The role of pectin degradation in pathogenesis of *Botrytis cinerea*

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool Experimental Plant Sciences.

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M.J. Kropff,
in het openbaar te verdedigen
op vrijdag 21 september 2007
des morgens te 11.00 uur in de Aula.

Title The role of pectin degradation in pathogenesis of *Botrytis cinerea*
Author Ilona Kars
Thesis Wageningen University, Wageningen, NL (2007)
with references, with summaries in Dutch and English

ISBN 978-90-8504-707-0

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Chapter 1

General introduction

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This chapter is published as: Kars, I., and van Kan, J.A.L. (2004) Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In *Botrytis*: Biology, Pathology and Control (Elad, Y., Williamson, B., Tudzynski, P. and Nelen, N., eds). Dordrecht: Kluwer Academic Publishers, pp. 99-118.

Abstract

The infection of host plants by *Botrytis* spp. is mediated by numerous extracellular enzymes and metabolites. Each of these compounds may play a role in different stages of the infection process. Cutinases, lipases and some cell wall-degrading enzymes may facilitate the penetration of the host surface, while toxins, oxalate and reactive oxygen species may contribute to killing of the host cells. Several cell wall-degrading enzymes contribute to the conversion of host tissue into fungal biomass, but also other enzymes, such as laccases and proteases are potentially involved in pathogenesis. The cloning of the corresponding genes in recent years has facilitated studies on gene expression and targeted mutagenesis. This chapter gives an updated overview of the research performed on these secreted enzymes and metabolites and the role they play in pathogenesis.

Introduction

Botrytis cinerea is able to infect a wide spectrum of host plant species, whereas other *Botrytis* species are confined to a single host species. All *Botrytis* species, whether specific or not, are necrotrophs implying they are able to kill host cells during the infection process. De Bary (1886) observed that carrot cells were killed in advance of invading hyphae of the soft rot fungus *Sclerotinia*. He also noted that fluid from rotten tissue could degrade healthy host tissue, while boiled fluid had no effect. This led to his conclusion that the fungus produced heat-labile enzymes and toxins that kill and degrade plant cells. The same is true for *Botrytis* species. They are equipped with a set of enzymes and/or metabolites that enable the pathogen to invade host tissue, kill host cells and eventually convert host tissue into fungal biomass. Many of these enzymes and metabolites act extracellularly at the plant-fungus interface, or even in the host tissue at some distance from the growing hyphae. This chapter will deal with pathogenicity factors, i.e. effector molecules that cause damage to the host thereby enabling the pathogen to complete its disease and life cycle. The emphasis is placed on fungal extracellular enzymes, but we will also discuss the biosynthetic pathways of metabolites secreted during pathogenesis. This chapter discusses neither differentiation of infection structures nor the production of phytohormones. Most data originate from the research on *B. cinerea*, but we will also discuss other *Botrytis* species where relevant information is available.

Penetration of the host surface

The disease cycle starts with a conidium landing on the host surface. Upon attachment, it germinates on the host surface and produces a germtube that develops into an appressorium that facilitates penetration of the host surface. Invasion of host tissue can be achieved by active penetration or passive ingress. *B. cinerea* is an opportunist that can initiate infection at wound sites, or at sites previously infected by other pathogens. Nevertheless, *Botrytis* spp. are perfectly able to penetrate intact host surfaces. Only direct, active penetration of the epidermal surface is discussed in this section. For reasons of simplicity the penetration of dead or wounded tissue is regarded as an expansion process rather than a penetration process and is dealt with in later sections.

The first barrier to breach is the host cuticle covering all aerial parts of the plant. The cuticle consists of cutin, a polyester of hydroxylated and epoxidised C_{16}- and C_{18}-fatty acids, in many cases covered with a hydrophobic wax layer consisting of fatty alcohols. Physical damage or brute mechanical penetration of the cuticle by *B. cinerea* is not usually observed (Cole et al., 1996; Williamson et al., 1995) indicating that enzymatic activity is involved in penetrating intact host surfaces (Salinas and Verhoeff, 1995).

The role of lipase in wax layer penetration and surface adhesion

The wax layer does not seem to pose a serious barrier, although removal of the wax layer by abrasion was reported to increase the infection incidence (Sutton et al., 1984). No correlation was observed between the wax layer dry weight of rose or gerbera petals and their susceptibility to *B. cinerea* (Kerssies and Frinking, 1996).

It is conceivable that *B. cinerea* produces surfactants: proteins or metabolites that reduce surface hydrophobicity and "dissolve" the wax layer, thereby providing access to the underlying cutin polymer. The polysaccharide cinerean, covering *B. cinerea* germ tubes might fulfill a role as surfactant. Alternatively, the reduction of host surface tension may be achieved enzymatically. Cutinases, serine esterases, lipases and other non-specified esterases are reported to be involved in the adhesion of several plant pathogenic fungi: *Alternaria brassicola* (Berto et al., 1997; Fan and Köller, 1998; Köller et al., 1995; Yao and Köller, 1995), *Colletotrichum graminicola* (Pascholati et al., 1993), *Erysiphe graminis* (Pascholati et al., 1992), *Uromyces viciae-fabae* (Deising et al., 1992; Clement et al., 1993a, b).

