4°C) and apple fruit (20°C) when compared to infection by control strain (T132)

	n	Radial growth ± SD in mm		T test	Growth reduction
		T 132	Δ39		
Apple	38	28.0 ± 3.97	21.8 ± 5.81	3.9E-5	22%
Tomato	38	17.2 ± 4.23	13.3 ± 3.35	1.34E-6	23%

[a] Fruits were inoculated with, from each strain indicated, 5 µl droplets containing 5×10^3 conidia. Upon inoculation tomato fruits were incubated at 4°C in the dark, apple fruits at 20°C with a photoperiod of 16h. Radial growth of the lesions was determined as the radial growth of the lesions at the fruit surface after the lesions had established beyond the inoculation spot over a 56 hour timespan for apple fruits (48-104 HPI) and over a 94 hour timespan for tomato fruits (112-206 HPI). SD means standard deviation. Statistical analysis was performed using a T test. Germination rate of conidia was always between 70 and 80%.

Inoculated tomato fruits incubated at 4°C, developed lesions of soft rot at 112 HPI. In a first experiment the growth rate of the lesions, formed by the *Bcpg*1 elimination mutant between 112 HPI and 206 HPI, was significantly reduced ($P < 0.001$), when compared to the control, T132 (see Table 3). In two subsequent experiments, lesion growth rate was also significantly reduced ($P < 0.01$). In a fourth experiment, growth of the mutant appeared less aggressive but the reduction was statistically not significant.

Discussion

*Bcpg*1 encodes an endopolygalacturonase

The *Bcpg*1 gene has all features of a secreted endoPG, including a preprosequence and all amino acids strictly conserved among exo- and endopolygalacturonases (Benen *et al.,* 1996). The predicted amino acid sequence displays high identity with PGI from *A. niger* and PG1 from *S. sclerotiorum*. For *B. cinerea* up to 13 endoPG isozymes have been described of which only two have a basic pI. A constitutive endoPG, PG-II, was shown to have a pI of 8.8 and a molecular mass of 36 kDa ((Johnston and Williamson 1992a; 1992b). An isozyme denoted as PG-A, was shown to have a pI of approximately 9 (van der Cruyssen *et al.,* 1994). These reports are in reasonable agreement with the data calculated for the mature BcPG1 (pI = 8.07 and MW = 33.7 kDa). PG-A was the only isozyme which seemed to be produced constitutively, albeit in a growth dependent manner (Leone and van den Heuvel 1987, van der Cruyssen *et al.,* 1994). This is in agreement with our observation that *Bcpg*1 is constitutively expressed in planta and in liquid medium containing PGA or sucrose (Fig. 3), or both (data not shown). We conclude that *Bcpg*1 is likely to encode the PG-A isozyme. Since Southern analysis indicates the presence of more endoPG genes (Fig. 1C) we are currently in the process of cloning and characterising additional members of the endoPG gene family.

The *B. cinerea* PG1 has a functional role in pathogenesis

EndoPG BcPG1 is involved in pathogenesis. Although the initial infection frequency was not notably influenced by the replacement of the *Bcpg*1 gene, a significant reduction in lesion growth rate on tomato leaves and fruits as well as on apple fruits was observed (Table 1-3). Radial growth on sucrose as a carbon source was not influenced by the elimination of the *Bcpg*1 gene.

No difference was observed in the initial steps of infection, i.e. during the first 16-32 hours of infection, even although *Bcpg*1 is expressed in the wild type. Therefore it is unlikely that BcPG1 has an important function in penetration as was suggested by a number of authors (Mansfield and Richardson 1981; Elad and Evensen 1995).

Targeted replacement mutants have been constructed in a variety of presumed pathogenicity genes of several fungi, and have confirmed their involvement in pathogenesis (e.g. Shi and Leung 1995; Perpetua *et al.,* 1996; Wassmann and VanEtten 1996). However, except for one case, the elimination of CWDEs in plant pathogenic fungi has thus far not indicated that these enzymes play any significant role in pathogenesis. This was studied most extensively in *Cochliobolus carbonum* by Walton and co-workers. Eliminations of a cellulase (Sposato *et al.,* 1995), an exo-β-1-3-glucanase (Schaeffer *et al.,* 1994), *MLG1* encoding two β-1,3-1,4-glucanases (Görlach *et al.,* 1998), an endoPG (Scott-Craig *et al.,* 1990) and an exopolygalacturonase (Scott-Craig *et al.,* 1998) did not result in reduced virulence on maize. Even the elimination in one strain of both an endo- and an exopolygalacturonase (Scott-Craig

et al., 1998) as well as of three different β-1,4-xylanases (Apel-Birkhold and Walton 1996) had no effect on virulence.

Also in other plant pathogenic fungi, genes encoding cell wall degrading enzymes have been eliminated. A pectin lyase deficient mutant of *Glomerella cingulata* (Bowen *et al.,* 1995) and an endoPG (*enpg-1*) mutant of *Cryphonectria parasitica* (Gao *et al.,* 1996) did not display a phenotype different from the corresponding wild type. The β-1,4-xylanase mutant of *C. carbonum* showed wild type growth on the specific substrate whereas substantial residual enzyme activity was found in all other mutants. Only gene disruption of *pecA,* encoding the endoPG P2c from the saprophytic fungus *Aspergillus flavus,* resulted in a reduced aggressiveness, i.e. a decreased invasion of cotton bolls (Shieh *et al.,* 1997). Interestingly, this gene is constitutively expressed, analogous to the *Bcpg*1 gene of *B. cinerea*. Since the *Bcpg*1 mutants also exhibit a reduced radial growth on PGA as sole carbon source, the absence of the BcPG1 isozyme apparently results in a reduced capability of degrading the complex pectin structures present in the cell wall. Therefore, the retarded invasion of the tissue surrounding the inoculation spot (Tables 1-3, Fig. 5) is most probably caused by a reduced maceration capability. In concordance with this, it has been reported that PG-A is the only *B. cinerea* endoPG isozyme which is secreted in the outer regions of the lesions (Kamoen and van der Cruyssen, 1996). Several other endoPG isozymes, of which some are induced by the breakdown product of polygalacturonase action, galacturonic acid (van der Cruyssen *et al.,* 1994), might serve in the further degradation of tissue which is already colonised. Therefore it cannot be excluded that the reduced virulence displayed by the *Bcpg*1 mutants, is not solely the result of the absence of BcPG1 degrading activity, but could also be attributed by an altered induction of other polygalacturonases.

Our observation that a CWDE of *B. cinerea* is required for full virulence may be related to the fact that the fungus causes soft rot on many plant tissues. There is substantial evidence that CWDEs from bacterial plant pathogens causing soft rot, are involved in pathogenesis. Eliminations of several pectinases in *Erwinia chrysanthemi*, independently influence maceration of potato tubers (reviewed by Barras *et al.,* 1994). Similar results were obtained in studies on CWDEs of *Erwinia carotovora* (Barras *et al.,* 1994) and *Ralstonia solanacearum* (Schell *et al.,* 1988).

Oligogalacturonides have been identified as elicitors of plant defence responses (de Lorenzo *et al.,* 1990). A decrease of the release of oligogalacturonides, as a result of the elimination of BcPG1, might therefore reduce the plant response. An extensive study on the expression of other members of the endoPG gene family, presumably corresponding to the weakly hybridising bands in Figure 1C, will be performed in several hosts as well as in liquid cultures will provide more insight in the function of these enzymes in pathogenesis. Elimination of other endoPGs, with different regulatory characteristics and other types of CWDEs in *B. cinerea* as well as in other soft rot causing fungi such as the related *S. sclerotiorum*, will provide further insight into the role of CWDEs in fungal pathogenesis..

Acknowledgements

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Materials and Methods

Growth of fungal strains and tomato plants. Fungal strains (SAS56, (van der Vlugt-Bergmans 1993)), B05.10 (haploid derivative of SAS56 (van Kan *et al.,* 1997)) and T 132 (B05.10 transformant containing a single copy of a hygromycin resistance gene cassette in the *cut*A locus (van Kan *et al.,* 1997)) were maintained and conidia were isolated as described (Chapter 2). Tomato plants (Moneymaker genotype *Cf4*) were grown for four to six weeks in 1 litre pots in potting soil in a climate chamber at 24°C with a 16 hour photoperiod. Additional nutrients per pot were 1 ml FeNaEDTA (35g l^{-1}) (Fluka, Buchs, Switzerland) at seed germination, 1 ml of micronutrients (2.86 g l^{-1} H_3BO_3, 1.558 g l^{-1} $MnSO_4.H_2O$, 0.08 g l^{-1} $CuSO_4.H_2O$, 0.22 g l^{-1} $ZnSO_4$, 0.0196 g l^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$ (Merck, Darmstadt, Germany)) at three days after seed germination and 5 ml Hoagland (5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4 (Merck)) on a weekly basis.

Isolation of the *Bcpg*1 gene and Southern analysis. A genomic λ-EMBL3 library of *B. cinerea* strain SAS56 (Kusters-van Someren *et al.,* 1992) was screened using the 0.7 kb *Sal*I fragment from the *pgaII* gene from *A. niger* (Bussink *et al.,* 1990) as a probe. Positive phages were purified and characterised. Hybridisations were performed at 65°C in 50 mM Tris pH 7.5, 10mM EDTA, 0.5% SDS, 0.9M NaCl, 0.09M Na-citrate containing 1 g l^{-1} Ficoll (Type 400, Pharmacia, Uppsala, Sweden), 1 g l^{-1}polyvinylpyrrolidone, 1 g l^{-1} bovine serum albumin (Boehringer, Mannheim, Germany). Subsequent washings were performed at 65°C in 0.5 × SSC, 0.1% SDS for high stringency conditions and in 2 × SSC, 0.1% SDS for low stringency.

Inoculation of tomato leaves. Harvest and preincubation of conidia, and tomato leaf-handling were performed as described (Chapter 2). Virulence assays were performed by droplet inoculations of leaflets of compound leaves. The left part of each leaflet, except the apical, was inoculated with five 1µl droplets, containing 10^3 conidia, of a *Bcpg*1 elimination transformant, whereas the right part was identically inoculated with the control (either recipient strain B05.10 or vector control T132). Incubations were performed at 20°C and 4°C as described (Chapter 2). Lesion diameters were measured using a calliper. Viability of conidial suspensions was determined by plating dilutions of the preincubation suspensions on Malt Broth Agar (Oxoid, Basingstoke, Hampshire, UK). Germination frequencies and radial outgrowth were measured. Foliar spray inoculations and incubations were performed as described (Chapter 2).

Fruit inoculations. Tomato fruits were a gift from De Ruiter Seeds (Bergschenhoek, The Netherlands)(cv. Carlita) or commercially obtained. Apple fruits (cv. Jonagold or cv. Braeburn) were commercially obtained. Conidia were harvested and preincubated as described (Chapter 2). Fruits were surface-disinfected with 70% ethanol and inoculated in small punctures with 5 µl droplets containing 5×10^3 conidia. A BcPG1 mutant and a control strain were inoculated onto each fruit, at two positions each. Fruits were subsequently incubated in a 30×45 cm plastic box on a plastic grid. Wet paper was placed beneath the grid in order to maintain a high humidity. Boxes were covered with a plastic, transparent lid. Apples were incubated at 20°C using a 16 hour photoperiod whereas the tomato fruits were incubated at 4°C in the dark. Lesion diameters were measured using a calliper.

RNA analysis. RNA was extracted as described (van Kan *et al.,* 1992), denatured by glyoxylation and subsequently electrophoresed (Sambrook *et al.,* 1989). Blotting and Hybridisations (van der Vlugt-Bergmans *et al.,* 1997a) were performed with a radiolabelled *Pst*I-*Bam*HI fragment of the *Bcpg*1 coding sequence or a radiolabelled 320 bp PCR fragment from the *Bcact*A gene (Chapter 2). Fragments were radiolabelled using the Random Primers DNA Labelling System according to the manufacturer's description (Life Technologies Inc., Gaithersburg, MD, US) and Redivue α^{32}P-dATP (Amersham, Buckinghamshire, UK). Autoradiographs were made by 48 hour exposure of KODAK-S films at -70°C with two intensifying screens.

Construction of *Bcpg*1 replacement plasmid. All cloning steps were done by blunt ended ligation of fragments, using standard methods. A *Sst*I/*Hin*dIII cassette from pOHT, conferring resistance to hygromycin (Hilber *et al.*,, 1994), was ligated into p25#BP, a 5' *Bam*HI/*Pst*I *Bcpg*1 subclone comprising 5' flanking sequence as well as a part of the coding sequence, resulting in plasmid pΔ1 (see Step 1, Figure 4A). A *Kpn*I/*Sal*I fragment from *Bcpg*1 subclone p25#9H, comprising the 3' terminal part of the *Bcpg*1 gene as well as 3' flanking sequence was cloned downstream of the resistance cassette in pΔ1 (see Step 2, Figure 4A). In the resulting plasmid, pΔPG1 (Fig. 4B) 700 bp of coding sequence are replaced with the hygromycin resistance conferring cassette from pOHT.

Transformation of *B. cinerea.* A linear DNA fragment, excised from pΔPG1 using *Sst*I (Fig. 4B), was used for transformation. Strain B05.10 was transformed as described (Hamada *et al.,* 1994) with slight modifications. 1 μg DNA was added to 10^7 protoplasts in 50 μl 0.6 M KCl, 50 mM $CaCl_2$. 2.5 μl 50 mM spermidine (Sigma, St. Louis, USA) was added prior to a 5 minute incubation on ice. 50 μl 25% (w/v) PEG 3350 (Sigma) in 10 mM Tris pH 7.4, 50 mM $CaCl_2$, was added and the mixture was incubated at 20°C for 20 min. An additional aliquot of 250 μl PEG was added followed by 10 minute incubation at 20°C. 750 μl 0.6 M KCl, 50 mM $CaCl_2$ was added prior to plating on agar plates containing 0.6 M Sucrose, 5mM Hepes pH 6.5, 1mM $(NH_4)H_2PO_4$ (Merck) and 25 μg/ml hygromycin B (Sigma). Emerging colonies were transferred to Malt Broth Agar plates containing 100 μg/ml hygromycin B.

Hyphal tips of hygromycin B resistant colonies were transferred to Malt Broth Agar containing 100 μg/ml hygromycin B prior to a transfer to Potato Dextrose Agar (Merck) supplemented with 300 g/l pulped tomato leaves. Conidia were isolated and replated on Malt Broth Agar plates containing 100 μg/ml hygromycin B. Germlings from single conidia were isolated and transferred to tomato leaf supplemented Potato Dextrose Agar. For Southern analysis, conidia were isolated as previously described (Chapter 2), and subsequently incubated for 16 hour at 20°C in a rotary shaker at 180 rpm in B5 medium including vitamins (Duchefa, Haarlem, The Netherlands), supplemented with 10 mM sucrose and 10 mM $(NH_4)H_2PO_4$. Mycelium was harvested by filtration on Miracloth (Calbiochem, La Jolla, USA), dried on Whatman paper and subsequently grinded under liquid nitrogen. Genomic DNA was isolated as described (Möller *et al.,* 1992), digested with restriction enzymes and electrophoresed on 0.6% agarose gels. Genomic DNA was alkali blotted onto Hybond N^+ (Amersham) according to the manufacturer's description. Hybridisations and washings were performed as described (van der Vlugt-Bergmans *et al.,* 1997a). Autoradiographs were made by 24 hour exposure of KODAK-S films at -70°C using two intensifying screens.

Chapter 4

The *Botrytis cinerea* endopolygalacturonase gene family constitutes a flexible pectate degrading machinery[1]

Arjen ten Have, Jos P Wubben, Wietse Mulder, Jan A.L. van Kan and Jaap Visser

Abstract

Endo**p**oly**g**alacturonases (endoPGs) have been suggested to be actively involved in the infection of many plant pathogenic fungi. The ascomycetous plant pathogen *Botrytis cinerea* has a gene family encoding endoPGs that consists of at least six genes denoted *Bcpg*1-6. Homologues of the six genes are present in *Botrytis* species with a broad as well as a narrow host range, indicating that they are not required for a broad host range. Phylogenetic analysis, using a group of 35 fungal endoPGs and a plant endoPG as an outgroup, indicates that the six *B. cinerea* genes fall into three out of five monophyletic groups. The members of the gene family are differentially expressed in *B. cinerea*. A basal constitutive expression level was observed for two genes encoding basic isozymes, *Bcpg*1 and *Bcpg*2. Galacturonic acid (GA) was shown to induce the expression of *Bcpg*4 and *Bcpg*6. Furthermore, low pH of the culture medium resulted in expression of the *Bcpg*3 gene, irrespective of the carbon source present. Finally, GA-induced expression of the *Bcpg*4 gene was inhibited by the presence of glucose. *Bcpg*5 is expressed under certain conditions but the regulation of this gene is not yet clear. The regulation of the *Bcpg* gene family allows the degradation of the cell wall component pectate under various environmental conditions and control in respect to the catabolic demands of the fungus. This flexible pectate degrading machinery is believed to be active in the concerted degradation of plant cell walls. Therefore it is hypothesised that, besides the *Bcpg*1 gene (see Chapter 3), other members of the endoPG gene family are involved in pathogenicity of *B. cinerea*.

[1] **This chapter is a contraction of two manuscripts:**

-Wubben J.P., Mulder, W., ten Have A., van Kan, J.A. L. and Visser, J.; Cloning and partial characterisation of the *Botrytis cinerea* endopolygalacturonase gene family. Applied and Environmental Microbiology 65:1596-1602, (1999).
-Wubben, J.P., ten Have, A., van Kan, J.A.L. and Visser, J. Regulation of endopolygalacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. Current Genetics 37:152-157, (2000).

Introduction

Botrytis cinerea Pers.: Fr. *Botryotinia fuckeliana* (de Bary) Whetz. is an ascomycetous necrotrophic pathogen that can infect at least 235 plant species (Jarvis, 1977). It causes fruit rot and leaf as well as flower blight in the field and in greenhouses, but the fungus is also renowned for causing devastating disease in the post harvest chain (Berrie, 1994). The fungus produces a white woolly mycelium at the surface of the macerated tissue from which dark conidia develop. The disease is therefore also known as gray mould. There is no broad understanding of how *B. cinerea* penetrates the epidermis, how it kills plant cells and how it spreads through the host tissue.

Enzymatic degradation of pectin polymers in plant cell walls is considered important during the infection of plants by pathogenic fungi. During all stages of infection *B. cinerea* produces a broad set of **c**ell **w**all **d**egrading **e**nzymes (CWDEs) among which pectin methylesterase (Reignault *et al*., 1994), pectin lyase (Movahedi and Heale, 1990), exo- and endopolygalacturonases (Johnston and Williamson, 1992a) and cellulase (Barkai-Golan *et al*., 1988), which all are believed to degrade the cell wall in concerted action. Endopolygalacturonase (endoPG, EC3.2.1.15) seems to be the most prominent CWDE that is secreted by *B. cinerea*. A large set of endoPG isozymes is produced by this fungus, which is regarded as an indication for their importance in maceration of plant tissue (Leone and van den Heuvel, 1987; van der Cruyssen *et al*., 1994). One of the endoPG isozymes is expressed constitutively (Leone *et al*., 1990; van der Cruyssen *et al*., 1994) and is therefore usually the first CWDE that is detected during infection. A gene encoding a constitutively expressed endoPG, denoted as *Bcpg*1, has been cloned and the targeted mutation of this gene resulted in a strain with reduced virulence (Chapter 3). Southern analysis of the wild type strain, using *Bcpg*1 as a probe, indicated the presence of multiple sequences that hybridise under low stringency conditions. This is in correspondence with a large gene family encoding endoPG isozymes, as was anticipated from biochemical studies that report up to 13 isoforms of endoPG in *B. cinerea* (van der Cruyssen *et al*., 1994). *Sclerotinia sclerotiorum*, a closely related plant pathogen, possesses a family of seven endoPG genes of which three have been characterised in detail (Reymond *et al*., 1994; Fraissinet-Tachet *et al*., 1995). Biochemical studies on the pectinolytic system in *B. cinerea* suggested that the proposed gene family is delicately regulated. The endoPG isozymes can be grouped in four classes on the basis of regulatory aspects as catabolite repression and induction or repression by end product (van der Cruyssen *et al*., 1994). The individual endoPG genes of *S. sclerotiorum* are not expressed synchronously. A distinction was made between constitutively expressed genes and genes that are only expressed in the presence of galacturonic acid (GA). Fraissinet-Tachet and Fèvre (1996) stated that the action of constitutive isozymes on the pectin substrate would release GA, which would subsequently activate the inducible gene family members. In addition, one of the endoPG genes was shown to be subject to carbon catabolite repression (Reymond-Cotton *et al*., 1996).