B. cinerea produces an extracellular triacylglycerol lipase, with a molecular mass of 60 kDa during culture in the presence of a fatty acid ester. The enzyme is able to hydrolyse unsaturated long chain fatty acid esters (Comménil et al., 1995), known to be components of cutin and waxes. Lipase production *in vitro* was induced by wax esters and free fatty acids (Comménil et al., 1999). The lipase possesses cutinolytic activity, although its kinetic properties (Comménil et al*.,* 1998) are clearly distinct from those of a 'typical' cutinase that will be discussed below. It was proposed that the enzyme plays a role in modifying the waxes and cuticle, and in adhesion of conidia to the plant surface (Comménil et al., 1997, 1998). Studies with polyclonal antibodies blocking the active site suggested that the lipase plays an important role in the infection process. When antibodies were applied on to intact tomato leaves prior to inoculation with *B. cinerea* conidia, the fungal germ tubes were unable to penetrate the cuticle (Comménil et al*.,* 1998). The antibodies did not affect germination of *B. cinerea* conidia nor did they inhibit the infection of wounded tissue, suggesting a role for the lipase specifically during host surface penetration. These antibodies also inhibited a lipase purified from *Alternaria brassicicola* and they were able to reduce by 90% the occurrence on intact cauliflower leaves of blackspot lesions caused by *A. brassicicola*. The antibodies did not prevent *A. brassicicola* infection on dewaxed cauliflower leaves, again indicating a crucial role for lipase in the penetration phase of the infection (Berto et al., 1999).

The *B. cinerea* triacylglycerol lipase was partially sequenced (Comménil et al., 1999). The corresponding gene (*Lip1*) was cloned and targeted mutants were made by insertion of a hygromycin resistance cassette. *Lip1*-deficient *B. cinerea* mutants did not produce extracellular lipase under inducing conditions, but remained able to infect intact primary

leaves of *Phaseolus vulgaris* (H. Reis and M. Hahn, Univ. Kaiserslautern, Germany, pers. comm.), indicating that the lipase is not essential in host surface penetration.

Penetration of the cutin network by cutinase

Below the wax layer lies cutin, a highly complex three-dimensional network of chemically heterogeneous esterified hydroxylated, partly unsaturated fatty acids. How does *B. cinerea* breach the cutin network? In the 1980s and early 1990s several groups studied the role of cutinases of a number of plant pathogenic fungi in penetration (reviewed by Köller et al., 1995). Evidence was presented that cutinase was important for cuticle penetration by *Nectria haematococca* (*Fusarium solani* f.sp. *pisi*; Rogers et al., 1994), although this conclusion was firmly rebutted by others (Stahl and Schäfer, 1992; Stahl et al., 1994).

Salinas (1992) purified a 18-kDa cutinase from *B. cinerea* and raised monoclonal antibodies against the protein. Application of the antibody to gerbera flowers prior to inoculation reduced the number of lesions formed, suggesting that indeed the 18-kDa cutinase was important for penetration. This conclusion was however rejected on the basis of molecular-genetic studies. The *Bccut*A gene, encoding the 18-kDa cutinase, was cloned (van der Vlugt-Bergmans et al., 1997). The gene was expressed during pathogenesis from the onset of germination on the host surface onwards (van Kan et al., 1997). Gene replacement mutants were made that lacked the enzyme activity. The mutants were equally virulent as the wild type isolate both on gerbera flowers and tomato fruits, and the fungus remained able to penetrate intact cuticle surfaces (van Kan et al., 1997). Further experiments on other host species and different tissues have never revealed any reduction of virulence of the *BccutA*-deficient mutant (van Kan et al., unpubl.). Although the results of Salinas (1992) remain to be explained, it is evident that this cutinase is not essential in penetration.

The role of pectinases in penetrating the anticlinal epidermal wall

Over the years many studies have dealt with the secretion of cell wall-degrading enzymes (CWDEs) by *B. cinerea* during the early stages of infection. Swelling of the anticlinical epidermal cell wall (Mansfield and Richardson, 1981) suggested active involvement of CWDEs in penetration. Enzymes that attack pectic substances in the plant cell wall are thought to play a major role in pathogenicity (Clark and Lorbeer, 1976; Cole et al., 1998; Collmer and Keen, 1986). Endopolygalacturonase activity was detected in ungerminated *B. cinerea* conidia (Verhoeff and Warren, 1972) and two polygalacturonase isozymes were associated with the penetration stage of the infection process (van den Heuvel and Waterreus, 1985). It was suggested that the early, constitutive production of polygalacturonases enables fast penetration of the host tissue (Kapat et al., 1998), although no evidence was presented to support this hypothesis. Conclusions can only be drawn by

studying mutants in pectinolytic genes during early stages of infection. In all of the mutants generated in the course of our work on *B. cinerea* CWDEs, we have never observed a mutant incapable of penetrating intact host tissue (Kars et al., unpubl.).

Killing of host cells

Once through the cuticle, *B. cinerea* kills underlying epidermal cells before they are invaded by hyphae (Clark and Lorbeer, 1976). Invasion of plant tissue by *B. cinerea* triggers processes indicative of programmed cell death at a distance from the hyphae (Govrin and Levine, 2000), implying that diffusible factors have a direct or indirect phytotoxic activity. Also *B. elliptica* triggers programmed cell death in its host plant lily (van Baarlen and van Kan, 2004b). The inducing factors may be proteins or low molecular weight compounds secreted by the fungus into its environment. The induction of (programmed) cell death facilitates *B. cinerea* invasion and may in fact be essential for successful infection (Govrin and Levine, 2000). Most studies on the induction of cell death were performed with *B. cinerea*.