Here we extend our molecular genetic study on *B. cinerea* with the isolation of additional endoPG encoding genes. The deduced amino acid sequences are subjected to a phylogenetic analysis and the expression of the genes is studied. We used different carbon sources as well as changes of the pH in the medium to study the regulation of pectinolysis in *B. cinerea*, based on the biochemical work that has been performed on *B. cinerea*, and the molecular genetic work reported for *S. sclerotiorum* and *Aspergillus* species.

Results

Cloning of the *Bcpg* gene family encoding endopolygalacturonases

The isolation and characterisation of the *Bcpg*1 gene has been described in Chapter 3. Screening of the genomic library of *B. cinerea* (strain SAS56) using the *Bcpg*1 gene as probe resulted in the identification of numerous hybridising phages of which 100 phages were chosen randomly for DNA isolation. Twenty-one clones appeared to contain the *Bcpg*1 gene as determined using PCR analysis. Eighteen hybridising clones (not containing *Bcpg*1) were further characterised using Southern and nucleotide sequence analyses. The phages were assigned to five groups of clones, each covering a separate region of the *B. cinerea* genome, based on the structural organisation of the flanking DNA. This resulted in the identification of five additional sequences encoding endoPGs, denoted as *Bcpg*2-6. PCR screening of all isolated phage DNA showed that the phages all contained one of the cloned *Bcpg* genes. The complete nucleotide sequences of these genes have been determined and deposited in GenBank.

Organisation of the endopolygalacturonase gene family

Intron positions in the nucleotide sequences were predicted based on codon usage using the program Testcode (Ficket, 1982) and amino acid sequence alignment with homologous fungal endoPGs from related species. Between 1 and 4 introns are present in the different *Bcpg* genes, with the exception of *Bcpg*1 which is intron-less (Fig. 1). The border sequences of the introns in the *Bcpg* genes, and the putative internal consensus for lariat formation, correspond with previously reported 5' and 3' splice sites in fungal genes (Unkles, 1992). The position of intron 3A was confirmed by sequencing of RT-PCR products whereas indirect evidence for the presence of introns was obtained for 2C, 4A and 4B, 5B and 5C, and 6B by RT-PCR using primers extending over the introns (ten Have and Wubben, unpublished). The introns in the *Bcpg* genes varied in size between 46 and 60 nucleotides. Conservation of intron positions was observed for an intron in *Bcpg*2, 4 and 5 (2C, 4B and 5B respectively in Figure 1). Amino acid sequences were deduced from the predicted open reading frames

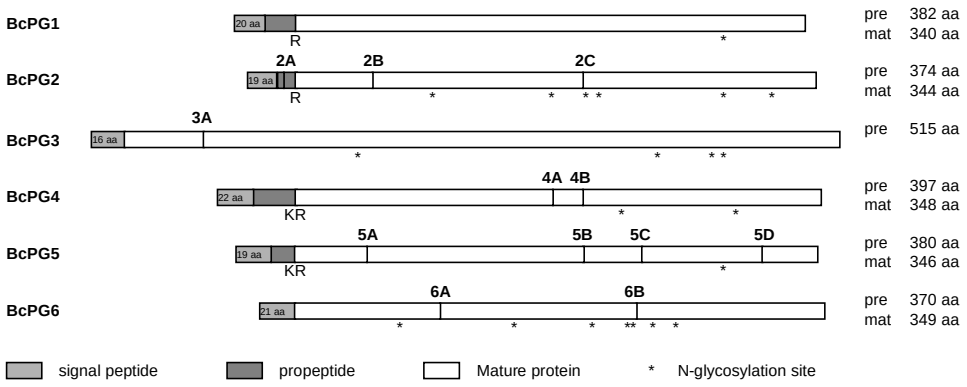


***Figure 1: Genomic organisation of the endopolygalacturonase gene family of* Botrytis cinerea**

Indicated are the positions of the introns in the original DNA sequence (2A-6B), the presence of putative mono-(R) or dibasic- (KR) cleavage sites, and the presence of N-glycosylation signals (*). Also depicted in the figure are the derived lengths of unprocessed proteins (pre) and mature processed proteins (mat). The lengths of predicted signal peptides for each of the proteins are indicated in the respective boxes.

present in the genomic sequences of each of the five endoPG genes. The predicted endoPG proteins, denoted BcPG1-6, ranged in length from 370 to 515 amino acids (Fig. 1) and contained all the amino acid residues that are strictly conserved in endopolygalacturonases (Benen *et al*., 1996). All protein sequences contain a predicted signal sequence as determined according to Nielsen *et al*. (1997). Either a mono- (Arg) or a dibasic (Lys-Arg) propeptide cleavage site was present in four of the six *B. cinerea* endoPGs (Arg for BcPG1 and BcPG2; Lys-Arg for BcPG4 and BcPG5), analogous to *Aspergillus niger* endopolygalacturonases (Benen *et al*., 1996). No propeptide cleavage site could be predicted for BcPG3 and 6. All endoPGs contain N-linked glycosylation signals (Fig. 1) indicating that the enzymes might be excreted as glycosylated enzymes. Glycosylation was reported to result in multiple endoPG-isoforms, which primary structure is encoded by one coding sequence in *Fusarium moniliforme* (Caprari *et al*., 1993).

Table 1: Sequence pair distance (percentage of identity) of the endoPG family of* Botrytis cinerea *using the ClustalW method

	BcPG2	BcPG3	BcPG4	BcPG5	BcPG6
BcPG1	72.0	38.7	65.8	72.7	55.0
BcPG2		34.8	59.2	63.7	54.3
BcPG3			33.6	36.7	48.3
BcPG4				67.7	48.5
BcPG5					55.2

Protein sequence alignment of fungal endoPGs

Sequence identity between the unprocessed endoPGs of *B. cinerea* varied between 34 and 73 % (Table 1). BLAST protein sequence similarity searches, using deduced amino sequences of the *Bcpg* genes revealed striking homology with amino acid sequences of endoPGs from other filamentous fungi. This prompted us to perform a detailed phylogenetic

analysis using a large number of fungal and yeast endoPGs. A total of 35 endoPGs from 17 species of yeasts and fungi (Table 2) were used to generate a protein sequence alignment that was used for phylogenetic analysis. A general heuristic search was performed using PAUP 3.1 and clade stability was assessed by bootstrap replications. Gaps in the alignment were treated as missing values. The *Arabidopsis thaliana* polygalacturonase was used as an outgroup. Figure 2A shows the consensus tree generated from the three most parsimonious trees found in the analysis.

Table 2: Endopolygalacturonases used for the construction of the protein sequences alignment

Organism	Protein	Accession	length	reference
Arabidopsis thaliana	GBGATHAL	S34266	445 aa	unpublished
Aspergillus flavus	PGAASPFL	P41749	363 aa	(Whitehead *et al*., 1995)
	PGBASPFL	P41750	366 aa	(Whitehead *et al*., 1995)
Aspergillus niger	PG1ASPNG	P26213	368 aa	(Bussink *et al*.,1991a)
	PG2ASPNG	P26214	362 aa	(Bussink *et al.,* 1991b)
	PGAASPNG	-	370 aa	(Pařenicová *et al.,* 2000a)
	PGBASPNG	-	362 aa	(Pařenicová *et al.,* 2000a)
	PGCASPNG	X64356	383 aa	(Bussink *et al*., 1992)
	PGDASPNG	-	495 aa	(Pařenicová *et al*., 2000b)
	PGEASPNG	Y14386	378 aa	(Pařenicová *et al*., 1998)
Aspergillus oryzae	PGRASPOR	P35335	363 aa	(Kitamoto *et al*., 1993)
Aspergillus parasiticus	PGRASPPA	P49575	363 aa	(Carey *et al.,*, 1995)
Aspergillus tubingensis	PG2ASPTU	P19805	362 aa	(Bussink *et al*., 1990)
Botrytis cinerea	BcPG1	U68715	382 aa	(Chapter 3)
	BcPG2	U68716	374 aa	this paper
	BcPG3	U68717	514 aa	this paper
	BcPG4	U68719	397 aa	this paper
	BcPG5	U68721	380 aa	this paper
	BcPG6	U68722	370 aa	this paper
Claviceps purpurea	CPPG1	Y10165	369 aa	(Tenberge *et al*., 1996)
	CPPG2	Y10165	369 aa	(Tenberge *et al*., 1996)
Cochliobolus carbonum	PGRCOCCA	P26215	364 aa	(Scott-Craig *et al*., 1990)
Colletotrichum lindemuthianum	CLPG1END	X89370	363 aa	(Centis *et al*., 1996)
	CLPG2END	X95475	366 aa	(Centis *et al*., 1997)
Cryphonectria parasitica	CP47910	U49710	369 aa	(Gao *et al*., 1996)
Fusarium moniliforme	PGRFUSMO	Q07181	373 aa	(Caprari *et al*.,1993)
Fusarium oxysporum	FOAB124	AB000124	371 aa	unpublished
	FOU96456	U96456	370 aa	(di Pietro *et al*., 1998)
Kluyveromyces marxianus	KMAJ76	AJ000076	361 aa	unpublished
Saccharomyces cerevisiae	PGXSACSE	P47180	361 aa	unpublished
Sclerotinia sclerotiorum	PG1SCSCL	L12023	380 aa	(Reymond *et al*., 1994)
	PG2SCSCL	S62742	380 aa	(Fraissinet-Tachet *et al*., 1995)
	PG3SCSCL	S63743	380 aa	(Fraissinet-Tachet *et al*., 1995)
	PG5SCSCL	Y13669	387 aa	unpublished
Stereum purpureum	SPD072	D45072	404 aa	(Miyairi *et al*., 1997)
Trichosporon penicillatum	TPD650	D89650	367 aa	(Iguchi *et al*., 1997)

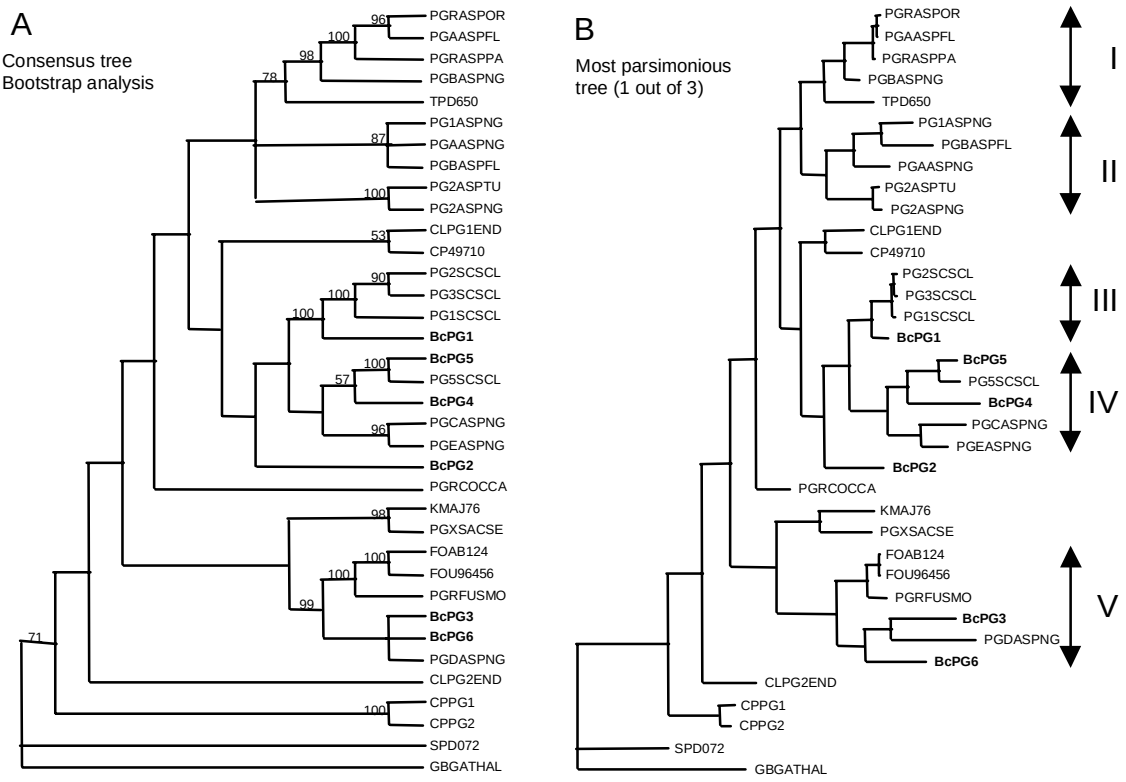


Figure 2: Phylogenetic analysis of fungal endopolygalacturonases
The analysis was performed using an optimal alignment generated from the polygalacturonases depicted in Table 2. Panel A shows the consensus tree derived from three most parsimonious trees calculated using PAUP 3.1. The different values represent percentage occurrence obtained after bootstrap analysis (1000 iterations) of the phylogenetic analysis. Panel B shows one most parsimonious tree and identifies the different monophyletic groups that were defined as a result of the analysis. The abbreviations of protein names are indicated in Table 2. B. cinerea *endoPGs are indicated in bold.*

Figure 2B shows one of the three most parsimonious trees. The phylogenetic analysis indicated the presence of several groups of related endoPGs as predicted from the BLAST protein sequence similarity searches. Five different monophyletic groups were distinguished, each containing a minimum of 3 endoPGs originating from more than one fungal species. Among the fungal species represented in the tree, several possessed endoPGs belonging to more than one group, for example, *Aspergillus niger* (groups I, II, IV, V), *Sclerotinia sclerotiorum* (groups III and IV), *Aspergillus flavus* (groups I and II) and *B. cinerea* (groups III, IV, V). With respect to the endoPGs of *B. cinerea*, BcPG1 belongs to group III together with three endoPGs of *S. sclerotiorum*, with sequence identities around 90 %. BcPG3 and BcPG6 cluster with PGD (*A. niger*) and several endoPGs isolated from different *Fusarium* species (group V). PGD from *A. niger* (Pařenicová, 2000b) and BcPG3 are distinct from most endoPGs because of the presence of a N-terminal extension of approximately 150 amino acids. BcPG4 and BcPG5 were assigned to group IV together with PG5 of *S. sclerotiorum* and PGC and PGE of *A. niger*. BcPG5 and PG5 of *S. sclerotiorum* are

89.5% identical at the amino acid level. BcPG2 was related to BcPG1 and BcPG5, however, it was assigned neither to group III nor to group IV, and resolved in a separate branch of the tree.

Table 3: Strains of* Botrytis cinerea *and other* Botrytis *species used

Strain	**Type**	**Mating type**	**Origin Parental Strains**
SAS56	Mono-ascospore isolate	*MAT1-1*	Bari, Italy
SAS405	Mono-ascospore isolate	*MAT1-2*	Bari, Italy
B05.10	Haploid derivative SAS56	Unknown	Not applicable
Isolate	**Host from which isolated**	**Year of isolation**	**Origin**
Bc7	Tomato	1970	The Netherlands
Bc12	Gerbera	1986	The Netherlands
Bc21	Rose	1990	The Netherlands
Bc29	Gerbera	1991	The Netherlands
J13	*Vitis vinifera*	before 1985	Jena, Germany
M15	*Vitis vinifera*	1993	Freiburg, Germany
Species	**Known hosts**		
Botrytis allii	onion		
Botrytis gladiolorum	gladiolus		
Botrytis paeoniae	peony		
Botrytis squamosa	onion		

The endopolygalacturonase gene family is present in different *Botrytis cinerea* strains and *Botrytis* species

A high stringency DNA blot analysis was performed with DNA obtained from 9 different strains (Table 3) in order to examine the presence of the genes among the species *B. cinerea*. All strains tested showed a hybridising fragment, indicating that the gene family isolated from SAS56 is likely to be present throughout the species *B. cinerea* (Fig. 3). Some restriction fragment length polymorphisms were observed. In addition we performed a low stringency DNA blot analysis with DNA isolated from *Botrytis allii*, *Botrytis gladiolorum*, *Botrytis paeoniae* and *Botrytis squamosa*. All *Botrytis* species tested displayed at least one hybridising fragment specific for each of the probes used (Fig. 4). The signal was not strong with some probes, however, comparison of each individual hybridisation pattern with those of the other genes showed clearly distinct patterns for each probe. Apparently, the whole endoPG gene family that is found in *B. cinerea* is present in other *Botrytis* species as well.

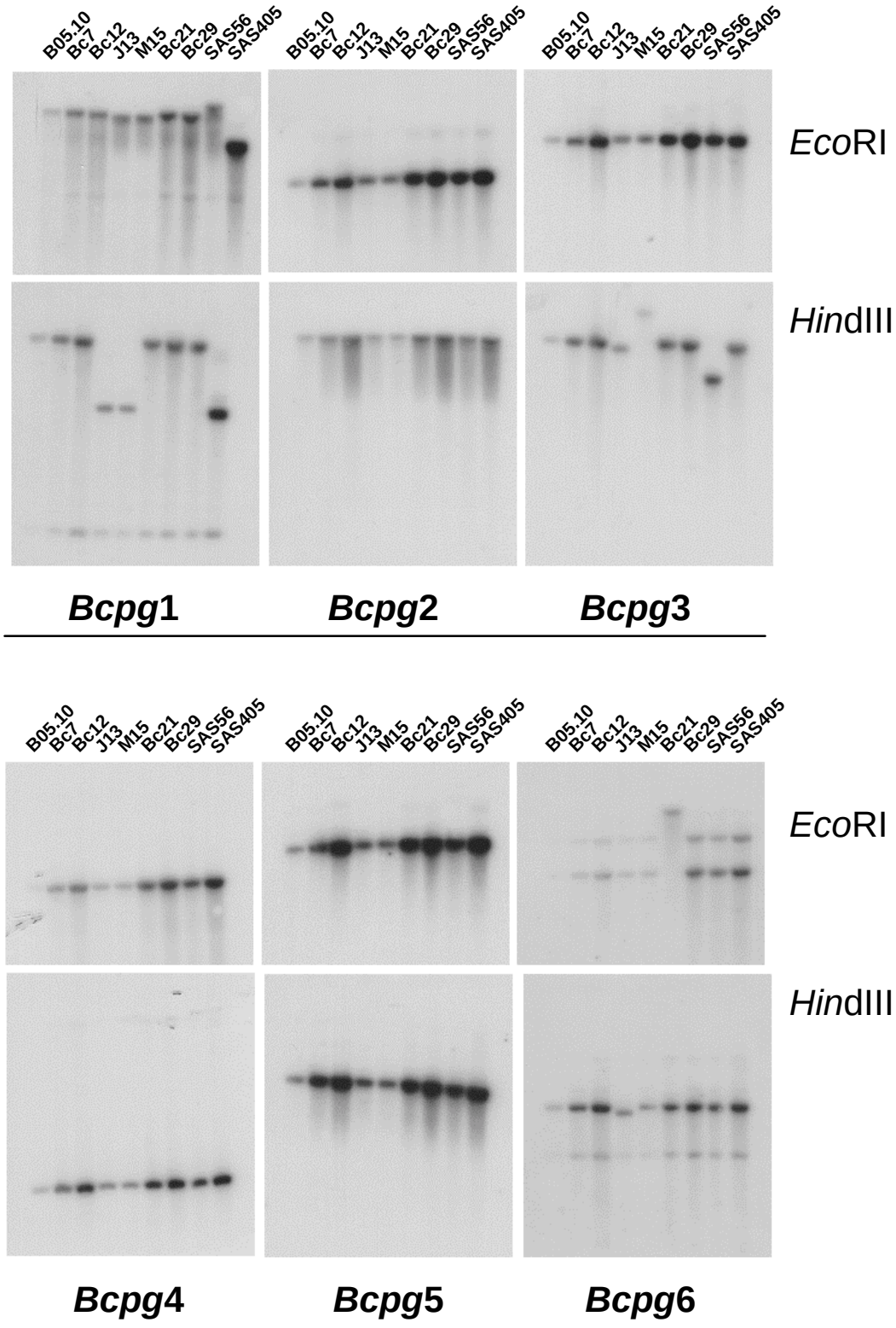


Figure 3: Southern blot analysis of* Botrytis cinerea *strains using the* Bcpg *genes as probe

Fungal DNA isolated from the different strains of B. cinerea *was digested with* Eco*RI (top panel) or* Hind*III (lower panel) and subjected to Southern hybridisation using gene specific probes (*Bcpg *1 - 6) at high stringency conditions, as described in Materials and Methods.*

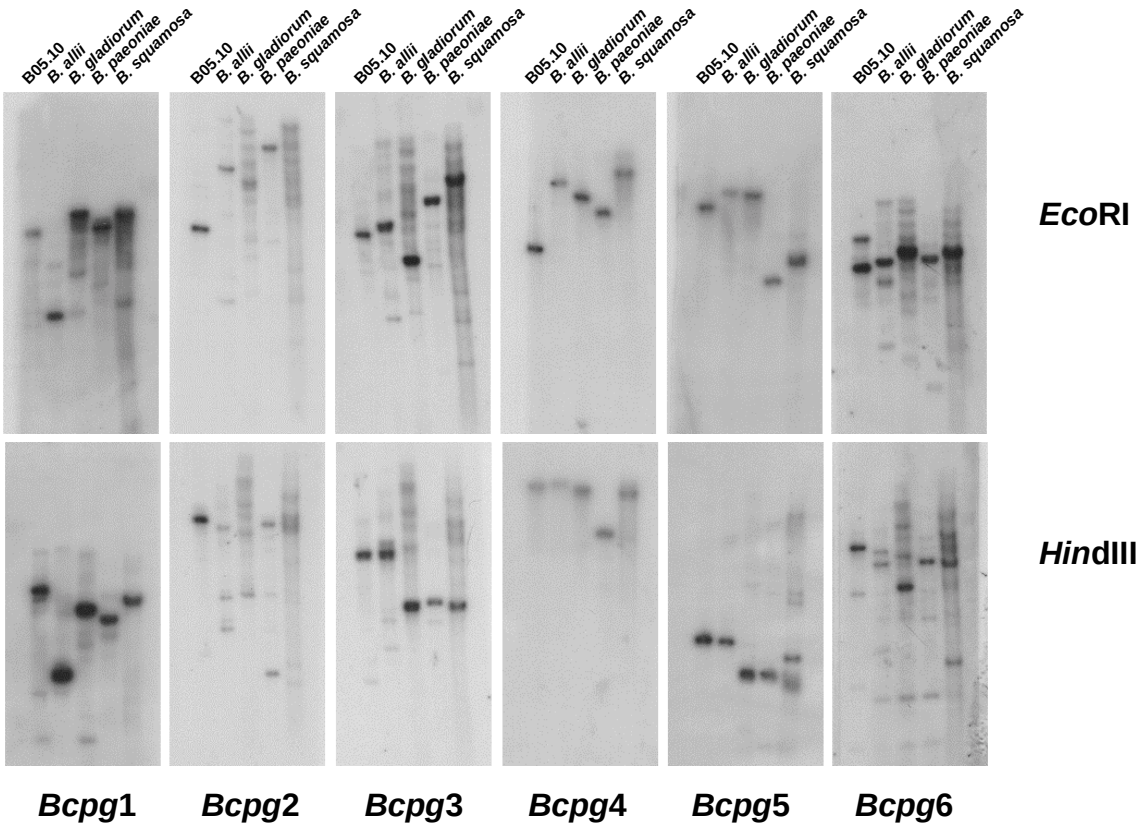


Figure 4: Southern blot analysis of* Botrytis *species using the* Bcpg *genes as probe
Fungal DNA was isolated from the different Botrytis *species, digested with* Eco*RI (top panel) or* Hind*III (lower panel), and subjected to Southern hybridisation using gene specific probes (*Bcpg *1 - 6) at low stringency conditions, as described in Materials and Methods.*

Induction of gene expression by glucose, pectin, polygalacturonic acid and galacturonic acid

The presence of a family of six endoPG-encoding genes prompted us to perform a detailed expression analysis. On the one hand it can be envisaged that one or more genes are not expressed and, hence, are redundant. On the other hand, differences in substrate specificity as well as putative regulatory systems might explain the presence of six genes encoding a similar enzyme. Biochemical studies, using zymogram analysis of *B. cinerea* culture filtrates, have indicated four types of regulation based on patterns of induction as well as patterns of repression by either substrate or products released by pectate cleavage, or by carbon catabolites (van der Cruyssen *et al*., 1994). RNA blot analysis showed constitutive expression of the *Bcpg*1 gene in a time course infection experiment with detached tomato leaves (Chapter 3). Here we investigate the expression of the complete *Bcpg* gene family at RNA level in liquid cultures. The expression of a *B. cinerea* ribosomal 27S RNA was used as a control to monitor equal loading.

Mycelium was pre-cultured for 16 h on glucose containing medium, harvested, washed and transferred to media containing either glucose, polygalacturonic acid (PGA),

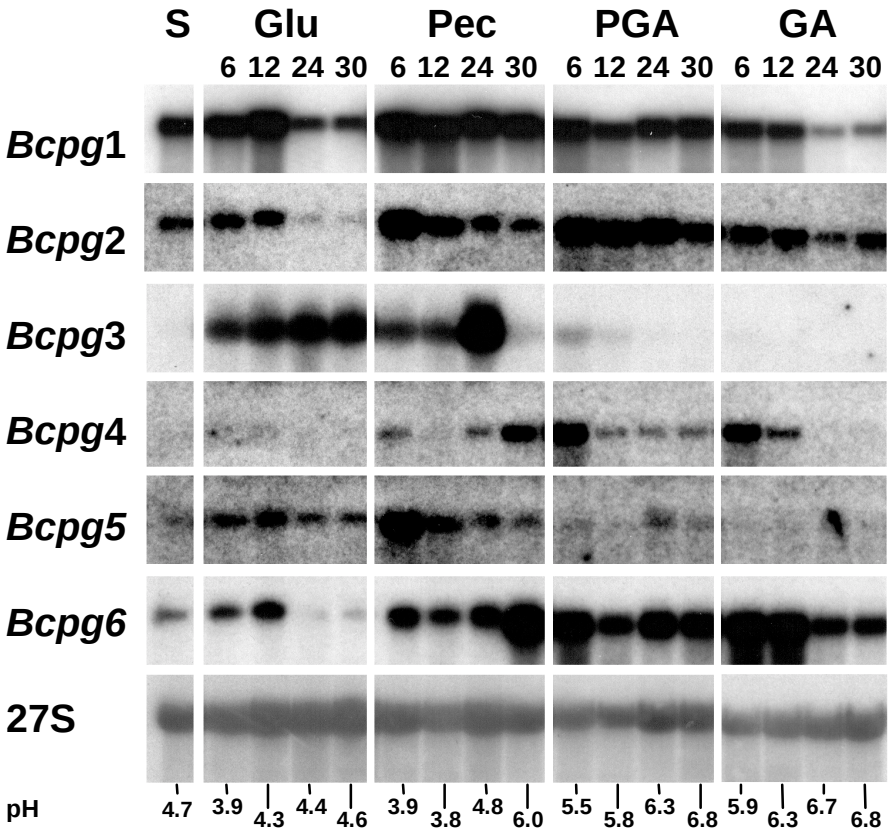


Figure 5: EndoPG gene expression of* Botrytis cinerea *strain B05.10 in a medium shift experiment

*Fungal RNA was isolated from mycelium grown in liquid culture on glucose (Glu), pectin (Pec), PGA and GA, harvested 6, 12, 24, and 30 hours after transfer from glucose (S). RNA was subjected to RNA blot hybridisation using gene specific probes (*Bcpg*1-6) at high stringency conditions, as described in Materials and Methods. As loading control RNA was hybridised with a 27S rDNA probe from* B. cinerea*. Exposure times for autoradiography after hybridisation with the different probes were adjusted to obtain signals with similar intensities.*

pectin (Pec) or mono-galacturonic acid (GA) as carbon source. Figure 5 shows the expression at the RNA level. *Bcpg*1 and *Bcpg*2 are expressed on all four carbon sources. Although the temporal expression pattern was similar for both genes, *Bcpg1* transcript levels were much higher than B*cpg*2 transcript levels.

Both *Bcpg*1 and *Bcpg*2 transcript levels decreased at late time points after transfer (24 and 30 h), especially in mycelium that was grown on glucose or GA. Surprisingly, this phenomenon was also observed for the *B. cinerea* actin gene (results not shown), even though hybridisation with the rRNA probe confirmed equal loading (Fig. 5). Expression of the *Bcpg*3 gene is highest on glucose and pectin. The *Bcpg*4 transcript was expressed at 6 hours after transfer on both **p**oly**g**alacturonic **a**cid (PGA) and GA, and at later stage of growth on pectin. Expression of *Bcpg*4 on glucose was not detected in these experiments. *Bcpg*5 gene expression was detected on glucose and pectin, especially six hours after transfer on pectin. *Bcpg*6 gene expression was observed on all four carbon sources, with high transcript levels on pectin, PGA and GA, a pattern distinct from *Bcpg*1 and *Bcpg*2 expression.

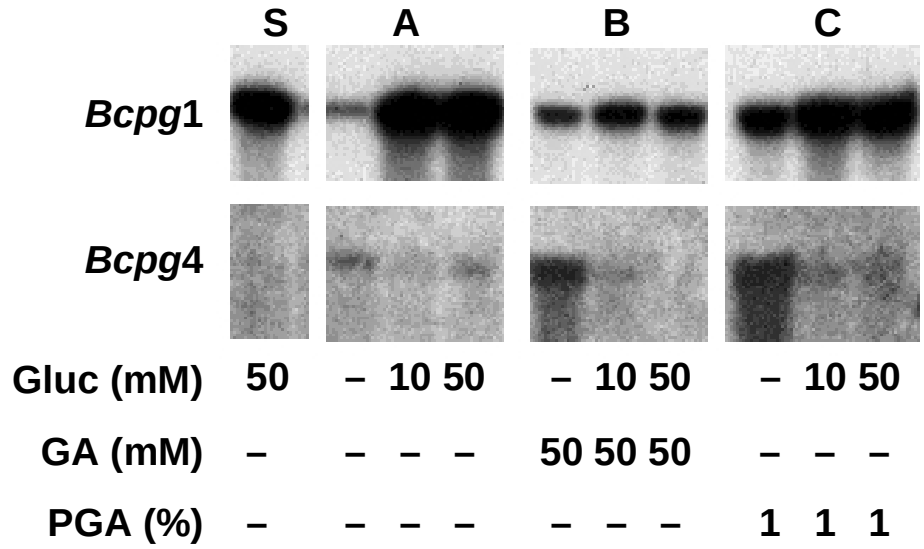


Figure 6: Carbon catabolite repression of endoPG gene expression of* Botrytis cinerea *strain B05.10 in a medium shift experiment
*Fungal RNA was isolated from mycelium grown in liquid culture with glucose (A), GA (B), and PGA (C) as initial carbon sources, harvested six hours after transfer from glucose (S). Exact amount and combination of carbon sources supplied to the culture medium is indicated in the bottom rows of the figure. RNA was subjected to RNA blot hybridisation using gene specific probes (*Bcpg*1 and* Bcpg*4) at high stringency conditions, as described in Materials and Methods. Exposure times for autoradiography after hybridisation with the different probes were adjusted to obtain signals with similar intensities.*

Carbon catabolite repression of *Bcpg* gene expression

The results obtained with medium shift experiments showed that the endoPG gene family was differentially regulated by carbon sources in the culture medium. The *Bcpg*4 gene shows a low expression on glucose as well as on pectin, which contains approximately 10% glucose, during early time points, indicating that this gene is subject to carbon catabolite repression. Several groups have reported carbon catabolite repression on endoPG production by *B. cinerea* (Leone and van den Heuvel, 1987; Johnston and Williamson, 1992b; van der Cruyssen *et al*., 1994). We studied the possibility of carbon catabolite repression by combining different inducing carbon sources with glucose as repressing sugar. Mycelium was pre-cultured for 16 h on glucose-containing medium, harvested, washed and transferred to different inducing media with and without glucose (10 or 50 mM). Six hours after transfer, mycelium was collected and expression of the different *Bcpg* genes was analysed. Figure 6 shows the expression of the *Bcpg*1 and *Bcpg*4 genes in mycelium obtained from media containing either glucose, GA or PGA only, and combinations of glucose with GA and PGA, as indicated. Repression of *Bcpg*4 gene expression was observed both on GA and PGA in the presence of 10 or 50 mM glucose. No effects were observed on the expression of the *Bcpg*1 gene (Fig. 6). Other gene family members did not reveal significant repression in the presence of glucose (data not shown).

pH regulation of *Bcpg* gene expression

Culture filtrates of all the mycelial cultures used in the medium shift experiments, were collected and chemically analysed. The pH was measured (Fig. 5) and the sugar composition determined (results not shown). Acidification of the culture medium was found when *B. cinerea* was grown on glucose and pectin. In these two cultures the initial pH (5.7) dropped within six hours to pH 3.9 (Fig. 5). The pH returned to 6.0 after 30 h of growth on pectin. The pH of the culture medium with PGA or GA never decreased, but increased steadily,

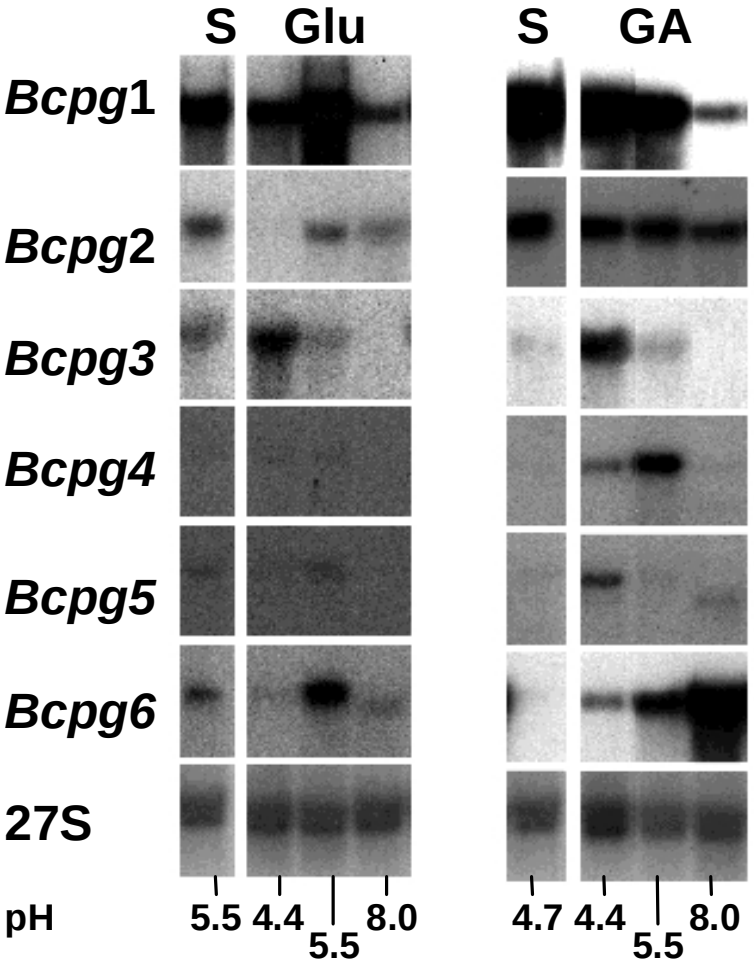


Figure 7: pH modulation of endoPG gene expression of* Botrytis cinerea *strain B05.10 in a medium shift experiment

*Fungal RNA was isolated from mycelium grown in liquid culture on glucose (Glu), or galacturonic acid (GA), harvested 6 hours after transfer from glucose (S). RNA was subjected to RNA blot hybridisation using gene specific probes (*Bcpg*1-6) at high stringency conditions, as described in Materials and Methods. As loading control RNA was hybridised with a 27S rDNA probe from* B. cinerea*. Exposure times for autoradiography after hybridisation with the different probes were adjusted to obtain signals with similar intensities. Bottom row of the figure shows the measured pH values of the culture medium when the mycelium was collected.*

stabilising after 30 h of growth at pH 6.8 (Fig. 5). This prompted us to study the effect of ambient pH on the expression of the *Bcpg* gene family in liquid cultures. Media with two different carbon sources (glucose or GA) were strongly buffered at pH 4, 6, or 8 (4.4, 5.5 and 8.0 after sterilisation) and tested for the effect of the pH on the expression of individual *Bcpg* genes (Fig. 7). The pH value did not change during the course of the experiment. *Bcpg*1 gene expression was higher around pH 4.4 and 5.5 than at pH 8, both on glucose and GA. On glucose, the expression of the *Bcpg*2 gene was highest at pH 5.5 and 8.0, however, on GA, no pH effect was observed. The *Bcpg*3 gene showed expression only in acidic media (pH 4.4)

on both carbon sources. *Bcpg*4 gene expression was highest on GA at pH 5.5. *Bcpg*5 gene expression was highest on glucose at pH 5.5 and on GA at pH 4.4. Finally, *Bcpg*6 gene expression on glucose was highest at pH 5.5, while on GA the highest expression was observed at pH 8.0.

Effects of pectin constituents on *Bcpg* gene expression

The pectin used for the medium shift experiments was isolated from apple fruit and might contain other sugars that act as inducing or repressing factors. Besides glucose and GA, apple pectin contains several other monosaccharides such as galactose, xylose, rhamnose, and L-arabinose (information supplied by the manufacturer). These sugars were added to liquid cultures in order to analyse their effects on *Bcpg* gene expression. Apart from the *Bcpg*4 gene, all gene family members showed low levels of expression on the different carbon sources (results not shown). In general, only minor differences were observed in *Bcpg* gene expression levels between the cultures with various carbon sources.

Discussion

The *Botrytis cinerea* endopolygalacturonase gene family is not a crucial determinant for broad host range

B. cinerea secretes a range of pectinolytic enzymes (Leone *et al*., 1990; Movahedi and Heale, 1990; Reignault *et al*., 1994), including up to 13 different polygalacturonase isoforms (van der Cruyssen *et al*., 1994). A thorough screening of a genomic library from *B. cinerea* strain SAS56 resulted in the isolation of six different endoPG encoding genes, denoted as the *Bcpg* gene family. BLAST protein sequence similarity searches yielded endoPGs from other fungal species with sequence identity to BcPG1 of more then 80%, whereas sequence identity between the different members of the endoPG family of *B. cinerea* varied between 34 and 73%. Phylogenetic analysis indicated the existence of distinct monophyletic groups of endoPGs originating from different species. Several of these species have endoPGs belonging to different monophyletic groups. This suggests that ancestor genes for these groups existed prior to the divergence of these fungal species. For other species only a single endoPG is known yet. The monophyletic groups clearly indicate the close evolutionary relationship between *B. cinerea* and *S. sclerotiorum*. Group III consists only of genes from these two species (see Figure 2). This is, however, biased by the presence of three genes from *S. sclerotiorum* that are probably the result of recent gene duplications (Fraissinet-Tachet *et al*.,1995). Group IV consists of two subgroups of which one consists only of genes from these two species. Identification of the sequences of four additional endoPG genes from *S. sclerotiorum*, as well as from other fungi, will enable us to understand possible evolutionary relationships. Enzymes produced by the saprophytic fungus *Aspergillus niger* show considerable differences with respect to substrate specificity, cleavage rate and optimal pH

for activity (Kester and Visser, 1990; Pařenicová *et al*., 1998; Benen *et al*., 1999). This phenomenon could explain the presence of endoPG families in so many saprophytic and necrotrophic fungi. However, information on endoPG characteristics of other fungal species is required to show whether enzymes belonging to the same monophyletic group share biochemical properties, and whether such properties are related with a biological function.

We analysed different *B. cinerea* strains and different *Botrytis* species for the presence of DNA homologous to the endoPG-encoding genes of *B. cinerea* strain SAS56. Without exceptions, each member of the gene family was present in all strains and species tested, although for some of the genes the hybridising signal was not strong. Since the different *Botrytis* species tested can only infect a single host plant genus, the presence of the endoPG gene family in *B. cinerea* is probably not crucial for its broad host range. It remains to be determined whether the *Bcpg* homologues in the other *Botrytis* species are expressed during the infection process. The observation that the saprophytic fungus *A. niger* produces a spectrum of endoPGs that does not enable this fungus to infect living plant tissue, further supports that endoPGs can probably at best be virulence factors, rather than determinants of host range.

Expression of the *Botrytis cinerea* endopolygalacturonase gene family is regulated by environmental factors

EndoPG enzyme production by *B. cinerea* is differentially regulated in liquid culture. Most of the isoforms produced are only found when the fungus is cultured on pectin-related carbon sources (Leone and van den Heuvel, 1987; Johnston and Williamson, 1992b; van der Cruyssen *et al*., 1994). Here we show that the endoPG-encoding genes are differentially expressed in liquid culture, when tested on different carbon sources and different pH's of the culture medium. We were able to distinguish four mechanisms that regulate gene expression. Firstly, two genes, *Bcpg*1 and 2 show a basal level of expression although *Bcpg*2 is not expressed in glucose medium at a low pH. Secondly, GA could induce the expression of both *Bcpg*4 and 6 whereas thirdly, glucose repression was observed for *Bcpg*4 only. Fourthly, induction of expression of *Bcpg*3 by low pH occurred largely irrespective of the carbon source present. We could not confirm end product repression, as has been reported for endoPG production by GA or di-galacturonic acid (Leone and van den Heuvel, 1987; van der Cruyssen *et al*., 1994), at the RNA level.