Toxins

Culture filtrates of *B. cinerea* may be phytotoxic when applied to plant tissue (Rebordinos et al., 1996). Compounds with phytotoxic potential were identified as botcinolide, a highly substituted lactone (Cutler et al., 1993) and botrydial, a tricyclic sesquiterpene (Colmenares et al., 2002). Several compounds related to botcinolide and botrydial have been identified that may either be precursors or conversion products, but these compounds generally have a lower toxicity (Colmenares et al., 2002; Durán-Patrón et al., 2000). Botrydial requires light for phytotoxic activity (Colmenares et al., 2002), but the reason for light dependence remains to be clarified. The observation that botcinolide and botrydial types of secondary metabolites were only secreted by *B. cinerea* in medium with high glucose levels initially raised doubts about their production *in planta*. However, with analytical chemical methods it was demonstrated that botrydial accumulates in infected tissue (Deighton et al., 2001) at concentrations that are presumably physiologically relevant. No evidence has yet been presented for production of botcinolide *in planta*. The role of botrydial in the infection of host plants needs to be evaluated by constructing mutants in the botrydial biosynthetic pathway. Resolution of this pathway is in progress, but the relevant genes have not been identified.

The *B. cinerea* toxins described above have a general phytotoxic activity. There is no evidence for production by *B. cinerea* of host-specific toxins, i.e. molecules produced by a pathogen that are specifically and exclusively toxic to its host and essential for the pathogen to achieve successful infection (Walton, 1996). This may not be surprising in view of the broad host range of *B. cinerea*. Recently, however, a protein was identified that is secreted

by *B. elliptica* and able to trigger programmed cell death in its host plant lily, but not in non-host plant species (van Baarlen et al., 2004b). This protein meets the criteria of a host-selective toxin and may turn out to be a determinant of compatibility for *B. elliptica*. By analogy, it is worth considering the possibility that the other, specialized *Botrytis* species may also be equipped with host-selective toxins, but this remains to be studied.

Oxalic acid

Secretion of oxalic acid (OA) occurs in fungi from various taxonomic classes (reviewed by Dutton and Evans, 1996). A key role has been postulated for OA in pathogenesis of *Sclerotinia sclerotiorum* (Godoy et al., 1990), a close relative of *Botrytis* spp. Mutants of *S. sclerotiorum,* deficient in OA production, were unable to infect *Arabidopsis* plants (Dickman and Mitra, 1992) and the deficiency could be restored by supplementing inoculum with OA. The oxalate-mediated acidification facilitates induction of gene expression by the ambient pH-dependent regulator *pac1*, which is required for virulence of *S. sclerotiorum* (Rollins, 2003).

B. cinerea produces OA both *in vitro* (Gentile, 1954; Germeier et al., 1994) and *in planta* (Verhoeff et al., 1988). OA forms calcium oxalate crystals within the host tissue (Figure 3 in Prins et al., 2000b). The sizes of lesions induced by different strains of *B. cinerea* on grapevine and bean leaves correlated with the amount of OA that these strains secreted *in vitro* (Germeier et al., 1994). It remains unclear whether the levels of OA produced *in planta* are sufficient to cause host cells to directly collapse. OA may in fact be a co-factor in pathogenesis rather than the primary phytotoxic agent. Culturing *B. cinerea* in low ambient pH resulted in the enhanced production of secreted endopolygalacturonase, aspartic protease and laccase activity (Manteau et al., 2003). Fungal endopolygalacturonases, aspartic proteases and laccases have an optimal activity at low pH and are therefore stimulated by the simultaneous secretion of OA (Manteau et al., 2003; ten Have et al., 2002). Moreover, OA may stimulate pectin degradation resulting from endopoly-galacturonase action by sequestering the Ca^{2+} ions from (intact or partially hydrolyzed) Ca-pectates in the cell walls. The removal of Ca^{2+} ions disturbs intermolecular interactions between pectic polymers and disrupts the integrity of the pectic backbone structure. Consequently, the pectic structure absorbs water and swells, as described by Mansfield and Richardson (1981).

The oxalate biosynthetic pathway in *B. cinerea* remains to be established. Several metabolites may serve as precursors of oxalate: glyoxal, oxaloacetate, erythroascorbic acid. Glyoxal oxidase is an enzyme that converts glyoxal into OA and H_2O_2. In the white rot fungus *Phanerochaete chrysosporium* this enzyme serves to generate H_2O_2, required as substrate for lignin peroxidases involved in lignin degradation (Kersten and Kirk, 1987). *B. cinerea* contains a gene encoding a glyoxal oxidase homologue, which is expressed *in vitro*

and *in planta* at a constitutive level. The gene product is predicted to possess a secretion signal peptide, suggesting extracellular localization. However, mutants in the glyoxal oxidase gene retained the ability to produce OA (van Kan et al., unpubl.), ruling out an important role of this enzyme in OA production. A second candidate enzyme that can generate OA is oxaloacetate hydrolase, converting oxaloacetate into acetate and OA. An oxaloacetate hydrolase gene was cloned from *Aspergillus niger* (Pedersen et al., 2000) and *B. cinerea* expresses a homologous gene. Its role in OA production and pathogenesis is under investigation (van Kan et al., unpubl.). Finally, several compounds were identified as potential OA precursors in *Sclerotinia sclerotiorum*: L-ascorbic acid, D-erythroascorbic acid, and D- and L-arabinose (Keates et al., 1998; Loewus et al., 1995; Loewus, 1999). The enzymes involved in this pathway remain to be identified. It seems plausible to speculate that *B. cinerea* possesses a similar biosynthetic pathway, but this question remains to be addressed.