The expression of the *Bcpg*5 gene was highest on pectin. This could be due to an inducing factor present in apple pectin, possibly rhamnogalacturonans, xylo-galacturonan or combinations of saccharides. Experiments have not been conclusive to identify the inducing factor. *Bcpg*5 showed only a very low level of expression when the fungus is grown on PGA or GA (see Figures 5 and 7). However, in separate experiments aimed at studying glucose repression, we observed a high *Bcpg*5 expression on PGA and GA. Thus, no conclusion can be drawn about the regulation of this gene by PGA and GA.

Our expression studies support previous observations by others that *B. cinerea* endoPG activity is greatly affected by the carbon sources available for growth of the fungus (Leone and van den Heuvel, 1987; van der Cruyssen *et al*., 1994). Similar results were found for the plant pathogenic fungus *S. sclerotiorum* (Fraissinet-Tachet and Fèvre, 1996). For most plant pathogenic fungi, including *B. cinerea,* regulation of endoPG gene expression is not yet well understood. This process has been studied in some detail in the genus *Aspergillus,* saprophytes renowned for their cell wall degrading capability. A CAAT box is an important promoter sequence in *Aspergillus* where it is believed to account for a basal level of transcription. CAAT boxes were identified in the promoters of all six *Bcpg* genes. A number of transcription factors (*Cre*A, *Pac*C and *Xln*R*)* regulating *Aspergillus* CWDE gene expression has been identified. Their target sequences in a number of promoters have been determined (Cubero and Scazzocchio, 1994; Tilburn *et al*., 1995; MacCabe *et al*., 1996; van Peij *et al*., 1998b). Although the promoters of all *B. cinerea* endoPG genes, with the exception of the *Bcpg*2 gene, contain the sequence SYGGGG, the target site of the *Aspergillus* CREA protein, glucose repression was only found for *Bcpg*4. In order to resolve the mechanism of catabolite repression in *B. cinerea,* it remains to be determined whether the recognition motif of the *B. cinerea Cre*A homologue, which has recently been cloned and characterised *(*Liu *et al*., 2000), is similar to that of the *A. nidulans* protein. The modulation of expression levels of the different *Bcpg* genes at different pH values strongly suggests the involvement of pH regulatory elements, as has been reported for other filamentous fungi (MacCabe *et al*., 1996). The recognition site of the *A. niger pac*C pH regulatory element (GCCARG) is present in the promoters of all six endoPG encoding genes of *B. cinerea*. The relevance of the elements in *B. cinerea* remains to be determined. Not much is known about induction by end products at the transcriptional level. So far, regulatory sequences in the promoter elements of these genes that could be involved in the GA-induced gene expression have not been identified. Transcription factors involved in specific regulation of cell wall degrading enzymes have been identified in other fungi. The *Xln*R gene of *A. niger* encodes a transcriptional activator which regulates both xylanolytic and endoglucanase gene expression (van Peij *et al*., 1998a; 1998b). Analogously, a transcription factor is likely to be involved in the GA-induced gene expression of some of the *Bcpg* genes.

It is envisaged that differential regulation of gene expression occurs during infection of plants. EndoPGs encoded by constitutively expressed genes release products of pectin degradation that might induce the expression of other endoPG-encoding genes. Other genes could be induced during infection of different plant tissues or organs, e.g. *Bcpg*3 could well be expressed during the infection of acidic fruit tissue. Our final aim is to unravel the role of the induced and combined action of endoPGs of *B. cinerea* during infection of plants. We have reported that gene replacement of *Bcpg*1 in *B. cinerea* resulted in reduced virulence (Chapter 3). Similar experiments are in progress with the other *Bcpg* gene family members. It will be interesting to find out whether each of the endoPGs of *B. cinerea* has a specific function during the course of infection. Differential gene expression in combination with specific

enzyme activity of the endoPGs would clarify the need of *B. cinerea* for several enzymes, in order to optimally degrade pectin polymers under different environmental conditions.

Acknowledgements

We acknowledge Mrs. Dr. M.A. Kusters-van Someren for her advice at the start of the project, D.K. Aanen for his assistance and advise on the phylogenetic analysis, T. Prins for construction of the genomic library, Prof. Dr. P. Tudzynski and Dr. B. Tudzynski for providing *B. cinerea* isolates J13 and M15, and M. Hulst who contributed in gene cloning and sequencing. The authors also thank Dr J.A.E. Benen and L. Pařenicová for critical reading of the manuscript.

Materials and Methods

DNA recombinant techniques. Standard DNA recombinant protocols were used as described before (Sambrook *et al*., 1989). Host strains used were *Escherichia coli* LE392 for λEMBL3 phages and DH5α for plasmid propagation. The plasmid vectors pBluescript II SK (Stratagene, La Jolla, CA, USA) and pGEM-T-Easy (Promega, Madison, WI, USA) were used for DNA fragment cloning.

Table 4: Carbon sources used in medium shift experiments

Compound	Concentration	Manufacturer
D(+)glucose	10 mM, 50 mM, 1 % (w/v)	Merck, Darmstadt, Germany
Apple pectin	1 % (w/v)	Copenhagen Pectin, Copenhagen, Denmark
Polygalacturonic acid	1 % (w/v)	U S Biochemical Corp., Cleveland, Ohio, USA
D(+)galacturonic acid	10 mM, 50 mM	Fluka Chemie AG, Neu-Ulm, Switzerland
D(+)xylose	50 mM	Merck, Darmstadt, Germany
L(+)arabinose	50 mM	Merck, Darmstadt, Germany
L(+)rhamnose	50 mM	Janssen Chimica, Geel, Belgium
D(+)galactose	50 mM	BDH Chemicals Ltd, Poole, England

Fungal strains and standard culturing methods. *B. cinerea* strains and isolates of four other *Botrytis* species, indicated in Table 3, were grown on malt extract agar (OXOID, Basingstoke, UK) at 20 °C. For liquid cultures conidia were harvested from ten day-old plates and used to inoculate Gamborg's B5 medium (Duchefa Biochemie BV, Haarlem, the Netherlands) supplemented with 1 % (w/v) glucose and 10 mM$(NH_4)H_2PO_4$. Cultures were incubated in a rotary shaker at 180 rpm and 20 °C. Depending on the growth rate of the different strains and isolates used, cultures were grown between 16 and 48 hours post inoculation prior to harvesting of the mycelium.

Medium shift experiments and RNA blot analysis. The expression of the endoPG gene family was analysed on different carbon sources (Table 4). Gamborg's B5 medium supplemented with 1 % (w/v) glucose, 0.05 % yeast extract and 10 mM NaH_2PO_4-Na_2HPO_4 pH 6.0, was inoculated with 10^6 conidia ml^{-1} as described above. After 16 hours of growth in a rotary shaker at 180 rpm and 20°C the mycelium was harvested using Miracloth membrane (Calbiochem, La Jolla, USA), and washed thoroughly with Gamborg's B5 medium supplemented with 10 mM NaH_2PO_4-Na_2HPO_4 pH 6.0. Wet mycelium was transferred to fresh Gamborg's B5 medium with 10 mM NaH_2PO_4-Na_2HPO_4 pH 6.0, and supplemented with the carbon source of interest (Table 4). A pH shift experiment was performed using glucose and galacturonic acid as carbon sources and different buffers set at pH 4.0 (0.1 M Sodium citrate), pH 6.0 (0.1 M MES buffer), and pH 8.0 (0.1 M Tris buffer). After transfer, the fungus was grown for different

time periods (3 up to 30 h) prior to harvest of the mycelium using Miracloth membrane. The harvested mycelium was blotted dry on filtrepaper, quickly frozen in liquid nitrogen, and stored at - 70°C prior to extraction of the RNA. RNA was extracted from frozen mycelium using the TRIzol reagent (Life Technologies Inc., Gaithersburg, USA). 10 µg of total RNA was denatured using glyoxal as described before (Sambrook *et al*., 1989) separated on 1.2 % (w/v) agarose gel, and blotted onto Hybond N membranes using 10×SSC, according to the manufacturers instructions (Amersham, UK). Equal loading and blotting of the gel was checked by staining of the RNA on the Hybond filters with 0.2 % (w/v) methylene blue solution. Membranes were hybridised as described (van der Vlugt-Bergmans *et al*., 1997a) at 65°C and washed with 2×SSC / 0.1 % (w/v) SDS (two times 30 min) and 0.5×SSC / 0.1 % (w/v) SDS (30 min). Autoradiographs were made by exposure of KODAK-LS/KODAK-AR films at -70°C with two intensifying screens. DNA fragments used for probe preparation were similar as described above for DNA blot analysis. A *B. cinerea* 27 S rDNA fragment was used as probe to demonstrate equal loading of the gels.

DNA blot analysis. DNA was isolated as described (Möller *et al*., 1992), digested with *Eco*RI or *Hin*dIII, separated on a 0.7% (w/v) agarose gel and subsequently alkali blotted onto Hybond N^+ membranes according to the manufacturer (Amersham, Buckinghamshire, UK). Membranes were hybridised as described (van der Vlugt-Bergmans, 1997a) at 65°C and 55°C for high and low stringency, respectively. High stringency hybridisations were followed by washing in 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 (2×SSC) / 0.1% (w/v) sodium dodecyl sulphate (SDS), 0.5×SSC / 0.1% (w/v) SDS, and 0.2×SSC / 0.1% (w/v) SDS at 65°C for 30 min each. Low stringency hybridisations were followed by washing in 2×SSC / 0.1% (w/v) SDS, 0.5×SSC / 0.1% (w/v) SDS at 55°C for 15 min each. Autoradiographs were made by 96 h exposure of KODAK-LS/KODAK-AR films at -70°C with one intensifying screen. The following fragments were used for probe preparation for the different genes (numbers indicate the position of restriction site relative to the translation start site): *Bcpg*1, *Pst*I-BamHI (+161, +862); *Bcpg*2, *Nco*I-*Eco*RI (+858, +1413); *Bcpg*3, *Kpn*I-*Kpn*I (+766, +1313); *Bcpg*4, *Bam*HI-*Bam*HI (+384, +891); *Bcpg*5, *Bgl*II-*Hind*III (+92, +1072); *Bcpg*6, *Cla*I-*Cla*I (+308, +980).

Screening of genomic library. A genomic library (λEMBL3) of *B. cinerea* strain SAS56 was screened (1×10^5 phages) using an internal *Pst*I/*Bam*HI fragment (0.7 kb) of *Bcpg*1 as a probe. Hybridisations and washings were performed as described (van der Vlugt-Bergmans, 1997a) at 60 °C, and resulted in the isolation of positive phages. Hybridising fragments of these phages were subcloned in pBluescript II SK plasmids and further characterised by restriction analysis and Southern hybridisations. This resulted in the identification of different classes of hybridising clones. Within each class, DNA fragments were further characterised by sequence analysis.

Nucleotide sequence analyses. Sequencing reactions were performed using the ThermoSequenase fluorescent-labelled primer cycle sequencing kit (Amersham) with universal sequencing primers, and the Cy5 Autoread Sequencing kit (Pharmacia Biotech, Uppsala, Sweden) using gene specific oligonucleotides. The sequencing reactions were analysed on an ALF express sequencer (Pharmacia Biotech). Nucleotide sequence data were analysed using the Lasergene Biocumputing Software for Windows (DNASTAR Inc, Madison, WI, USA). BLAST database searches were performed using the National Centre for Biotechnology Information BLAST WWW server. Phylogenetic analyses were performed using PAUP 3.1 (Swofford, 1993).

Nucleotide sequence accession numbers. The nucleotide sequences for the endoPG-encoding genes of *B. cinerea* are in the GenBank database under accession numbers U68715 [*Bcpg*1], U68716 [*Bcpg*2], U68717 [*Bcpg*3], U68719 [*Bcpg*4], U68721 [*Bcpg*5] and U68722 [*Bcpg*6].

Chapter 5

Botrytis cinerea endopolygalacturonase genes are differentially expressed in various plant tissues[1]

Arjen ten Have, Wendy Oude Breuil, Jos P. Wubben, J. Visser and Jan A.L. van Kan.

Abstract

Botrytis cinerea, causal agent of leaf blight, fruit rot and gray mould on many plant species, secretes various endopolygalacturonases during all stages of infection (van der Cruyssen *et al*., 1994, Med. Fac. Landbouwwet. Rijksuniv. Gent. 59:895-905). The encoding genes, denoted as *Bcpg*1-6, are differentially expressed *in planta*, depending on the stage of infection and the host species that is infected. *Bcpg*1, reported previously to be constitutively expressed (Chapters 3 and 4) is expressed in all tissues tested although strong differences in transcript levels are observed. *Bcpg*2, is expressed early in the infection of most plant tissues, suggesting a function in lesion expansion. *Bcpg*3 is expressed in acidic environments such as in apple fruit. The expression pattern of *Bcpg*4 and 6 on all hosts tested is in agreement with the induction of these genes by monogalacturonic acid (Chapter 4), suggesting a role in nutrient provision rather than lesion expansion. *Bcpg*5 expression is found under varying circumstances. The regulation of *Bcpg* gene expression indicates that *B. cinerea* is equipped with a flexible enzymatic pectate degradation machinery throughout infection of its hosts. *Bcpg*1 was previously shown to be required for full virulence (Chapter 3). In addition, *Bcpg*2 and *Bcpg*3 might have discernible contributions to virulence.

[1] **A slightly modified version of this chapter was published by Arjen ten Have, Wendy Oude Breuil, Jos P. Wubben, Jaap Visser and Jan A.L. van Kan in Fungal Genetics and Biology 33:97-105 (2001)**

Introduction

Botrytis cinerea Pers.: Fr. *Botryotinia fuckeliana* (de Bary) Whetz. is a plant pathogenic ascomycete that causes pre- and post harvest diseases on many economically important crops. Leaf and petal blight occur on a number of ornamental crops as well as on blossom, whereas rot occurs on a number of vegetables and fruits (Jarvis, 1977). A heavily sporulating gray mould can develop in two or three days, hence the common name of this disease, gray mould.

For the primary infection of *B. cinerea*, a number of mechanisms has been suggested. *B. cinerea* is often regarded as a wound pathogen that may penetrate through wounds or natural openings (Staples and Mayer, 1994). However, ramification of the plant epidermis using appressorium-like bodies has also been reported (Rijkenberg *et al*. 1980). Cell wall degradation is often observed during the initial steps of pathogenesis and has been suggested to be instrumental in penetration (Mansfield and Richardson, 1981). Since **c**ell **w**all **d**egrading **e**nzyme**s** (CWDEs) are found in all steps of the infection process, they may also be involved in later steps of infection. Degradation of plant cell walls by fungal CWDEs may facilitate fungal growth and may provide the fungus with nutrients. It has been shown that bacterial CWDEs can contribute to virulence of a number of bacterial pathogens (reviewed by Barras *et al*., 1994; Hugouvieux-Cotte-Pattat *et al*., 1996). It can be envisaged that CWDEs secreted by *B. cinerea* contribute to virulence in a similar way as these bacterial CWDEs.

There are indications that pectinases, which are among the CWDEs secreted by *B. cinerea*, play a role in the infection process. Firstly, the broad host range of *B. cinerea* is confined to plants that contain high pectin levels in their cell wall, i.e. dicotyledons and non-graminaceae (Jarvis, 1977; Carpita and Gibeaut, 1993). Furthermore it has been reported that penetration often takes place at the anticlinal cell wall (Mansfield and Richardson, 1981), followed by intercellular growth through the middle lamella, a part of the cell wall containing a high proportion of pectins (Bateman and Basham, 1976). In addition, a swelling of the epidermal cell wall was observed upon penetration of the cuticle, which indicates that the coherence of the pectic matrix is disturbed (Mansfield and Richardson, 1981). Furthermore, numerous biochemical studies on pectinases from *B. cinerea* reported that an endo**p**oly**g**alacturonase (endoPG) is the first CWDE detected in the infection process (e.g. Johnston and Williamson 1992a; 1992b; van der Cruyssen *et al*. 1994; Kamoen and van der Cruyssen, 1996). Up to 13 endoPG isozymes have been described for *B. cinerea*, and the activity is found in all steps of the infection process (van der Cruyssen *et al*., 1994). It can therefore be envisaged that endoPGs play a role in different steps of the infection process.

We started a molecular genetic approach by isolating six genes encoding endoPGs denoted as *Bcpg*1-6 (Chapters 3 and 4). We reasoned that, in order to be involved in pathogenesis, it is a prerequisite for a *Bcpg* gene to be expressed in some stage of the infection process. *Bcpg*1 was shown to be constitutively expressed and to be required for full virulence on a number of host tissues (Chapter 3). Here we present data on the expression of

all six *Bcpg* genes in time course experiments in tomato leaves as well as broad bean leaves at moderate and low temperature, using RNA blot hybridisations and RT-PCR. Infections of apple fruit and courgette fruit were used to determine whether transcript levels differ in different zones of the infected tissue. The expression patterns that were observed *in planta* are discussed in relation to the regulation that was observed in liquid cultures (Chapter 4).

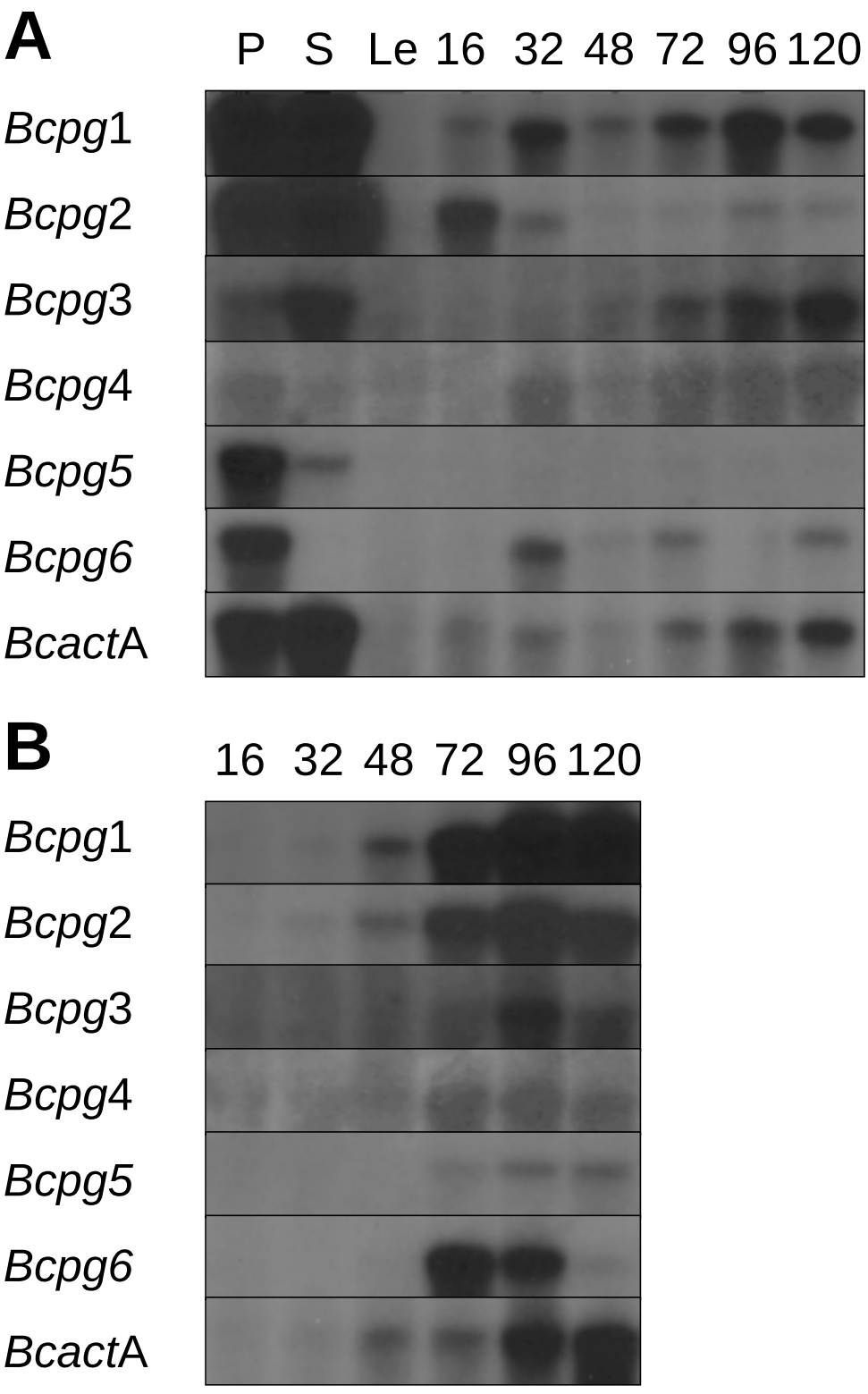
Results

Expression of the *Bcpg* gene family during infection of tomato leaves

The expression of the endoPG gene family was studied in time during infection of detached tomato leaves, as previously described for the *Bcpg*1 gene (Chapter 3). Infection was established by inoculating the leaves with a conidial suspension and subsequent incubation at 20°C or 4°C. Primary infection occurs within 16-20 **h**ours **p**ost **i**noculation (HPI) for both incubation conditions. The necrotic plant response restricts the outgrowth of approximately 80-90% of the lesions at 20°C, while the remaining 10-20% of the primary lesions expand, eventually resulting in complete leaf colonisation at 120 HPI (Chapter 2). At 4°C, when the plant defence responses are strongly reduced, 80-90% of the primary lesions expand, resulting in full leaf maceration at 144 HPI. Samples of infected plant material from both incubations were taken for RNA isolation at six time-points after inoculation.