Induction of Active Oxygen Species

Recent studies have focused on Active Oxygen Species (AOS) production in relation to *B. cinerea* pathogenicity (Govrin and Levine, 2000; Patykowski and Urbanek, 2003; von Tiedemann, 1997). AOS is the joint term for the superoxide anion, hydroxyl radical and hydrogen peroxide. An oxidative burst and enormous perturbances in the redox status are observed at the host-fungal interface, as well as in plant tissue at some distance from the infection front. A large part of the H_2O_2 production seems to occur in plasma membranes of host cells adjacent to fungal hyphae (Govrin and Levine, 2000; Schouten et al., 2002a). There are a number of fungal enzymes that potentially contribute to the H_2O_2 production. Fungal extracellular sugar oxidases (Edlich et al., 1989; Liu et al., 1998) or superoxide dismutase (SOD) were considered as candidate enzymes potentially responsible for generating the H_2O_2. Genes encoding an extracellular glucose oxidase and SOD were cloned. The targeted deletion of the SOD gene significantly reduced virulence and extracellular H_2O_2 accumulation at the host-fungus interface whereas deletion of the glucose oxidase gene did not affect virulence (Rolke et al., 2004).

Conversion of host tissue into fungal biomass

Plant cell walls function as barriers to biotic and abiotic agents. The strength and flexibility of cell walls depend on their composition. Plant cell walls are made up of different types of polysaccharides: the primary cell wall consists of cellulose and hemi-cellulose, while the middle lamella has a high pectin content. Pectin, a complex network of various polygalacturonans, also extends into the primary wall.

Once it has penetrated the anticlinal epidermal cell wall, *Botrytis* grows through the middle lamella and produces a range of CWDEs. Enzymatic breakdown of the plant cell wall releases carbohydrates which form a major carbon source for consumption. Cell wall degradation by *Botrytis* is mediated by pectinases, cellulases and hemicellulases. These CWDEs each have specific features and are involved in different steps of host tissue colonization and maceration. In the next sections we review research performed in recent decades on different types of *Botrytis* CWDEs.

Pectinases

Pectin is a major component of the plant cell wall and consists of three main types of polygalacturonans: homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II. Homogalacturonans are made of α-1,4-linked chains of D-galacturonic acid that can be methylated and/or acetylated. Highly methylated homogalacturonan is referred to as pectin, while homogalacturonan with a low degree of methylation is called pectate. Enzymes that are able to degrade pectic components are denoted as pectinases. During infection *Botrytis* produces various types of pectinases, each having distinct roles in pectin degradation.

Pectin methylesterase

Most pectinolytic enzymes cannot degrade highly methylated pectin. Therefore, the action of pectin methylesterase, which de-methylates pectin to pectate, is required. The biochemical characteristics were described of two *B. cinerea* pectin methylesterase (BcPME) isozymes with different molecular mass and identical pI values (Marcus and Schejter, 1983). In contrast, Reignault et al. (1994) identified two isozymes in a different *B. cinerea* strain (Bd90), which were of identical molecular mass (42 kDa) but had different pI values (pI 7.0 and 7.4, respectively). Levels of BcPME activity in liquid cultures were neither enhanced by pectic derivates nor subject to glucose repression. In *B. fabae*-infected broad bean tissue, the content of methyl-esterified homogalacturonan decreased in the vicinity of hyphae, indicative of either pectin methylesterase action or pectin degradation (Cole et al., 1998). Pectin methylesterase activity was detected in *B. cinerea*-infected bean leaves and its level was correlated with disease severity (Zimand et al., 1996).

Disruption of the *B. cinerea* pectin methylesterase gene *Bcpme1* in strain Bd90 revealed that this gene encodes the isozyme with pI 7.4, and confirmed that this strain possesses more than one pectin methylesterase-encoding gene. Pectin methylesterase activity in the *Bcpme1* mutant was reduced by 75% and the mutant was less virulent on apple, grapevine and *Arabidopsis* (Valette-Collet et al., 2003). Only a single mutant was obtained; complementation of the mutant with the wildtype gene restored virulence. In our laboratory, however, multiple *Bcpme1* mutants were generated in strain B05.10 using a gene-replacement strategy. None of these mutants showed a reduction in virulence on five

different host plants tested, including apple (Kars et al., 2005b). The mutants did not grow differently on plates containing 75% methyl-esterified pectin, as compared to B05.10. The differences in virulence between *Bcpme1* mutants in the two different strains must be further investigated. Zymograms of pectin methylesterase activity in plant tissues infected by either Bd90 or B05.10 were similar (Reignault et al., 2000). No differences were detected between the two strains by Southern analysis of genomic DNA (Valette-Collet et al., 2003). This raises the possibility of the involvement of another pectin methylesterase isozyme. A second BcPME isozyme (pI 7.1) was detected in strain Bd90 (Valette-Collet et al., 2003), but was proposed to play a less prominent role than *Bcpme1*. This isozyme could be encoded by the recently identified gene *Bcpme2*. *Bcpme2* might be more important for pathogenicity of strain B05.10 than of Bd90. It will be of interest to generate mutants lacking both genes and study the effect on pathogenesis.

Endopolygalacturonase

Endopolygalacturonases are endo-acting enzymes catalysing the hydrolysis of homogalacturonan, resulting in substrate fragmentation. These enzymes are not able to hydrolyse highly methylated pectin, but first require the action of pectin methylesterase to de-methylate pectin to pectate. Over the years, many researchers have tried to unravel the complex mechanism of action of *Botrytis* endopolygalacturonases (BcPGs). Research mainly focused on the production, purification and characterization of *Botrytis* endopoly-galacturonase isozymes *in vitro* and *in planta* (Chilosi and Magro, 1997; Drawert and Krefft, 1978; Hancock et al., 1964a, b; Johnston and Williamson, 1992a, b; Kapat et al., 1998; Leone, 1990; Leone et al., 1990a, b; Manteau et al., 2003; Marcus and Schejter, 1983; Movahedi and Heale, 1990b; Pashkoulov et al., 2002; Reignault et al., 2000; Tobias et al., 1993, 1995; Urbanek and Zalewska-Sobczak, 1984; van den Heuvel and Waterreus, 1985; van der Cruyssen et al., 1994; Verhoeff and Warren, 1972; Zimand et al., 1996). Nevertheless, these studies have not revealed the exact function of individual endopolygalacturonase isozymes in pathogenesis. In the late 1990s the first BcPG encoding genes were cloned and characterized (Wubben et al., 1999). A gene family was discovered which encoded six isozymes, each having different characteristics. The expression pattern of each gene family member was studied and indicated how these genes are regulated *in vitro* and *in planta* (ten Have et al., 2001; Wubben et al., 2000). Characterization of five heterologously expressed, pure BcPGs revealed that each isozyme differs in specific activity, protein stability, substrate preference and end-products (Kars et al., 2005a).

The availability of *Bcpg* genes facilitated the analysis of function of individual isozymes in pathogenesis. Disruption of *Bcpg1* resulted in a reduction in virulence on tomato leaves and fruit, as well as on apple (ten Have et al., 1998). Disruption of other members of the endopolygalacturonase gene family was achieved and mutants were characterized. Assays

on various host species showed that *Bcpg2* also plays an important role in virulence (Kars et al., 2005a).

It was recently reported that BcPG1 displays elicitor activity, triggering an oxidative burst in grapevine cell suspensions (Poinssot et al., 2003). Mild heat treatment drastically reduced enzyme activity without strongly affecting elicitor activity, suggesting that defense responses result from BcPG1 protein recognition rather than from its enzyme activity. It was hypothesized that BcPG1 triggers a defense reaction through a gene-for-gene interaction, in which *Bcpg1* acts as an *avr* gene (Poinssot et al., 2003). This novel hypothesis, however, seems to contradict the proposed role of endopolygalacturonases in tissue colonization and maceration (ten Have et al., 2002). Unfortunately, Poinssot et al. (2003) did not present evidence that the elicitor activity of BcPG1 (i.e. the amount of H_2O_2 produced upon incubation of grapevine cells with BcPG1) was linearly correlated with the amount of protein added, or demonstrate that enzyme activity (i.e. the amount of reducing sugar ends released from the model substrate polygalacturonic acid) was linearly correlated with the amount of protein added. It can be envisaged that grapevine cell suspensions still mount an equally strong oxidative burst even with lower amounts of BcPG1. Furthermore, it remains questionable whether an oxidative burst in cell suspensions reflects physiological responses in *B. cinerea* challenged green tissue.

We favor the explanation that the main (if not exclusive) role of endopolygalacturonases is in tissue colonization and maceration. Firstly, all *Botrytis* species studied thus far seem to possess a gene family encoding multiple endopolygalacturonases (Wubben et al., 1999). This seems redundant for *Botrytis* spp. specialized on a single host species if their function was to act as an elicitor. Secondly, if the function of endopolygalacturonases was to act as an avirulence protein, one would predict that the genes evolve in such a way that enzyme activity of the gene product is lost. Yet, all *B. cinerea* endopolygalacturonase genes studied encode active enzymes (Kars et al., 2005a). Thirdly, the presence in plants of an R gene that recognizes BcPG1, and thereby confers susceptibility to *B. cinerea*, would put strong selection pressure on the host for loss of R gene function.

Exopolygalacturonase

Exopolygalacturonases cleave monomeric or dimeric glycosyl groups from the pectic polysaccharide, thereby providing the fungus with potential nutrients. *B. cinerea* and *B. allii* produced exopolygalacturonases in necrotic leaf sections of onion, while *B. squamosa* did not (Hancock et al., 1964a, b). Exopolygalacturonase activity was also detected in *B. cinerea*-inoculated tomato petiole and fruit (Verhoeff and Warren, 1972). The total exopolygalacturonase activity in *B. cinerea*-infected bean plants remained at a similar level for 6 days whereas the symptom development progressed (Kapat et al., 1998). Johnston and Williamson (1992a) were the first to purify and characterize two *B. cinerea*

exopolygalacturonases with molecular weights of 65 and 70 kDa, respectively. The same two isozymes were detected in *B. cinerea* cultures containing as carbon sources either citrus pectin, polygalacturonic acid or its monomer, galacturonic acid (Lee et al., 1997; Rha et al., 2001). Tobias et al. (1993), however detected four exopolygalacturonase isozymes produced by *B. cinerea* grown on apple pectin. Secretion of exopolygalacturonases was detected in cucumber leaves from 9 h after inoculation with *B. cinerea*, using polyclonal antibodies (Rha et al., 2001), suggesting that these enzymes play a role in early stages of infection and in subsequent tissue maceration.