The results of the RNA blot hybridisations of the tomato leaf infections at 20°C and 4°C are shown in Figure 1A and 1B, respectively. The level of the constitutively expressed actin mRNA (*Bcact*A) reflects the progress of the infection (Chapters 2, 3 and 4). The expression patterns of *Bcpg*1, *Bcpg*4 and *Bcpg*6 in the case of infection at 20°C are similar to that of the actin gene. Previously, we observed that *Bcpg*2 is expressed under all circumstances tested in liquid culture, except when grown on glucose at low pH (Chapter 4). The RNA blot analysis in Figure 1A shows a strong transient increase of *Bcpg*2 transcript level during the tomato leaf infection at 20°C with a maximum at 16 HPI. Furthermore, a low *Bcpg*2 transcript level is found late during infection. *Bcpg*3 is expressed during late stages at 20°C whereas *Bcpg*5 expression is barely detectable.

During infection at 4°C no significant differences in the expression patterns of the endoPG genes are observed (Fig. 1B). All genes show expression late in infection although differences in relative hybridisation signals are observed. Differential expression is observed only for *Bcpg*6 mRNA, which shows a strong increase at 72 HPI followed by a strong decrease at 120 HPI.


A
P S Le 16 32 48 72 96 120
Bcpg1
Bcpg2
Bcpg3
Bcpg4
Bcpg5
Bcpg6
BcactA
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Bcpg3
Bcpg4
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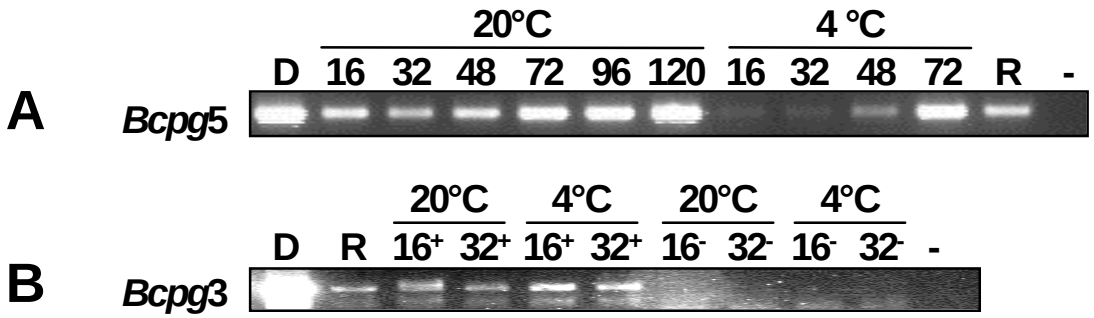


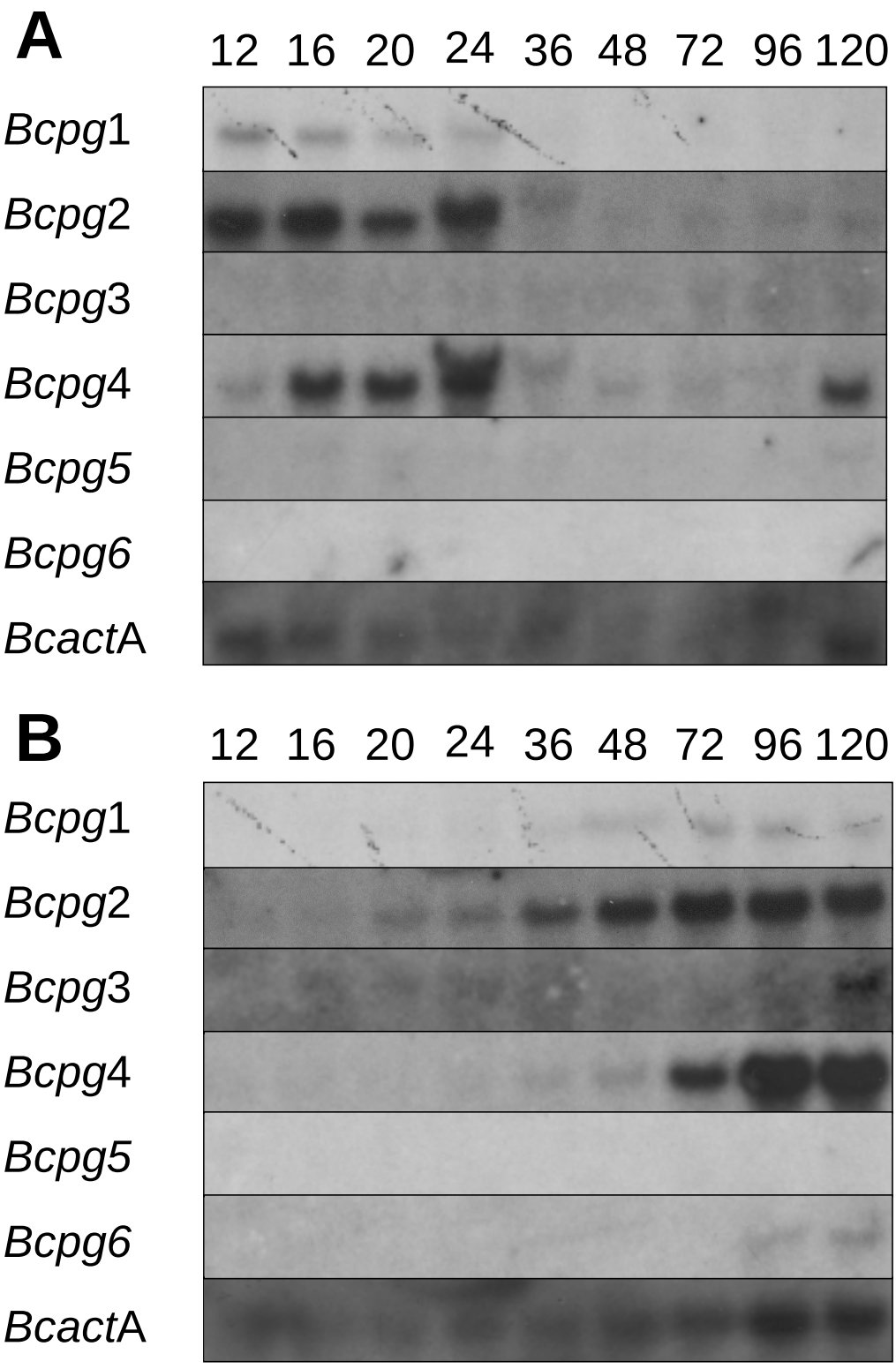
Figure 2: RT-PCR analysis of time course* Botrytis cinerea *infection of tomato leaves
2A: *RT-PCR with primers specific for* Bcpg*5 was performed on total RNA isolated from 20°C incubation (16, 32, 48, 72, 96, 120 hours post inoculation) and 4°C incubation (16, 32, 48, 72 hours post inoculation). The resulting bands reflect 1 µg total RNA. D: PCR control (1 ng pCPG5 DNA), R: reverse transcriptase control (0.1 pg synthetic* Bcpg*5 RNA), -: negative control (no input).*
2B: *RT-PCR with primers specific for* Bcpg*3 was performed on total RNA isolated from 16 and 32 hours post inoculation of both the 20°C and the 4°C incubations (16^-, 32^-, results reflect 1 µg total RNA) and on identical samples to which 0.5 pg of synthetic* Bcpg*3 RNA was added (16^+, 32^+). D: PCR control (1 ng pCPG3 DNA), R: reverse transcriptase control (0.5 pg synthetic* Bcpg*3 RNA), -: negative control (no input).*

RT-PCR enables to detect mRNA at lower detection limits and was therefore used to supplement the data obtained with RNA blotting. RT-PCR products derived from transcripts of *Bcpg*2, *Bcpg*4 and *Bcpg*6 were detected at all stages of infection at both 4 and 20°C (data not shown). *Bcpg*5 transcripts can also be detected throughout the infection process (Fig. 2A), although transcript levels are low at 4°C. *Bcpg*3 transcripts can not be detected during the first 32 hours of the infection at either temperature (Fig. 2B).

Expression of the *Bcpg* gene family during infection of broad bean leaves

The expression of the endoPG gene family was studied during infection of detached broad bean leaves using inoculation and incubation procedures similar to those used for the infection of tomato leaves. Primary infection of broad bean leaves at 20°C can be detected at 12 HPI when black necrotic spots develop at the inoculation site. Infection proceeds rapidly

Figure 1: RNA blot analysis of time course* Botrytis cinerea *infection of tomato leaves
1A: *P: 1% PGA, S: 1% sucrose (controls from liquid cultures), Le: uninoculated tomato leaf, and time course of infection at 20°C incubation (16, 32, 48, 72, 96, 120 hours post inoculation).*
1B: *Time course of infection at 4°C incubation (16, 32, 48, 72, 96, 120 hours post inoculation). The blots were consecutively hybridised with gene-specific fragments of Bcpg1-6 and BcactA, encoding actin. Each lane contains 20 µg of total RNA. Equal loading was confirmed by staining the blot with ethidium bromide (not shown).*


A
12 16 20 24 36 48 72 96 120
Bcpg1
Bcpg2
Bcpg3
Bcpg4
Bcpg5
Bcpg6
BcactA
B
12 16 20 24 36 48 72 96 120
Bcpg1
Bcpg2
Bcpg3
Bcpg4
Bcpg5
Bcpg6
BcactA

until approximately 36 HPI when the leaves become desiccated and the expansion rate decreases. At 4°C, primary infection is established around 20 HPI and the infection sites turn into black necrotic spots around 48 HPI. Expansion of the lesions is slow but eventually the whole leaf becomes infected at 144 HPI. Samples of infected plant material from both incubations were taken for RNA extraction at six time-points after inoculation.

The results of the RNA blot hybridisations of the broad bean leaf infections at 20°C and 4°C are shown in Figure 3A and 3B respectively. The expression pattern of the endoPG gene family during the infection of broad bean is differential. *Bcact*A transcript can be detected throughout the infection at 20°C. Early in the infection, the levels are just detectable above the relatively high background signal. A slight increase in *Bcact*A transcript level is observed at 120 HPI. Although the *Bcact*A gene is constitutively expressed, its mRNA level reflects actively growing mycelium rather than the total amount of fungal biomass, which includes degenerated or metabolically inactive mycelium (Prins *et al*., 2000). Apparently, during the leaf desiccation (36-72 HPI), fungal growth decreases, while in the late stages (96-120 HPI) fungal growth increases again. *Bcpg*1 can be detected early in the infection at 20°C only. *Bcpg*2 transcript seems the predominant *Bcpg* transcript and can be detected throughout the infection at 20°C, although a strong decrease in transcript level is observed at 36 HPI. *Bcpg*3 transcript is at or below the detection limit since only vague bands can be seen. *Bcpg*4 is expressed to significant transcript levels at 16-24 HPI as well as 120 HPI whereas *Bcpg*5 and *Bcpg*6 can hardly be detected in any of the samples. At 4°C (see Figure 3B), *Bcpg*2 is the first *Bcpg* gene that can be detected. Its transcript levels increase from barely detectable at 20 HPI up to its highest level at 96 HPI. *Bcpg*1 and *Bcpg*4 are detected at 48HPI, the latter showing increased transcript levels later in the infection. *Bcpg*3 hybridisation is under the detection limit throughout the first 72 hours of the infection at 4°C, whereas transcript levels are clear at 96 and 120 HPI. Also *Bcpg*6 transcript is detected at 96 and 120HPI whereas *Bcpg*5 transcripts can not be detected at any stage of the infection at 4°C.

***Figure 3: RNA blot analysis of time course* Botrytis cinerea *infection* of broad bean leaves**
3A*: Time course of infection at 20°C incubation (12, 20, 24, 36, 48, 72, 96, 120 hours post inoculation).*
3B*: Time course of infection at 4°C incubation (12, 20, 24, 36, 48, 72, 96, 120 hours post inoculation). The blot was consecutively hybridised with gene-specific fragments of* Bcpg*1-6 and* Bcact*A, encoding actin. Each lane contains 20µg of total RNA. Equal loading was confirmed by staining the blot with ethidium bromide (not shown).*

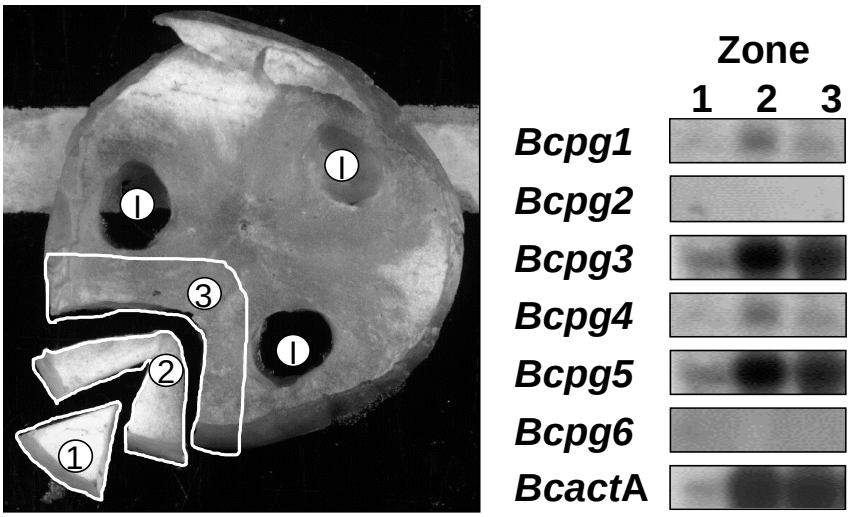


Figure 4: RNA blot analysis of* Botrytis cinerea *infected apple fruit
Apple slices were inoculated with conidia embedded in agar in the holes indicated by I. Three adjacent zones with varying degrees of infection were isolated at 48 hours post inoculation for RNA blot analysis: Zone 1: no disease symptoms; Zone 2: transition zone; Zone 3: fully macerated zone. The blot was consecutively hybridised with gene-specific fragments of Bcpg*1-6 and* Bcact*A, encoding actin. Each lane contains 10 µg of total RNA, no cross hybridisation to apple RNA sequences was found (not shown). Equal loading was confirmed by staining the blot with ethidiumbromide (not shown).*

Expression of the *Bcpg* gene family in apple and courgette fruit

The expression of the endoPG gene family during infection of apple fruit was determined in space rather than in time. Apple fruit disks were inoculated by embedding agar plugs containing germinating conidia into fresh punctures of identical size. Three days post inoculation, three adjacent zones of apple tissue were sampled, each reflecting a different degree of *B. cinerea* infection, as is indicated in Figure 4. RNA was extracted and analysed by hybridisation.

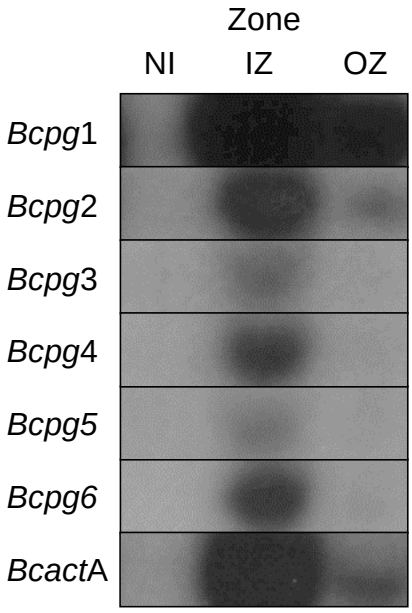


Figure 5: RNA blot analysis of* Botrytis cinerea *infection of courgette fruit
NI: non-infected fruit, IZ: tissue from the inner zone of infection, OZ: tissue from the outer zone of infection. Each lane contains 10 µg of total RNA. Identical blots were hybridised with gene-specific fragments of Bcpg*1-6 and* Bcact*A, encoding actin. The* Bcpg*1 panel is derived from a 16 hour exposure whereas the other panels are derived from a 72 hour exposure. Equal loading was confirmed by staining the blot with ethidiumbromide (not shown).*

Expression of *Bcpg*1, *Bcpg*3, *Bcpg*4 and *Bcpg*5 was found in all zones of the infected apple fruit whereas *Bcpg*2 and *Bcpg*6 could not be detected. The differences in hybridisation intensity between the three zones correlate with the proportion of fungal biomass in the infected tissue only. No cross-hybridisation with apple sequences was observed in non-infected tissue (data not shown).

A similar expression study was performed in courgette fruit from which two adjacent zones were sampled (see Figure 5). The hybridisation signal of the *Bcpg*1 gene is about 100 times higher than the hybridisation signals of the other *Bcpg* genes. All genes are expressed in the inner zone of the infection whereas only *Bcpg*1 and *Bcpg*2 can easily be detected in the outer zone of infection. Long exposures revealed a weak signal for *Bcpg*6 in the outer zone (data not shown). The hybridisation signals for *Bcpg*3 and *Bcpg*5 are low.

Discussion

The expression of the *Botrytis cinerea* endoPG genes *in planta* and in liquid cultures

The expression of the endoPG gene family *in planta* has a differential pattern and depends on the host tissue, the stage of infection and the temperature. In Chapter 4 we postulated four regulatory mechanisms based on the *Bcpg* gene expression patterns observed in liquid cultures. Firstly, two genes, *Bcpg*1 and *Bcpg*2 showed a basal level of expression although *Bcpg*2 was not expressed in glucose medium at a low pH. Secondly, **g**alacturonic **a**cid (GA) induced the expression of both *Bcpg*4 and *Bcpg*6. Thirdly, glucose repression was observed for *Bcpg*4 only. Fourthly, induction of expression of *Bcpg*3 by low ambient pH was observed largely irrespective of the carbon source present. These regulatory mechanisms presumably contribute to the observed expression patterns *in planta* (Figs.1-5), as will be discussed below.

It was reported that *Bcpg*1 is constitutively expressed in liquid cultures (Chapters 3 and 4) as well as during infection of tomato leaves (Chapter 3). Although this gene seems to be expressed under all circumstances, its expression level *in planta* shows strong differences between different hosts. The hybridisation intensity of the *Bcpg*1 gene in courgette fruit is 10-100 times higher than the hybridisation intensities of the other endoPG genes (Fig. 5), in analogy to what was reported for liquid cultures (Chapter 4). However, the *Bcpg*1 hybridisation intensity in the other infection studies is much lower (Figs. 1, 3 and 4). In broad bean (Fig. 3) the *Bcpg*2 transcripts show a much higher hybridisation level than *Bcpg*1 transcripts, which are barely detectable in late stages of infection at 20°C. The regulation of *Bcpg*2 expression remains unclear. The expression in liquid cultures suggests that this gene has a basal expression that might be modulated by environmental factors like low ambient pH (Chapter 4). The low pH of apple fruit tissue can explain the absence of detectable expression in this tissue (Fig. 3). The transient and high *Bcpg*2 expression in tomato leaf infections at 20°C (Fig. 1, 16 HPI) suggests a specific induction rather than a repression. Although it is

difficult to determine the actual pH in the vicinity of the fungal hyphae in leaf tissue, it is probably acidic rather than basic. The pH of apoplastic fluid of tomato leaf is approximately 6.3 (ten Have, unpublished). It can therefore probably be excluded that a high ambient pH is responsible for the high level of *Bcpg*2 expression. We tested whether the addition of plant derived factors such as ethylene and tomato or broad bean leaf apoplastic fluid would influence *Bcpg*2 transcript levels in liquid culture. No strong differences in transcript levels were observed. It is possible, however, that a combination of factors results in the onset of *Bcpg*2 gene expression. Another possible trigger is oxidative stress occurring in necrotic plant tissue or another necrosis-related factor. No elevated level of expression of *Bcpg*2 was observed in the 4°C incubation experiment with infected tomato leaves. Necrosis does not occur under these conditions in tomato leaves (Chapter 2). In broad bean, however, the infection at 4°C results in severe necrosis that appears to be accompanied with a strong expression of the *Bcpg*2 gene.

The regulation of expression of the other endoPG genes *in planta* can more readily be explained. *Bcpg*3 is induced at low pH in liquid cultures (Chapter 4). It is expressed in apple fruit tissue, which has a low pH, but is barely expressed in courgette, which has a more neutral pH. *Bcpg*5 transcript can be detected in infected apple fruit tissue, probably as a result of induction by a factor derived from apple pectin, as was discussed in Chapter 4. The low expression of *Bcpg*5 in courgette suggests that the postulated apple pectin derived factor (Chapter 4) is not present in courgette. *Bcpg*4 and *Bcpg*6 expression coincides with the constitutive expression of *Bcpg*1 in tomato leaf whereas *Bcpg*4 expression coincides with the basal expression of *Bcpg*2 in broad bean leaf. However, the zone expression pattern in apple shows that *Bcpg*4 is expressed in heavily macerated tissue only, suggesting that also *in planta* this gene is induced by GA. *Bcpg*6 expression is also inducible by GA but no signal was found for this gene in the inner zone of apple infection. This may still be in agreement with the results in Chapter 4 since this gene is also subject to repression by low pH. There is no apparent explanation for the low expression levels of *Bcpg*6 in broad bean. In courgette we investigated only heavily infected and poorly infected material. *Bcpg*4 and *Bcpg*6 are expressed in the inner zone of infected tissue.

The expression patterns that are observed for the 4°C infections differ significantly from the patterns observed for the 20°C infections (Figs. 1 and 3). The lack of differential expression at 4°C cannot easily be explained with the current knowledge. *B. cinerea* is generally believed to have its optimal growth *in vitro* at 15-20°C, although infection at 4°C can be aggressive. However, it is unclear whether the *Bcpg* transcripts are translated, whether the translation products, if any, are modified properly and whether the produced isozymes are active at this temperature. It can be envisaged that actual enzyme activities are low at this temperature which may result in a distinct regulation of *Bcpg* gene expression. The effect of starvation on the expression of the endoPG gene family members is as yet unknown.

The pectate degrading machinery of *B. cinerea* has a role in pathogenesis

The presence of six genes encoding endoPGs raises questions about the function of such a seemingly redundant set of enzymes. Differences in substrate and in environmental conditions may explain the requirement of such a set of isozymes. It has been shown that the number of genes in the genome of a *Botrytis* species is not correlated to the width of its host range (Chapter 4). During the last three decades there has been a debate on the function of CWDEs in plant pathogens. There is substantial evidence for functions of CWDEs in bacterial pathogenesis (reviewed by Barras *et al*., 1994; Hugouvieux-Cotte-Pattat *et al*., 1996). There are no individual CWDEs that are absolutely required for pathogenicity, yet several CWDEs contribute to the aggressiveness of a bacterial infection. Studies with targeted mutants in fungi (Scott-Craig *et al*. 1990; Schaeffer *et al*., 1994; Bowen *et al*., 1995; Sposato *et al*., 1995; Apel-Birkhold and Walton, 1996; Gao *et al*., 1996; Görlach *et al*., 1998; Scott-Craig *et al*. 1998) have not shown any fungal CWDE that contributes to virulence, except for the saprophytic fungus *Aspergillus flavus* (Shieh *et al*., 1997). We showed that the constitutively expressed *B. cinerea* endoPG BcPG1 is required for full virulence (Chapter 3). The identification of five additional genes encoding endoPG enzymes that are differentially regulated *in vitro* (Chapter 4) raised the question if any of these endoPGs have a role in the infection process. The observed *Bcpg* gene expression pattern *in planta* enables one to make predictions about the possible contributions of individual genes to virulence. *Bcpg*1 is expressed constitutively, although the transcript level in most plant tissues is lower than in liquid cultures. The targeted *Bcpg*1 deletion mutant is indeed less virulent on tomato and apple (Chapter 3) but also on broad bean (ten Have, unpublished data). *Bcpg*2 shows a very distinct expression pattern, especially in tomato leaves. It can be envisaged that a targeted gene replacement in this gene would show a reduction in the number of expanding lesions on tomato leaves. Since the *Bcpg*2 gene seems to be expressed in most host tissues, it is a good candidate for mutational studies. *Bcpg*3 might play a role in the infection of fruit that has a low pH. *Bcpg*5 might function in the breakdown of a specific pectin such as xylogalacturonan and possibly in the infection of hosts, like apple, that contain a significant amount of a specific pectin like xylogalacturonan. *Bcpg*4 and *Bcpg*6 complete the spectrum; both are inducible by GA while *Bcpg*4, is subject to glucose repression. It can be envisaged that these genes, which are expressed mainly in tissue that has already been invaded, have a function in nutrient provision rather than growth facilitation. The entire regulatory spectrum of the *Bcpg* gene family provides the fungus with a pectate degrading machinery that has a low basal activity, is induced under different environmental conditions and has a feedback control that is adjusted to the demands required for fungal growth. The predictions that are made on the role of various genes in pathogenesis remain to be confirmed by targeted mutagenesis, as was previously carried out for the *Bcpg*1 gene (Chapter 3).

Materials and Methods

Fungal strains, plants and inoculation of leaf material. B05.10 (van Kan *et al*., 1997), was maintained and conidia were isolated and preincubated as described (Chapter 2). Detached tomato (*Lycopersicon esculentum)* leaves, Moneymaker genotype *Cf4* grown as described (Chapter 3), were inoculated by spraying as was previously reported (Chapters 2 and 3). Broad bean (*Vicia faba*) plants were grown in a climate chamber at 24°C with a 16-hour light period in 1-liter pots in potting soil without additional nutrition. Compound leaves, obtained from 4-6-week-old plants by cutting the stem at approximately 1 cm under the petiole, were put in a block of water-saturated floral foam. Inoculation and incubation conditions were identical to those used for the tomato leaves (Chapter 2).

Infection of fruits. Inoculations for expression studies were performed with conidia embedded in agar. Conidia were preincubated as described (Chapter 2) and mixed with nine volumes 1.5% purified agar (Oxoid, Basingstoke, Hampshire, UK) of 42°C in Gamborg's B5 medium including vitamins (Duchefa, Haarlem, The Netherlands) supplemented with 10mM sucrose and 10 mM $(NH_4)H_2PO_4$, and directly poured in petridishes. Fruits of apple (*Malus domestica* Borkh. cv. Elstar) and courgette *(Cucurbita pepo*) were obtained from a local grocery. Stalks, if any were removed from apple fruits and both apple and courgette fruits were surface disinfected with 70% ethanol in 1% H_20_2. Slices, approximately 1/2 cm thick, were inoculated in 4 mm punctures with three equally thick agar discs (D = 4 mm) containing *B. cinerea* conidia (10^6 ml^{-1}) . Inoculated slices were sandwiched between their neighbouring slices to prevent desiccation and incubated in a sterile plastic box at 20°C, 100% RH in the dark.

RNA extraction and blotting. RNA from leaves and liquid cultures was extracted as described (van Kan *et al*., 1992). Apple and courgette tissue was grinded in liquid nitrogen in a Waring blender followed by further pulverisation by mortar and pestle. RNA was extracted from the powder by addition of 2 volumes extraction buffer (8.0M Guanidine hydrochloride, 20mM MES, 20mM EDTA, 50mM 2-mercaptoethanol, pH 7.0), incubation at room temperature for 10 min and mechanical disruption on a Vibrofix (Janke und Kunkel, Staufen, Germany) for 5 min. The extract was centrifuged at 1600 x g and the supernatant was subsequently filtered over Miracloth (Calbiochem, La Jolla, CA, USA). The flow through was purified using RNeasy maxi spin column, as described by the manufacturer (QIAGEN, Hilden, Germany).

Electrophoresis, blotting and hybridisation of the blots with gene specific probes for *Bcact*A (Chapter 2), *Bcpg*1 (Chapter 3), *Bcpg*2-6 (Chapter 4) were performed as described (van der Vlugt-Bergmans *et al*, 1997a). Fragments were radio-labelled using the Random Primers DNA Labelling System according to the manufacturer's description (Life Technologies Inc., Gaithersburg, MD, USA) and Redivue α^{32}P-dATP (Amersham, Buckinghamshire, UK). Autoradiographs were made by exposure of KODAK-S films at -70°C with two intensifying screens.

RT-PCR, cloning of partial cDNAs and RNA synthesis. Table 1 shows the sequences of oligonucleotides that were used in reverse transcriptase (RT) and PCR reactions. cDNA synthesis was performed with 10 pmol 1st strand synthesis primer (RT). The primer was annealed to 20 µg total RNA as described (Pařenicová *et al*., 1998). The nucleic acid was precipitated and dissolved in reverse transcription reaction mixture (Pařenicová *et al*., 1998). Primer elongation was performed for 2 hours using 200 U of M-MLV reverse transcriptase, 1.25mM of each dNTP according to the manufacturer's description (Life Technologies Inc.) in the presence of 40 U RNasin (Promega Madison, WI, USA). The reaction mixture was then extracted with phenol, precipitated with ethanol. The nucleic acid was subsequently dissolved in 20 µl of milliQ water (Millipore, Bedford, MA, USA), and thereafter referred to as cDNA template. PCR was performed using 1 µl cDNA template with 10 pmol of each PCR primer and 2U Taq polymerase (Perkin Elmer) according to the manufacturer's description. PCR products were separated on 3% NuSieve agarose gel (Biozym, Heerlen The Netherlands) in TAE buffer (Sambrook *et al*, 1989). For cloning into pGEM-T (Promega), RT-PCR fragments from *Bcpg*3 and *Bcpg*5 were isolated from gel using Glassmax (Life Technologies Inc.). The resulting plasmids were denominated pCPG3 and pCPG5.

Table 1: Oligonucleotides used for RT-PCR

Primer	Direction	*Bcpg*3	*Bcpg*5
RT[1]	antisense	5'CCGGTAATGGTGATATCGG3'	5'GGTGCAAGAACCACTTCCAC3'
PCR	sense, cDNA specific[2]	5'TGTCTTCAAGTAGCAACCAC3'	5'CAACTCCGGAACTGGCATT3'
PCR	antisense	5'CCGGTAATGGTGATATCGG3'	5'CAATAAGAATGTTGGTGGCGG3'[3]

[1] RT indicates 1st strand Reverse Transcriptase synthesis primer, PCR indicates PCR primer. [2] sequences of oligonucleotides were based on sequences spanning both sides of an intron [3] primer nested 5' to 1st strand Reverse Transcriptase synthesis primer.

Control RNA (cRNA, i.e. the RNA strand corresponding to the mRNA) was synthesised from plasmids pCPG3 and pCPG5 using T7 or SP6 polymerase using 200 ng and 100ng of plasmid DNA, respectively according to the manufacturer (Life Technologies Inc.). Plasmid DNA was digested in order to obtain length-defined cRNA. The synthesised cRNA was repeatedly treated with 1 U DNase which is free of RNase, according to the manufacturer (Promega) in the presence of 40 U Rnasin (Promega). The cRNA was purified using the clean up protocol of QIAGEN RNeasy mini kit (QIAGEN). Upon precipitation of the flow through the concentration and purity was determined using a Beckman Spectrophotometer (Fullerton, CA,USA) by measuring absorption at 260 nm and 280 nm respectively. Finally the cRNA was diluted in solutions of tomato leaf RNA in milliQ water (1 µg/µl) and used for RT-PCR in order to determine the detection limits for both transcripts (data not shown). RT-PCR was performed as described above on total RNA samples with or without the amount of cRNA that was shown to be just above the detection limit.

Chapter 6

General discussion

The plant cell wall and plant pathogens

The plant cell wall functions as an effective barrier that protects the cell from invasion by pathogens. Plant pathogens have various mechanisms to cope with the cell wall. Viruses utilise modified plasmodesmata to bridge the cell wall structure in order to spread to neighbouring cells (recent review see Reichel *et al*., 1999). Bacteria have developed a specific (so-called Type III) secretion system that may directly inject compounds that contribute to pathogenesis into the plant protoplast (Mudgett and Staskawicz, 1998). Bacteria may also use **c**ell **w**all **d**egrading **e**nzyme**s** (CWDEs) to cause rot and therewith destroy plant tissue (Hugouvieux-Cotte-Pattat *et al*., 1996). Although many plant pathogenic fungi produce CWDEs, our knowledge on the actual function(s) of fungal CWDEs in pathogenesis is rather limited. A minority of fungi, like *Cladosporium fulvum*, grows in the apoplastic space without visible effects on the cell wall (Joosten *et al*., 1990b). However, CWDEs may play a role in the majority of fungal infections. Several indications for contributions of fungal CWDEs exist. Firstly, CWDEs are ubiquitously detected in many plant-fungus interactions (Walton, 1994; de Lorenzo *et al*., 1997). Secondly, cell-free fungal CWDE preparations have been reported to mimic symptoms that are observed during infection by the organism from which the CWDEs originate (Urbanek *et al*., 1991; de Lorenzo *et al*., 1997 and references therein). Thirdly, changes in cell wall thickness, morphology, and apparent chemical constitution occur in a number of interactions between plants and fungal pathogens (Mansfield and Richardson, 1981; Benhamou *et al*., 1991; Xu and Mendgen, 1997).

Functional molecular-genetic evidence that fungal CWDEs may play an important role in infection processes was not available in 1994, at the start of the work described in this thesis. All targeted mutations that had been made in fungal genes encoding CWDEs at that time, had yielded strains unaffected in virulence, indicating that those particular CWDEs are not essential (Walton, 1994; de Lorenzo *et al*., 1997). However, only one plant pathogenic fungus had been studied by functional molecular genetics until then. Furthermore, no data on the expression of the genes during the infection process was available. It was anticipated that *Botrytis cinerea* is a good target organism to study the function(s) of fungal CWDEs in plant pathogenesis for a number of reasons. The fungus is renowned for its cell wall degrading capacity since it causes fruit and vegetable rot. The types of symptoms and diseases caused by *B. cinerea* are similar to those caused by *Erwinia* species, which demonstrably require CWDEs for full virulence (Barras *et al*., 1994; Hugouvieux-Cotte-Pattat *et al*., 1996). Furthermore, *B. cinerea* penetrates the epidermis preferably at the anticlinal position (Mansfield and Richardson, 1981), indicating a preference for cell walls. This thesis describes the molecular genetic studies performed on the endo**p**oly**g**alacturonases (endoPGs) which are secreted by *B. cinerea* (BcPGs). For one of the six endoPG-encoding genes isolated, it was shown that it contributes to virulence on a number of host species (Chapter 3). The expression of the complete *Bcpg* gene family is regulated in a sophisticated manner that enables the fungus to efficiently hydrolyse the heterogeneous substrate pectin under various

environmental conditions (Chapters 4 and 5). In this chapter the functions of endoPGs, additional pectinases and other CWDEs involved in pathogenesis of *B. cinerea* are discussed. Finally I will discuss possible implications for research on other plant pathogenic fungi as well as implications for control of gray mould.

The functions of *Botrytis cinerea* endoPGs in the infection process

The biochemical function of an endoPG (poly(1,4-α-D-galacturonide) glycanohydrolase) is to hydrolyse **p**oly**g**alacturonic **a**cid (PGA) (Nasuno and Starr, 1966; Rombouts and Pilnik, 1980). The biological function(s) of endoPGs was (were), however, unknown and was (were) subject of this thesis. As shown in Chapter 3, at least one endoPG of *B. cinerea* contributes to virulence and expression studies performed both in liquid cultures (Chapter 4) and *in planta* (Chapter 5) suggest similar contributions for at least two of the other endoPGs. The exact nature of the contribution of endoPGs and other CWDEs remains to be determined. Four possible functions, acting alone or in combination, should be considered:

(1) CWDEs degrade the plant cell wall thereby facilitating intercellular fungal growth through a physical barrier.
(2) CWDEs degrade the plant cell wall thereby providing the fungus with nutrients.
(3) CWDEs degrade the plant cell wall thereby decreasing its strength resulting in plant cell death.
(4) CWDEs degrade the plant cell wall thereby producing toxins or elicitors of cell death.

These proposed functions might have different biological effects (Ad. 1) Growth facilitation is a pathogenic function and may be affected by elimination of one individual CWDE encoding gene. The mutant fungus will have a reduced intercellular growth capacity and hence be less virulent. This hypothesis predicts that a plant with a thick cell wall would be more resistant to *B. cinerea* than a plant with a thin cell wall of identical chemical structure. (Ad. 2) Nutrient provision is a saprophytic function but has a clear impact for the fungus and may therefore be involved in virulence. A mutant fungus with reduced endoPG activity will have access to less nutrients and hence be less virulent. (Ad. 3 and 4) Both these functions result in cell death and are therefore necrotrophic functions. Pectinase activity, especially from plant pathogenic bacteria, has been shown to induce severe maceration of potato tuber slices accompanied with cell collapse (Quantick *et al*., 1983; de Lorenzo *et al*, 1991 and references therein). Cell death induced by a toxic compound liberated by CWDE activity has been reported in a number of hosts (reviewed in de Lorenzo *et al*., 1997). Targeted mutation of a CWDE might result in a strain that causes less frequent cell death, which would negatively influence the virulence of *B. cinerea*, a renowned necrotroph. Although all proposed functions may contribute to pathogenesis, the different effects that a single mutation can have on virulence

makes it difficult to assess the overall role of CWDEs. Main challenges for future research on endoPGs in *B. cinerea* and other endoPG-producing plant pathogens, will be to evaluate the relevance of the four proposed functions.

A similar situation, as described in the above section, holds for pectinases from *Erwinia* species. Targeted mutations of genes encoding CWDEs in *Erwinia chrysanthemi* resulted in mutants with a reduced macerating activity on potato tuber slices and reduced systemic growth on *Saintpaulia* (Beaulieu *et al*., 1993; Hugouvieux-Cotte-Pattat, *et al*., 1996). Although the latter shows that these bacterial CWDEs are involved in virulence and not in saprophytic ability only, it does not exclude that the CWDEs provide nutrients only. Although at least some of the CWDE mutants in *Erwinia* species are available for over a decade, there is no conclusive evidence on whether the CWDEs facilitate intercellular growth. A different experimental design is required in order to demonstrate this hypothesised function for pectinases and CWDEs in general. *Erwinia spp.* mutants that are unable to import or metabolise mono- or di-galacturonides would be helpful in elucidating the biological function(s) of endoPGs. Aldohexuronate transport in *E. carotovora* is however not specific for galacturonates (Hugouvieux-Cotte-Pattat *et al.,* 1983) and galacturonate degradation mutants of *E. chrysanthemi* can degrade other hexuronates as well (van Gijsegem *et al*., 1985). This strategy seems therefore inappropriate in bacterial pathogens. However, mutants of *Aspergillus nidulans* were obtained that were unable to degrade only D-galacturonate (Uitzetter *et al*., 1986). The availability of a similar mutant in *B. cinerea* will provide a helpful tool in obtaining insight in the relevance of the different biological functions of CWDEs.

Concerted action and functional overlap of CWDEs during infection

CWDEs are believed to act in concert and some CWDEs may show a mutual functional overlap. These two aspects impede the elucidation of the function of CWDEs in the infection by *B. cinerea*. The concerted action of **p**ectin **m**ethyl**e**sterase (PME) and endoPG will obviously contribute more to degradation of the cell wall than the action of the endoPG alone. However, it will be difficult to envisage the effect of concerted action of an endoPG and a glucanase. EndoPG and **p**ectat**e** **l**yase (PeL) act on the same substrate but have different pH activity profiles, providing a means for functional overlap during infection of different tissues but also during different stages of infection. Functional overlap will most probably also occur within the endoPG gene family. The BcPGs are isozymes but this does not mean that the enzymes are identical; they may differ in stability, pH and temperature optimum, substrate specificity, kinetics and end product formed.