A *B. cinerea* exopolygalacturonase gene sequence was deposited in a database (Kim and Rha, Gyeongsang National University, Korea, unpubl.), but transcripts of this gene in *B. cinerea*-infected tomato leaves were only detected at low levels in late stages of infection (Kars et al., unpubl.).

Pectin lyase and pectate lyase

Pectin lyase is a pectin-degrading enzyme that cleaves homogalacturonan with a high degree of methyl-esterification. Pectin lyase is inactive at acidic pH. Several groups investigated *B. cinerea* pectin lyase activity *in vitro* and *in planta* (Chilosi and Magro, 1997; Doss, 1999; Movahedi and Heale, 1990b). Pectin lyase isozymes were detected in extracts of ungerminated conidia and in the extracellular matrix of *B. cinerea* germlings. Pectin lyase was produced early after inoculation of soybean hypocotyls and zucchini fruits, but not in infected apple tissue (Chilosi and Magro, 1997). Tissue-specific enzyme production may be correlated to the ambient pH in uninfected tissue. Apple tissue is very acidic (pH 3-4), while zucchini fruit and soybean hypocotyls have a more neutral pH. Since *B. cinerea* acidifies its environment prior to pectin degradation, pectin lyases are in any case unlikely to contribute significantly to pectin degradation in the early stages of infection by *Botrytis*. A pectin lyase gene from *B. cinerea* was cloned (van Kan et al., unpubl.) that is expressed at low levels in *B. cinerea*-infected tomato leaves (chapter 4). The generation of mutants has been unsuccessful so far.

Pectate lyases catalyze the cleavage of pectate, i.e. unmethylated homogalacturonan. Pectate lyases strictly require Ca^{2+} ions for catalysis and are inactive at acidic pH. This enzyme is therefore also unlikely to contribute significantly to cell wall degradation in early stages of infection by *Botrytis*. *B. cinerea* strain B16 produced pectate lyase in liquid cultures and on French bean leaves (Kapat et al., 1998; Zimand et al., 1996). One pectate lyase gene was cloned from *B. cinerea*, but mutants in this gene have not yet been made.

Rhamnogalacturonan hydrolase

Rhamnogalacturonan hydrolase (RGase) specifically hydrolyses non-esterified galacturonosyl-rhamnosyl linkages in the modified hairy regions of pectin. RGase activity

was first detected in *B. cinerea* culture filtrates by Gross et al. (1995). Chen et al. (1997) cloned a rhamnogalacturonan hydrolase gene from *B. cinerea*, present in a single copy in the genome. The biochemical characteristics of the RGase gene product, including substrate specificity were determined (Fu et al., 2001), but its role in pathogenesis remains to be investigated.

Non-pectinolytic cell wall-degrading enzymes

Although *B. cinerea* is considered to be a pectinolytic fungus (ten Have et al., 2002), the degradation of plant cell walls may also require a number of non-pectinolytic CWDEs such as cellulases, xylanases and arabinases (Cole et al., 1998; Drawert and Krefft, 1978; Hancock et al., 1964a, b; ten Have et al. 2002; Urbanek and Zalewska-Sobczak, 1984; Verhoeff and Warren, 1972). *B. alli*, *B. squamosa* (Hancock et al., 1964a, b) and *B. fabae* (Cole et al., 1998) also produce non-pectinolytic CWDEs.

Cellulases

The cellulolytic complex comprises, among others endoglucanase, cellobiohydrolase and β-glucosidase and it degrades cellulose into cellobiose and glucose. Cellulase activity was neither detected in ungerminated nor in germinating conidia of *B. cinerea* (Verhoeff and Warren, 1972). Cellobiohydrolase activity was not detected in *B. fabae*-infected bean leaves (Cole et al., 1998). Transcripts of a *B. cinerea* cellobiohydrolase gene were not detected in infected tomato leaves (Kars et al., unpubl.). These observations indicate that cellobiohydrolase does not play an important role in infection. *B. cinerea* was found to produce three intracellular β-glucosidases (Gueguen et al., 1995), and one extracellular β-glucosidase (Sasaki and Nagayama, 1994) in liquid cultures. The extracellular β-glucosidase and one intracellular β-glucosidase were purified and characterized, but only the extracellular β-glucosidase was suggested to be involved in plant cell wall degradation.

Xylanase and arabinase

Xylan and arabinan are constituents of hemicellulose in the primary cell wall. The hemicellulolytic complex degrades these polymers by a range of enzymes including xylanases and arabinases. Urbanek and Zalewska-Sobczak (1984) reported that *B. cinerea* secreted one arabinase and three xylanases during apple cell wall degradation. No further studies were published on xylanases and arabinases in any *Botrytis* spp. Neither of the genes has been cloned nor were the enzymes purified. Involvement of these enzymes in pathogenesis remains to be resolved.