Based on the expression studies presented in Chapters 4 and 5 it is suggested that the *Bcpg* gene family provides *B. cinerea* with a set of endoPGs that may be active in a broad spectrum of environmental conditions and is possibly capable of degrading a spectrum of galacturonans present in the plant pectic compound network. E.g. the increase of *Bcpg*3

transcript level at low pH might reflect preferential activity of the BcPG3 isozyme at low pH. In the entomopathogenic fungus *Metarhizium anisopliae*, the pH induced regulation of expression of a range of genes encoding cuticle degrading enzymes correlates with the pH optima of these enzymes (St. Leger *et al*., 1998). An exo**p**oly**g**alacturonase (exoPG) from *Aspergillus tubingensis* was shown to be active on xylogalacturonan (Kester *et al*., 1999). Apparently, substitution of the terminal galacturonic acid by xylose does not prohibit enzyme activity. Similar situations may occur for endoPGs. The availability of different genes that encode isozymes with either different substrate specificities or intermediate degradation products, as was found in *Aspergillus niger* (Benen *et al*., 1999), might facilitate breakdown of the chemically heterogeneous pectin molecules present in the plant cell wall. Therefore, it can be envisaged that certain endoPGs can degrade galacturonans other than homogalacturonan. The induction of *Bcpg*5 by an unknown factor present in a pectin preparation obtained from apple fruit, might reflect the need for adapting the expression of the gene family to the presence of certain types of galacturonans. The inducing compound is not necessarily a pectin, but is presumably part of the pectic compound network. Arabinose is a non pectic component of the pectic compound network (see also Chapter 1) and is an inducer of the expression of an endoPG in *Colletotrichum lindemuthianum* (Hugouvieux *et al*., 1997). Similar mechanisms of gene expression for other types of CWDEs have been reported in *A. niger* (van Peij *et al*., 1998a). In *Aspergillus aculeatus* expression of a rhamnogalacturonan hydrolase gene (*Rhg*A) in a multicopy transformant occurs when the fungus is grown on either pectin or on the combination of galacturonic acid and rhamnose, the components of the rhamnogalacturonan I backbone. Expression of the *Rhg*A gene is not found on either monogalacturonic acid or rhamnose alone (Suykerbuyk *et al*., 1996).

One of the characteristics of a depolymerising enzyme is the ratio in which end-product or intermediates are formed (Robyt and French, 1970; Benen *et al*., 1999). Some endoPGs produce high amounts of monogalacturonic acid (GA) on PGA as substrate whereas others mainly produce **o**ligo**g**alacturonic **a**cid (OGA). The endoPGs of the first class are referred to as processive enzymes since after the initial hydrolysis, the substrate remains bound to the enzyme and additional hydrolysis reactions may be catalysed upon repositioning of the substrate. The latter hydrolysis results in the release of GA (Benen *et al*., 1999). The difference in catalytic characteristics of endoPG enzymes may have implications for the exact function of those endoPGs and may therefore have impact on virulence. Production of high amounts of GA will have a strong impact on the nutrient availability and therefore on the growth ability of the fungus. Breakdown of PGA into mainly OGAs seems to have a strong impact on the loss of coherence of the pectic compound network and hence, may be either more efficient in growth facilitation or more devastating to the mechanical strength of the cell wall. It can be envisaged that the function of BcPG1 is to decrease coherence of the cell wall whereas BcPG4 and 6 are involved in the breakdown of PGA, and possibly also OGA, into GA. This would predict that BcPG4 and 6, rather than BcPG1, are processive. Determination of the biochemical properties of the different BcPGs, their substrate preference, their released

products and their kinetic characteristics will be informative since the emerging data may be useful to make predictions about the exact function of individual BcPGs in the infection process. The information may reveal proposed concerted actions as well as functional overlap of the endoPGs from *B. cinerea*.

The contribution of CWDEs other than BcPG1 to virulence of *Botrytis cinerea*

Following our demonstration that BcPG1 contributes to virulence, it is conceivable that other CWDEs also play a role in the infection process of *B. cinerea*. Firstly, all members of the endoPG gene family are candidate virulence factors although most probably not under all circumstances since the expression patterns that we observed indicate functional overlap. Secondly, other pectolytic enzymes like pectin lyase, rhamnogalacturonan hydrolase and pectin methylesterase may act in concert with endoPGs in the degradation of the pectic compound network. Thirdly, non-pectolytic CWDEs, including proteases, could play an important role in the breakdown of other cell wall components. This section discusses the possible roles that the different CWDEs may play in the degradation of the plant cell wall and in the pathogenesis of *B. cinerea*.

It should first be emphasised that although there is no strict correlation between the level of expression of a gene and its functionality, the expression of a gene is at least a prerequisite for its involvement in pathogenesis. An extensive set of data has been collected on the expression of the *Bcpg* gene family. These data allow predictions on their possible contributions to saprophytic as well as parasitic fitness. Of all endoPG-encoding genes from *B. cinerea* that have not yet been mutated, *Bcpg*2 is the most likely candidate to encode a virulence factor. The transient expression of this gene during the infection of tomato leaves suggests a function early in pathogenesis. However, since the regulation of the expression of this gene remains unclear, predictions about the effect of a targeted mutation of this gene on the infection of other hosts can not be made. *Bcpg*3 is a likely candidate to encode a virulence factor, based on the expression data (Chapter 4 and 5). *Bcpg*3 is expressed at low pH in host tissues such as apple fruit. Furthermore, *B. cinerea* secretes acids during growth in liquid medium and *in planta* (Verhoeff *et al*., 1988; Germeier *et al*., 1994), which might result in the acidification and subsequent onset of *Bcpg*3 gene expression. A targeted *Bcpg*3 null mutant may therefore show a reduced virulence on a variety of hosts. It is less likely that *Bcpg*5 is an important virulence factor since the expression of this gene is low, as compared to the other members of the *Bcpg* gene family (see Chapters 4 and 5). A high enzyme activity could in theory compensate for low gene expression levels, but iso-electric focusing zymograms indicated only a weak activity for the acidic endoPGs, including BcPG5 (van der Cruyssen *et al*., 1994; ten Have, unpublished). Since *B. cinerea* has many different hosts, it

cannot be excluded that this gene plays a more important role during pathogenesis on other host plants than those currently tested.

For *Bcpg*4 and *Bcpg*6 we can make predictions for their possible functions, based on the regulation of their expression. *Bcpg*6 mRNA is induced in the presence of a high concentration of GA. The BcPG6 isozyme may contribute to the degradation of heavily infected tissue in the inner zone of a lesion. A targeted mutation in the *Bcpg*6 gene may therefore result in a mutant that is impeded in the complete consumption of the plant tissue, a mutant with reduced saprophytic ability. The demonstration of a reduced saprophytic ability and the effect thereof in parasitic ability, would require a different experimental set up than we have used thus far. GA can also induce *Bcpg*4 expression but the glucose repression of *Bcpg*4 (shown in Chapter 4) will possibly down regulate this transcript in a number of infections. Preventing excessive secretion of BcPG4 may be functional in maintaining a balance between production of monosaccharides and/or oligosaccharides by enzymatic degradation of cell wall polymers and the consumption thereof. The release of more monosaccharides than can be taken up by *B. cinerea* itself could be advantageous to certain competitors like true saprophytes.

Several *B. cinerea* genes encoding other pectolytic enzymes have been cloned and partially characterised. A rhamnogalacturonan hydrolase gene is expressed on apple pectin as well as on a rhamnogalacturonan I substrate and seems to be induced during carbon starvation (Chen *et al*., 1997). The authors hypothesise that this gene acts as a virulence factor since it can degrade the hairy regions of the pectin, thereby physically disturbing the pectic compound network. Unfortunately, the expression pattern of this gene *in planta* as well as its targeted mutation have not yet been reported. Sequences that encode a **p**ecti**n** **l**yase (PnL) (Mulder, unpublished), a pectate lyase (PeL) (Wubben, unpublished) and a PME (Prins, unpublished) have been cloned from *B. cinerea*. Both PnL and PME activities have been detected early in infection of carrot slices (Movahedi and Heale, 1990) but there are no reports on PeL activity in *B. cinerea.* Possibly the activity of the PeL gene product from *B. cinerea* is hard to detect. Both PnL and PeL enzymes have, in general, a high pH optimum and may show functional overlap with endoPG and PME activities during pathogenesis. This implies that these enzymes may function before the fungus has acidified the invaded plant tissue (i.e. early in pathogenesis), or after the acids have been neutralised (i.e. late in pathogenesis). In addition, PnL and PeL may function on certain hosts only, or only on certain organs and tissues within a host.

B. cinerea secretes a broad set of non-pectolytic CWDEs. Cellulose is degraded by a β-1,4-endoglucanase and a cellobiohydrolase. Verhoeff and co-workers reported cellulolytic activities in *B. cinerea* culture filtrates as well as in infected plants (Verhoeff *et al.*, 1983). Others have reported a cellulose-induced enzymatic complex in *B. cinerea* comprising 1,4-β-endoglucanase, cellobiohydrolase and β-glucosidase activity (Touzani and Doneche, 1996). Genes that may encode cellulolytic enzymes have been identified in an expression sequence tag (EST) library that was made with RNA isolated from a nitrogen starved culture of *B.*

cinerea (WWW-1). In order to evaluate whether one or more glucanases contribute to virulence, expression studies and functional molecular analysis with targeted mutants should be performed preferably on hosts that contain a high proportion of glucans in their cell wall.

The last class(es) of enzymes that may be involved in the degradation of cell walls are enzymes that degrade linkages between major cell wall components, e.g. proteases and feruloyl esterases. A role was reported for an aspartic protease of *B. cinerea* in the infection of carrot slices Movahedi and Heale, 1990) and it can be envisaged that proteases in general may function in the degradation of the cell wall. Feruloyl esterases are so far mainly described for saprophytes (de Vries *et al*., 1997; Donaghy and McKay, 1997). These enzymes act on feruloyl esters that are present in lignin networks, but also in the linkage between lignin, the pectic compound network and xylans (de Vries and Visser, 1999). It is believed that these crosslinks provide rigidity to the cell wall (see also Chapter 1; Ishii, 1997). Penetration of onion epidermis by *Botrytis allii* results in an increase in feruloyl-3'-methoxytyramine (McClusky *et al*., 1999), suggesting that enforcement of the cell wall by feruloyl esters is part of the defence of this host.

Botrytis cinerea endoPGs and polygalacturonase inhibiting proteins

In the last decade **p**oly**g**alacturonase-**i**nhibiting **p**roteins (PGIPs) have attracted attention since they might confer disease resistance to plants. Their inhibiting activity is believed to result in an increase of oligogalacturonides of a particular degree of polymerisation, that are capable of inducing plant defence responses because of their specific length. Incubation *in vitro* of homogalacturonan with endoPG from either *Fusarium moniliforme* or *A. niger*, in the presence of a PGIP from *Phaseolus vulgaris,* resulted in accumulation of OGAs with elicitor activity in soybean (Cervone *et al*., 1989). PGIPs have a domain that shows homology to the leucine-rich repeat domain present in the majority of plant resistance gene products, which made de Lorenzo and co-workers suggest that PGIP plays a role in defence signalling (de Lorenzo *et al*., 1997). There is, however, scarce evidence for the model in which PGIP acts as a component of the plant's signalling system. Crude endoPG preparations from *B. cinerea* have been incubated with PGIP from pear and inhibition of total endoPG activity was confirmed. However, neither an increase in OGA nor a change in elicitor activity was found (Sharrock and Labavitch, 1994). Recently, seven fungal endoPGs were tested *in vitro* with four PGIPs. Although there were clear differences in endoPG inhibiting activities of the PGIPs, not one of the endoPG-PGIP combinations did result in an increase of OGA with a specific length (Cook *et al.*, 1999). The reported function of *P. vulgaris* PGIP in generating elicitor-active OGAs is therefore probably not a general feature of PGIPs. All reports however, indicate that PGIPs can inhibit at least some endoPGs. PGIP from pear fruit is able to inhibit the BcPG1 isozyme (ten Have, unpublished) and transgenic tomato plants expressing this pear PGIP have an increased, yet incomplete, resistance against *B. cinerea* (Powell *et al*.,

2000). Even when only one endoPG, BcPG1, is inhibited, the effect of the inhibition could reduce fruit rot to an extent that is significant for improving product quality management in the post harvest chain. Recently it was shown that introducing a specific point mutation in PGIP I from *P. vulgaris* extended the endoPG inhibitory range of this protein (Leckie *et al*., 1999). A combination of a search in the natural gene pool and mutational analysis of the encoding genes could provide new strategies in crop protection. Similar strategies can be envisaged for the functional inhibition of other CWDEs. Besides PGIPs, inhibitors of fungal PnL (Bugbee, 1993) and fungal proteinase (Lorito *et al*., 1994) have been reported in plant tissues.

The role of CWDEs in plant pathogenesis: A matter of fungal lifestyle?

CWDEs have been regarded as virulence or pathogenicity factors for a long time. The application of functional molecular-genetics has not provided as much evidence for a role of CWDEs in fungal diseases as has been reported in bacterial diseases. The data provided at the start of the work presented in this thesis showed that fungal CWDEs do not play a significant role in pathogenesis. Targeted mutation, initially performed in one fungus and later in two other fungi, did not result in any detectable loss of virulence. In my opinion, however, CWDEs might be involved in pathogenesis, although possibly for certain types of fungal pathogens only. The two fungi in which endoPGs demonstrably contribute to virulence are related. *B. cinerea* is a pathogen with weak saprophytic abilities whereas *Aspergillus flavus* is predominantly a saprophyte with weak pathogenic abilities. This raises the question whether the lifestyle of a fungus determines the functionality of CWDEs or whether the function(s) of CWDEs predisposes the lifestyle of a fungus. It has been reported that certain mycorrhizal ericoid fungi show a low polygalacturonase activity when grown on artificial media and in their host. However, when grown saprophytically on non-host plants this activity is much higher (de Lorenzo *et al*., 1997), suggesting that saprophytic growth *in planta* is the result of high CWDE production or that high CWDE production is the result of saprophytic growth *in planta*. This section discusses several aspects of the functionality of CWDEs in a number of plant-microbe interactions as well as directions for further research.

Firstly, I would like to emphasise that, when a targeted mutant shows normal virulence, this does not necessarily mean that the eliminated gene product does not contribute to virulence. As stated earlier, functionality of a gene in pathogenesis at least requires expression during some stage of the infection process. Most reports on targeted mutations of fungal CWDEs lack information on the expression of the gene *in planta* (Schaeffer *et al*., 1994; Bowen *et al.,* 1995; Gao *et al*., 1996; Görlach *et al*., 1998). It might be that the mutated genes have another function than anticipated or that they function in a stage of the infection that was not examined. Therefore pathogenicity tests may need to be refined in order to evaluate the function of a particular CWDE. Later this will be discussed for *Cochliobolus carbonum*.

Secondly, the complexity of the apoplast as well as the broad spectrum of CWDEs that are secreted by fungal pathogens (discussed in Chapter 1) makes it difficult to detect minor contributions of certain CWDEs to virulence. The aspects of concerted action and functional overlap, discussed above, complicate this further. Scott-Craig and co-workers therefore constructed a double mutant of *C. carbonum*, deficient in both an endoPG and an exoPG (Scott-Craig *et al.,* 1998; Scott-Craig, personal communication). This mutant was normally virulent presumably as a consequence of the low proportion of pectins in maize cell walls. However, this result could also be explained by the presence of other, unidentified pectin degrading enzymes. *Erwinia* mutants in multiple pectinase genes appeared to possess additional, unrelated pectinase genes that were expressed *in planta* only (Kelemu and Collmer, 1993).

Thirdly, there is not enough knowledge that justifies any general statement on the role of CWDEs in fungal pathogenesis. Targeted mutations in genes encoding CWDEs have been made in five fungi. This did result in a reduction in virulence for two out of five fungi, *B. cinerea* and *Aspergillus flavus*, respectively. In two out of three fungi for which no effect of a targeted mutation was found, (i.e. *Cryphonectria parasitica* and *Glomerella cingulata*) only one enzyme has been studied so far. Only one out of the five fungi mentioned (i.e. *C. carbonum*) has been investigated thoroughly by targeted mutations of CWDE encoding genes. There are, however, recent indications that the genus *Cochliobolus* occupies a special position among fungal pathogens. The genus consists of weakly pathogenic species as well as species that are highly pathogenic on a very specific host (Turgeon, 1998). There are strong indications for multiple convergent gains of pathogenicity traits in the genus. It is hypothesised that there is a pool of weakly pathogenic *Cochliobolus* isolates containing basal genes for pathogenicity (Turgeon,1998). Gene clusters encoding highly specific virulence factors were acquired by some of these weak pathogens, probably by horizontal gene transfer. *Cochliobolus heterostrophus* obtained a gene encoding a polyketide synthase that causes the production of the T toxin, which enables the fungus to infect T cytoplasm corn (Yang *et al*., 1996). Similarly, *Cochliobolus victoriae* is believed to have acquired a gene cluster that is responsible for the production of victorin while *C. carbonum* is believed to have acquired a gene cluster for the production of HC toxin (Panaccione *et al*., 1992). The CWDEs of *C. carbonum* might therefore be regarded as weak virulence factors that can only modestly contribute to disease. For a better understanding of the role of CWDEs in virulence of *Cochliobolus* species, it would be recommended to perform mutational analysis of CWDE encoding genes in a HC toxin-deficient strain or a weakly virulent relative of *C. carbonum.*

CWDEs are likely to have important functions for pathogenic fungi with a strong saprophytic competitive ability. The functionality of CWDEs in certain real saprophytes like *Aspergilli*, is evident. It can therefore be expected that pathogens with a saprophytic stage, like *B. cinerea*, will depend at least partially on their CWDEs. A number of endoPG-encoding genes has been isolated from *Sclerotinia sclerotiorum* (Reymond *et al*., 1994; Fraissinet-Tachet *et al*., 1995) and targeted mutation of one of these genes may answer the question

whether *B. cinerea* is an exceptional case. Other fungi of interest are *Rhizopus* species, necrotrophs like *Alternaria* species and also hemibiotrophs like *Colletotrichum* species. Also among oomycetes there are candidates for further research. *Pythium* species can cause rot and damping off in which CWDEs may be involved. *Phytophthora infestans* has been shown to secrete a cellulase, a polygalacturonase as well as a PME (Jarvis *et al*., 1981; Bodenmann *et al*., 1985; Förster *et al*., 1988), enzymes that may have a function in the infection of either potato leaves or tubers.

Pathogens without a clear saprophytic stage, such as biotrophic fungi that form haustoria, may also depend on CWDEs. There are sufficient data to corroborate a role for CWDEs in the infection of bean by *Uromyces viciae-fabae*. This fungus forms an appressorium and penetrates through a stoma. Penetration of the stoma is presumably supported by a local softening of the guard cell wall since this process coincides with secretion of acidic cellulases and proteases (Mendgen *et al*., 1996). In a later stage of infection, the secretion of PME and PeL accompanies the onset of host wall penetration by the haustorial mother cell. PeL is only synthesised in the presence of its substrate PGA but, strikingly, addition of PGA to spores and appressoria cannot induce its expression in these infection structures. The authors suggests that the infection process is consists of a highly regulated secretion of pectic enzymes that might be accompanied by shifts in pH that would activate the at that point essential enzyme (Deising, 1995). Another indication for the importance of concerted action is the absence of extensive cell wall maceration in the bean-*U. viciae-fabae* interaction. Plant cell death, inflicted by severe cell wall degradation, as well as the release of increasing amounts of elicitor-active cell wall fragments would interfere with the biotrophic lifestyle of the fungus. This pathosystem has ample possibilities to study functions of CWDEs in a number of processes. Unfortunately, the molecular tools to create targeted mutants in *U. viciae-fabae* are not yet available. Also for other haustoria-forming fungi there are clear indications for the function of CWDEs. Cellobiohydrolase I from *Erysiphe graminis* f. sp. *hordei* is present at the tip of the appressorial germ tube whereas isoform II is present at the tip of the primary germ tube (Pryce-Jones *et al*., 1999). The availability of a transformation system for this fungus (Christiansen *et al*., 1995; Chaure *et al*., 2000) opens the way for functional molecular-genetic studies.

Botrytis cinerea shows an opportunistic way of life *in muro*

B. cinerea has been described as an opportunistic fungus (Elad and Evensen, 1995) as it causes losses in the field (real pathogen) but also in the post harvest chain (weak pathogen). A wide variety of symptoms is caused by *B. cinerea*. It causes rot on fruits, blight on leaves, spot on flowers and damping off on seedlings. As a result, *B. cinerea* is described as a necrotroph (Jarvis *et al.,* 1977), a pectolytic fungus (Wasfy *et al*., 1978; di Lenna *et al*., 1981) a saprophyte, an endophyte (Espinosa-Garcia and Langenheim, 1991), a secondary invader

(Hallet *et al*., 1990) and a post harvest pathogen (Hammer *et al*., 1990; Jarvis, 1994). The term "opportunist" seems therefore justified. I hypothesise that this opportunistic nature is predominantly determined in the second, quiescent phase of the infection rather than in the initial colonisation phase. During quiescence, *B. cinerea* presumably resides dormant and switches to an invasive stage at a particular point during host plant development. I propose that chemical compounds resulting from the degradation of the pectic compound network induce not only the expression of a wide variety of CWDEs but also other pathogenesis-related processes. The concerted action of both plant and fungal pectinases on the plant and the local composition of that cell wall determine the release of these compounds.

Quiescence occurs often during pathogenesis of *B. cinerea*. Ghost spot in tomato fruit (Verhoeff, 1970), post harvest disease in many hosts but also the three-phase infection process on tomato leaves as we have described in Chapter 2, most probably all involve a stage of quiescence. End-rot of fruits like apple and pear (de Kock and Holz, 1992; Berrie, 1994) is probably the most typical example of this phenomenon. Conidia infect the flower during blooming but symptoms remain limited or undetected and the disease stays quiescent. Fruits look healthy after fertilisation, without any detectable fungal growth or expansion (de Kock and Holz, 1992). At a particular point in the fruit ripening process the fungus starts its second attack that results in end-rot. A similar phenomenon occurs during flower senescence. Infection of gerbera flowers is arrested when the flowers are incubated at 20°C whereas at 4°C fungal growth proceeds rapidly (Salinas *et al*., 1989). A survey among cut roses from greenhouses in Israel showed that 50% of the symptomless roses was actually infected by *B. cinerea* (Elad,1988). Storage of cut roses at low temperature often results in increase of disease (Hammer *et al*., 1990). In all these cases, it seems that environmental conditions, rather than host tissues, determine the outcome of the infection.

Ripening as well as senescence is accompanied by changes in the cell wall: e.g. ripening of tomatoes coincides with an increase in non-esterified galacturonan (Koch and Nevins, 1989). It can be envisaged that plant PME activity on pectin results in an increase of PGA, which forms the substrate for the constitutive BcPG1 isozyme. *Bcpg*4 and 6 expression might be induced as a consequence of a release of GA by concerted action of a PME, of either plant or fungal origin, and BcPG1. Fruits of tomato mutants *nor* and *rin*, which have strongly reduced pectinase activity, are more resistant to *B. cinerea* (Barkai-Golan *et al*., 1988; Lavy-Meir *et al*., 1989). The authors suggested that the low accumulation of galacturonate during infection is the result of a reduced or delayed activity of fungal pectinases, probably BcPG4 and 6. In this hypothesis GA would act in a manner similar to homoserine lactones in quorum sensing, a signalling system that regulates the onset of production of pathogenicity factors of a number of bacteria (for review see Fuqua and Greenberg, 1998). In addition to the quantity of galacturonides it can also be envisaged that the exact chemical nature of the monomer is involved. E.g., acetylation, methylation or substitution with sugar residues might have strong impact on gene regulation and therefore on the onset of pathogenesis. Some endoPGs are able to act efficiently on methylated

substrate whereas others are not, as has been described in *A. niger* (Benen *et al.,* 1999*)*. Specific isozyme activity on pectin may release mono- or dimers that serve as inducers for other endoPGs and possibly other virulence factors. The transient expression of *Bcpg*2 during the infection of tomato leaves (see Chapter 5) suggests such a function.

The studies on the expression of fungal (Chapters 4 and 5) and plant (Chapter 3) genes during infection of detached tomato leaves at 20°C confirm the visual observation that the infection process consists of three phases:

(1) Primary lesion formation phase: *B. cinerea* penetrates the epidermis and the plant responds with the formation of a necrotic lesion that restricts the fungus (0-16 hours post inoculation (HPI)).
(2) Quiescent phase: no detectable fungal growth or plant decay can be observed (16-48 HPI).
(3) Lesion expansion phase: the fungus starts to invade surrounding tissue from a small proportion of the primary lesions, subsequently invading the whole leaf (72-120 HPI).

The constant level of fungal actin mRNA in infected tissue during the first 24-72 HPI indicates that there is no increase of fungal biomass, in agreement with quiescence. This is corroborated by the expression patterns of *Bcpg*1 (Chapters 3, 4 and 5). Subsequently, quiescence is broken in a proportion of lesions only. The disease expands from a number of primary lesions, which is accompanied by an increase in fungal transcripts. Similar observations have been made by others during infection of leaves (Mansfield and Richardson, 1981; Salinas *et al*., 1989; Hammer *et al*., 1990; de Meyer and Höfte, 1997;). The frequency at which lesions expand on leaves is used as a measure for virulence on tomato (Chapter 3), broad bean (V*icia faba*) (ten Have, unpublished), common bean (*Phaseolus vulgaris)* (Schouten, unpublished) and grapevine (*Vitis vinifera*) (Schoonbeek, unpublished). Quiescence is also observed during infection of *Arabidopsis thaliana* leaves. Upon inoculation only a few symptoms occur for up to ten days. Then, a proportion of the plants is completely colonised and killed by the fungus within one or two days (van Kan, unpublished; Thomma *et al*., 1998). Although the typical three phase infection process observed in these artificial leaf infections can be explained by different theories, it might be due to the presence of a quiescent phase. It may be needed to accumulate a signal derived from the plant cell wall above a certain threshold level. The reaching of the threshold may initiate the transition from the quiescent phase to the expansion phase.

It might seem farfetched that there is induction of pathogenic abilities by a pectic substrate but this might correspond with the lifestyle of *B. cinerea*. There are indications that compounds that are only indirectly related with a gene product can regulate the expression of the encoding gene. Arabinose and rhamnose, pectic compound network components (see Chapter 1), can induce the expression of an endoPG in *C. lindemuthianum*. Furthermore, in *A. niger* it has been shown that xylose, released from xylan by a constitutive xylanase, can

induce hemicellulases (Gielkens, *et al*., 1998; van Peij *et al*., 1998a; Gielkens *et al*., 1999) and galactosidases (de Vries *et al*., 1999). This suggests that xylose regulates the degradation of glucans in general rather than xylan only. *B. cinerea* is able to infect larch and black spruce trees (Dugan and Blake, 1989; Zhang *et al*., 1994). However, this has only been reported for seedlings and young plants, in which the plant cell wall consists mostly of pectins. No disease has been reported in mature trees, in which the cell walls consist mostly of celluloses. In conclusion, I hypothesise that *B. cinerea* is a pathogen that depends on pectins, for the induction of its pathogenic abilities and the acquisition of nutrients.

Concluding remarks

New insights in the role of CWDEs in pathogenesis of *B. cinerea* have been obtained by the work described in this thesis. The assumption that CWDEs are entirely redundant during infection by pathogenic fungi can now be refuted. Our results have made clear that generalised statements can not be made for all plant-fungus interactions. Each plant-fungus interaction and the function(s) of each individual CWDE in this interaction needs to be studied independently, taking functional overlap and concerted action into consideration. Conclusions on the involvement of fungal CWDEs in plant pathogenesis require detailed studies on three aspects of the interaction:

1 The plant cell wall: its spatial, developmental and cell-type specific composition; the amount of cross-linking before and after attempted invasions by pathogens.
2 The fungal CWDEs: the regulation of gene expression and enzyme activity (both in time and space) by substrates, reaction products and environmental factors; the biochemical characteristics of isozymes.
3 The interaction between the plant cell wall and CWDEs: the chemical nature of products that can be released from a plant cell wall as a consequence of CWDE action; the effect of these cell wall fragments on the induction of defence responses and on fungal gene expression.

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Summary

Cell **w**all **d**egrading **e**nzyme**s** (CWDEs) secreted by microbial plant pathogens have been suggested to function as virulence factors. Evidence that particular bacterial CWDEs contribute to virulence has emerged in the last two decades. Targeted gene replacement of different genes encoding CWDEs resulted in mutants with reduced virulence on a number of host plants. Similar molecular genetic approaches in plant pathogenic fungi have, until recently, been unsuccessful in elucidating a role for fungal CWDEs in pathogenesis. This thesis describes molecular genetic analyses of CWDEs secreted by the necrotrophic plant pathogenic fungus *Botrytis cinerea*, the causal agent of gray mould.

From literature it was known that *B. cinerea* secretes many CWDEs when grown in liquid culture. The number of CWDE encoding genes present in the *B. cinerea* genome was unknown and detailed expression studies were lacking. In order to fill this knowledge gap we used the following strategy:

(1) Cloning of genes encoding CWDEs
(2) Study of the expression of CWDE genes both in liquid cultures and *in planta*
(3) Targeted deletion of CWDE genes that have expression patterns that indicate a function in the infection process

Chapter 1 introduces the research area and gives an outline of the thesis. It describes a model of the chemical and structural composition of the plant cell wall and reviews various classes of microbial CWDEs. It summarises previously published data on the role of bacterial and fungal CWDEs in pathogenesis in general and on the CWDEs secreted by *B. cinerea* in particular. *B. cinerea* has a wide host range but prefers hosts that contain high amounts of pectin. Therefore the focus was on endo**p**oly**g**alacturonases (endoPGs), enzymes that cleave homogalacturonan, a major constituent of pectin.

In order to study gene expression of *B. cinerea in planta*, it was essential to develop a standardised inoculation procedure that enables reproducible infections both in time and space. The development of this inoculation procedure for tomato leaves is described in Chapter 2. The expression of two fungal genes and a number of plant PR-protein genes was investigated in time course experiments performed at two different incubation temperatures.

Subsequently, we set out to clone the genes of interest, analysed their expression and studied the effect in pathogenesis by targeted gene replacement. The genes were isolated by hybridisation with heterologous probes. The first gene that was cloned and characterised, *Bcpg*1, is constitutively expressed. Targeted replacement of this gene resulted in a mutant with reduced virulence on apple fruits and tomato (Chapter 3). Subsequently, five

additional endoPG genes were isolated (Chapter 4). The gene products were compared with other fungal endoPGs and it was shown that the members of the *B. cinerea Bcpg* gene family fall into at least three distinct monophyletic groups (Chapter 4).

The members of the endoPG gene family, denoted as *Bcpg*1-6, are differentially expressed in liquid cultures that differed in carbon source or pH (Chapters 4). The constitutive expression pattern of *Bcpg*1, as found in Chapter 3, was further confirmed. *Bcpg*2 is expressed under all circumstances tested except when *B. cinerea* is grown in glucose-containing medium at low pH. *Bcpg*3 is expressed at low ambient pH. *Bcpg*4 is induced by the pectin breakdown end-product galacturonic acid, and is repressed by glucose. *Bcpg*5 expression can be induced by a yet unknown factor present in apple pectin. *Bcpg*6 is, like *Bcpg*4, induced by galacturonic acid but is, unlike *Bcpg*4, not repressed by glucose. The expression of the endoPG gene family enables the fungus to degrade pectate in a flexible manner. It enables the fungus to respond to environmental signals like nutrient availability and pH.

The expression of the endoPG gene family during infection of tomato leaf, broad bean leaf, apple fruit and courgette fruit was studied (Chapter 5). Expression of the genes *in planta* is differential and most expression patterns can be explained by the results of expression studies in liquid cultures. *Bcpg*1 is expressed in all host tissues tested, whereas expression of *Bcpg*2 is evident in tomato, broad bean and courgette. *Bcpg*3 and *Bcpg*5 are expressed in apple fruit. *Bcpg*4 and *Bcpg*6 are expressed in all host tissues tested.

Chapter 6 discusses the results in a broader context. It is hypothesised that, besides *Bcpg*1, additional members of the *Bcpg* gene family contribute to virulence, albeit likely under specific circumstances. It is suggested that fungal CWDEs can play a role in plant pathogenesis but that this role also strongly depends on the lifestyle of the fungus. It is postulated that *B. cinerea* depends strongly on endoPGs for successful infection. The research described in this thesis may lead to novel disease control strategies that rely on **P**oly**G**alacturonase **I**nhibiting **P**rotein (PGIP) expression in transgenic host plants.

Samenvatting

Van celwandafbrekende enzymen (CWDEs), uitgescheiden door microbiële plantenpathogenen, wordt verondersteld dat ze een rol spelen als virulentiefactor. De afgelopen twintig jaar is aangetoond dat bepaalde bacteriële CWDEs inderdaad een rol spelen in de virulentie. Het gericht uitschakelen van een aantal genen die voor een CWDE coderen, resulteerde in mutanten met een verminderde virulentie op een aantal waardplanten. Middels dergelijke moleculair-genetische benaderingen kon voor plantenpathogene schimmels tot voor kort geen rol voor schimmel-CWDEs in virulentie aangetoond worden. Dit proefschrift beschrijft een moleculair genetische analyse van CWDEs die uitgescheiden worden door de necrotrofe plantenpathogene schimmel *Botrytis cinerea*, ook bekend als grauwe schimmel.

Het was reeds bekend uit literatuur dat *B. cinerea* een groot aantal CWDEs uitscheidt wanneer hij in vloeistof media wordt gekweekt. Het was echter nog onbekend hoeveel CWDE coderende genen in het *B. cinerea* genoom aanwezig zijn, en welk isozym wordt gecodeerd door welk gen. Bovendien waren nog geen gedetailleerde expressie studies uitgevoerd. Om dit hiaat in kennis op te vullen werd de volgende strategie in het onderzoek gevolgd:

(1) Klonering van de *B. cinerea* genen coderend voor CWDEs.
(2) Expressie-analyse van de CWDE genen, zowel in vloeistofcultures als *in planta*.
(3) Gerichte deletie van genen met expressiepatronen, die een functie in het infectieproces doen vermoeden.

Hoofdstuk 1 geeft een inleiding in het onderzoeksgebied en eindigt met een korte beschrijving van de opzet van het proefschrift. De inleiding beschrijft een model van de chemische en de structurele samenstelling van de celwand van een plant en geeft een overzicht van de verschillende klassen van microbiële CWDEs. Het geeft een samenvatting van reeds gepubliceerde resultaten aangaande de rol van bacteriële en schimmel CWDEs in de pathogenese alsmede in het algemeen en in het bijzonder de CWDEs die door *B. cinerea* worden uitgescheiden. *B. cinerea* heeft een groot gastheerbereik maar lijkt een voorkeur te hebben voor gastheren met een hoog pectinegehalte. Om die reden richtte het onderzoek zich op endopolygalacturonases (endoPGs), enzymen die homogalacturonanen, een belangrijke fractie van pectine, afbreken.

Voor het uitvoeren van expressie studies in *B. cinerea in planta*, moest eerst een goed gestandaardiseerde inoculatie procedure worden ontwikkeld. De ontwikkeling van deze inoculatie procedure, die een reproduceerbare infectie bewerkstelligt in zowel tijd als ruimte,

staat beschreven in Hoofdstuk 2. De expressie van schimmelgenen en een groep plantengenen coderende voor PR-eiwitten is onderzocht in een tijdreeks bij twee verschillende incubatietemperaturen.

We begonnen vervolgens met het kloneren van relevante genen, analyseerden hun expressie en bestudeerden hun functie in de pathogenese via gerichte deletie. Genen werden geïsoleerd middels hybridisatie met heterologe probes. Het eerste gen dat werd gekloneerd en gekarakteriseerd, *Bcpg*1, komt constitutief tot expressie. Uitschakeling van dit gen door gerichte deletie resulteerde in een mutant met een verminderde virulentie op appel en tomaat (Hoofdstuk 3). Vervolgens werden vijf additionele genen geïsoleerd (Hoofstuk 4). De producten van deze genen zijn vergeleken met andere schimmel endoPGs en er werd aangetoond dat de *B. cinerea* endoPG genen tot tenminste drie monofyletische groepen behoren.

De leden van de genfamilie, aangeduid met *Bcpg*1-6, komen differentieel tot expressie (Hoofdstuk 4). Het constitutieve expressiepatroon van *Bcpg*1 werd bevestigd. *Bcpg*2 komt tot expressie onder alle omstandigheden, behalve wanneer *B. cinerea* groeit op glucose medium met een lage pH. *Bcpg*3 komt tot expressie bij een lage pH. *Bcpg*4 expressie wordt geïnduceerd door het pectine-afbraakproduct galacturonzuur en wordt gerepresseerd door glucose. *Bcpg*5 expressie kan worden geïnduceerd door een nog onbekende factor die aanwezig is in appelpectine. *Bcpg*6 wordt, net als *Bcpg*4, geïnduceerd door galacturonzuur maar wordt, in tegenstelling tot *Bcpg*4, niet gerepresseerd door glucose. De differentiële expressie van de endoPG genfamilie stelt de schimmel in staat te reageren op omgevingssignalen, zoals de aanwezigheid van nutriënten en de pH.

De expressie van de endoPG-genfamilie is bestudeerd tijdens infectie van tomatenblad, tuinboonblad, appel en courgette (vruchten) (Hoofdstuk 5). De expressie van de genen *in planta* is differentieel en de meeste expressie-patronen kunnen worden verklaard met de resultaten van de expressiestudies in vloeistofcultures. *Bcpg*1 komt tot expressie in alle gastheerweefsels die getest zijn, terwijl *Bcpg*2 gedetecteerd wordt in tomaat, tuinboon en courgette. *Bcpg*3 en *Bcpg*5 komen tot expressie in appels. *Bcpg*4 en *Bcpg*6 komen tot expressie in alle gastheerweefsels die zijn getest.

Hoofstuk 6 bespreekt de resultaten in een bredere context. De hypothese is dat, naast *Bcpg*1, ook andere leden van de *Bcpg*-genfamilie een bijdrage leveren aan virulentie, zij het vermoedelijk alleen onder specifieke omstandigheden. Er wordt gesuggereerd dat schimmel-CWDEs een rol kunnen spelen in de pathogenese, maar dat het effect van deze enzymen sterk afhangt van de levensstijl van de schimmel. Er wordt gepostuleerd dat *B. cinerea* voor het bewerkstelligen van een geslaagde infectie sterk afhankelijk is van endoPGs. De verkregen inzichten in de infectie van *B. cinerea*, op grond van het onderzoek beschreven in dit proefschrift, bieden mogelijkheden voor nieuwe gewasbeschermingsstrategieën, gebaseerd op **P**oly**G**alacturonase **I**nhibiting **P**rotein (PGIP) expressie in transgenen planten.

Nawoord

Voor u ligt het resultaat van vier jaar werken en anderhalf jaar zwoegen. Vrijwel altijd heb ik met plezier gewerkt al waren er uiteraard ook zware momenten. Er was een goed gevoel toen de eerste mutant meteen raak bleek. Zware momenten waren er toen bleek dat na het screenen van 200 transformanten er niet één goed bleek en toen bleek dat die tweede “knock-out" mutant niets meer was dan een Fata morgana.

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Curriculum vitae

Arjen ten Have werd geboren op 13 juni, 1967 te Deurne. Hij behaalde zijn VWO diploma in 1986 aan het Rijksatheneum te Helmond. In hetzelfde jaar begon hij zijn studie Moleculaire Wetenschappen aan de toenmalige Landbouwuniversiteit Wageningen. Hij studeerde af met de vakken Moleculaire Biologie, waarin hij werkte aan Cowpea Mosaic Virus (Ir. Jan Verver en Dr. Ir. Joan Wellink), Moleculaire Genetica (vakgroep Erfelijkheidsleer, Drs. P. van den Broek) alwaar hij in contact kwam met de schimmelgenetica van *Aspergillus nidulans* en een derde afstudeervak, Toegepaste Filosofie (Drs. H. van den Belt). Na zijn afstuderen in 1992 voldeed Arjen ten Have zijn vervangende dienstplicht op het ATO alwaar hij bij Dr. Ernst Woltering werkte aan de fysiologische en moleculair biologische analyse van de verwelking van anjers (*Dianthus caryophyllus*). De 16 maanden dienstplicht werden gevolgd door een periode van een half jaar waarin Arjen ten Have zijn onderzoek aan de verwelking van anjer vervolgde. Oktober 1994 startte hij als OIO bij de vakgroep Fytopathologie (Dr. Jan van Kan) in een samenwerkingsproject met de Sectie Moleculaire Genetica van Industriële Micro-organismen (Dr. J Visser), gefinancierd door de Stichting Technische Wetenschappen (STW). Dit werk heeft geleid tot dit proefschrift, getiteld: “The *Botrytis cinerea* endopolygalacturonase gene family.” Sinds februari 1999 is Arjen werkzaam als post-doc bij de Leerstoelgroep Fytopathologie van de Wageningen Universiteit alwaar hij, gefinancierd door wederom STW, wederom onderzoek verricht aan *B. cinerea*, ditmaal in een samenwerking met de leerstoelgroep Plantenveredeling (Dr. P. Lindhout).