Other enzymes potentially involved in pathogenesis

Aspartic proteases

Already over a decade ago, Movahedi and Heale (1990a) demonstrated that *B. cinerea* produces aspartic protease (AP) both in liquid culture and during early stages of the infection process. AP activity was detected prior to the appearance of pectinases. When the inoculum was supplemented with the specific AP inhibitor pepstatin, AP activity was blocked and infection was strongly reduced. This led Movahedi and Heale (1990a) to propose that a secreted fungal AP is important for pathogenesis. This conclusion, however, did not consider the possible role of plant (aspartic) proteases in the infection. Expression of a tomato AP is induced by wound responses (Schaller and Ryan, 1996) and the infection of tomato leaves by *B. cinerea* was shown to induce the expression of wound responsive genes in the host (Diaz et al., 2002). The effect of pepstatin that Movahedi and Heale (1990a) observed may therefore have been caused by its inhibition of a plant rather than a fungal AP. Recent studies have identified important roles for various types of plant proteases in R-gene mediated defense responses (Tör et al., 2003).

In filtrates of liquid cultures of *B. cinerea*, the total protease activity was highest when the assay was performed at low pH and it was fully inhibited by pepstatin, indicating that all enzyme activity was due to an AP (Manteau et al., 2003; ten Have et al., 2004). Five genes encoding an AP were cloned. All were expressed *in vitro*, as well as *in planta*. Expression of these genes may be modulated by the *Bcg1* gene, because deletion of this gene resulted in reduced secreted proteinase production (Schulze Gronover et al., 2001). The AP isozyme sequences cluster in four distinct phylogenetic groups. One of the *B. cinerea* AP isozymes is presumably secreted, while a second one is undoubtedly a vacuolar protease. Two isozymes show characteristics of membrane-bound APs, although this remains to be verified (ten Have et al., 2004). Single and double mutants were generated for all five *Bcap* genes (van Kan et al., unpubl.). Analysis of the mutants is in progress.

Laccases

Extensive studies on laccase activity have been performed over at least two decades by Mayer and co-workers. The production of gallic acid-inducible laccases in a *B. cinerea* culture was suppressed by secondary metabolites from Cucurbitaceae called cucurbitacins, while the activity of other fungal enzymes was not affected (Viterbo et al., 1993a). Cucurbitacins protected cucumber fruit and cabbage leaves from infection by *B. cinerea* (Bar-Nun and Mayer, 1990), leading to the hypothesis that laccase plays an important role in pathogenesis (Viterbo et al., 1993a; Staples and Mayer, 1995). Some cucurbitacin forms were more effective than others in reducing secreted laccase activity (Viterbo et al., 1993b). Radiolabeled cucurbitacin was taken up by *B. cinerea* mycelium suggesting that it acts intracellularly. Cucurbitacin induced a lower production of laccase activity (Viterbo et al.,

1994), supposedly as a consequence of repression at the mRNA level (Gonen et al., 1996). These mRNA expression studies were however performed using a probe corresponding to the gene *Bclcc1*, whereas the gallic acid-inducible laccase gene is in fact *Bclcc2* (Schouten et al., 2002b). These two genes show poor cross-hybridization (Schouten et al., unpubl.).

Three laccase genes were cloned, of which one (*Bclcc2*) was expressed in several host species and the second (*Bclcc3*) was expressed only in ageing mycelium, both *in vitro* and *in planta*. The third gene, denominated *Bclcc1*, was never expressed at any detectable level. Deletion of either the *Bclcc1* gene or the *Bclcc2* gene did not result in detectable reduction of virulence on a range of host species tested (Schouten et al., 2002b). It can thus be concluded that at least the laccases BcLCC1 and BcLCC2 are not important virulence factors. Deletion of the *Bclcc3* gene has not (yet) been performed, but its expression pattern suggests that it is unlikely to play an important role in early steps of pathogenesis.

The biochemical data of A. Mayer and co-workers (Hebrew Univ. Jerusalem, Israel) seem to point to an important role for laccases in the infection process, while the molecular-genetic data exclude such a role. How can one reconcile these two seemingly contradictory conclusions? There are differences in the experimental procedures and the fungal strains used. In most of the biochemical work, *B. cinerea* was grown in pectin-containing medium in which inducers were present throughout the culture. Growth was generally over a great length of time, up to well over 14 days. Schouten et al. (2002b) grew pre-cultures in defined synthetic medium and showed that *Bclcc2* mRNA could be induced within 1 h after addition of tannic acid or resveratrol. Laccase activity was detected after overnight incubation in the presence of an inducer. It is possible that the enzyme activity detected by Mayer and co-workers throughout their work is encoded by the *Bclcc3* gene, or by a novel laccase gene that has not yet been identified.

Counteracting host defense responses

Throughout the course of an interaction between *B. cinerea* and its host, the plant (often vigorously) attempts to defend itself against pathogen invasion. Largely the same spectrum of defense responses is activated during an infection by *B. cinerea* as during a hypersensitive response (HR) to avirulent races of a biotrophic pathogen: lignification (Heale and Sharman, 1977; Maule and Ride, 1976), biosynthesis of phytoalexins (e.g. Bennett et al., 1994) and accumulation of PR proteins (e.g. Benito et al., 1998; Diaz et al., 2002). These defense responses are presumably local, being restricted to the infection site(s). Although *B. cinerea* is a necrotizing pathogen it does not trigger Systemic Acquired Resistance (Govrin and Levine, 2002). The total spectrum of defense responses results in a primary necrotic lesion in which the fungus is effectively restricted. Depending on the type of host tissue and yet unidentified physiological aspects of the host, fungal growth enters a lag phase in which the lesions do not expand. A proportion of the primary lesions eventually

develop into aggressive, expanding lesions (Benito et al., 1998; De Meyer and Höfte, 1997; van den Heuvel, 1981). The pathogen is not killed in the non-expanding lesions since viable fungal mycelium could be recovered from all lesions (Benito and van Kan, unpubl.). Thus, an active defense contributes to (temporarily) restricting the fungus within the primary lesions, giving rise to a period of quiescence. It is as yet unknown which factors determine the transition from quiescence to the aggressive, expanding infection phase. Van Baarlen et al. (2004a) describes in more detail the defense compounds (metabolites and enzymes) produced in plants in response to *Botrytis* infection and their effectiveness in restricting the outgrowth of the pathogen. In order to be a successful pathogen on their respective host species, *Botrytis* spp. obviously have to cope with these defense compounds, either by active suppression of their synthesis or by counteracting their growth inhibiting effect. Van Baarlen et al. (2004a) discusses mechanisms that *Botrytis* spp. have evolved to overcome the deleterious effects of pre-formed or induced host defense compounds.

The oxidative burst at the host-pathogen interface imposes stress on the host as well as the pathogen. *B. cinerea* is able to cope with external oxidative stress in order to survive in the necrotic tissue. Successful detoxification of H_2O_2 is mediated by an extracellular catalase BcCAT2 with glutathione S-transferase presumably functioning as intracellular back-up (Prins et al., 2000a; Schouten et al., 2002a). Targeted mutagenesis of the catalase gene *Bccat2* did not affect the survival within the oxidative environment of a necrotic lesion. Virulence of *Bccat2* deficient mutants was indistinguishable from wildtype (Schouten et al., 2002a).

Concluding remarks

This chapter illustrates that *Botrytis* is equipped with a large toolbox of enzymes and metabolites that enable the pathogen to infect a spectrum of host plants. There can be functional overlap between different types of enzymes attacking the same substrate and there is apparent redundancy within a family of isozymes. Some enzymes supposedly act in concert, while others may be irrelevant for a particular host. Altogether these tools are needed to fulfill their job: killing plant cells and facilitating the conversion of plant tissue into fungal biomass.

Research on *B. cinerea* pathogenicity factors has focused for many decades on the production of secreted enzymes and their correlation with pathogenesis. Such studies are still pursued by certain researchers up until today. With our current knowledge of the occurrence of gene families encoding multiple isozymes, often with quite distinct biochemical properties, we recommend not to initiate further studies on total enzyme activity. It will be much more informative to study individual isozymes, their biochemical characteristics and expression during pathogenesis. Information on their temporal and

spatial accumulation, substrate preference, end-product release and other isozyme-specific characteristics will substantially contribute to understanding enzymatic processes during pathogenesis.

Targeted mutagenesis provides an excellent tool to study the role of specific gene products in pathogenesis. The generation of single, double or even triple gene-specific mutants is feasible and will provide a better understanding of the complex role of the different secreted enzymes during *Botrytis*-host plant interactions. We look forward to witnessing the design of rational control strategies, based on such knowledge, in the upcoming decade.

Acknowledgements

We acknowledge Holger Reis and Matthias Hahn (Kaiserslautern University, Germany) and our colleague Sander Schouten for allowing us to incorporate in this chapter unpublished results. The research of Ilona Kars was supported by the Dutch Technology Foundation STW, Applied Science Division of NWO and the Technology Program of the Ministry of Economic Affairs (project WGC.5034). We thank Prof. Dr. Ir. P.J.G.M. de Wit for critical reading of the manuscript.

Chapter 2

Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10

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This chapter is published as: Kars, I., McCalman, M., Wagemakers, L., and van Kan, J.A.L. (2005) Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. Mol. Plant Pathol., 6, 641-652.

Abstract

Botrytis cinerea is a necrotrophic pathogen that produces an array of enzymes capable of attacking the plant cell wall components. We have previously shown that growth of the fungus *in planta* is accompanied by the degradation of pectin and that endo-polygalacturonase (*Bcpg*) genes are expressed during infection of different plant tissues. It was assumed that pectin demethylation by pectin methylesterases (PME) was essential for the subsequent, depolymerization by BcPGs to occur efficiently. We report here on the functional analysis of two *Bcpme* genes in strain B05.10, using a gene-replacement approach. The method used for the generation of constructs for gene replacement in *B. cinerea* circumvents the need for cloning and yielded a high proportion of homologous recombinants. Mutants lacking both *Bcpme* genes are neither affected in their growth on highly methylated pectin, nor did they show any reduction in virulence. The results suggest that *B. cinerea* strain B05.10 can efficiently degrade pectin without prior demethylation.

Introduction

Higher plants have different types of primary cell wall constituents. Pectin is one of the major structural components of the middle lamella of primary cell walls and is composed of rhamnogalacturonan and homogalacturonan. During plant development and infection by pathogens, pectin can be degraded by an array of enzymes that can either depolymerize pectin (polygalacturonases, pectin and pectate lyases; Kars and van Kan, 2004) or alter its structure (pectin acetylesterases and pectin methylesterases). Pectin methylesterase (PME) catalyses the hydrolysis of a methyl ester, thereby releasing methanol and pectate. When the degree of methylation (DM) of pectin decreases, pectin becomes more accessible to depolymerizing enzymes (Koch and Nevins, 1989). The DM of pectin varies, generally ranging from 13% to approximately 80% (Mohamed et al., 2003; Voragen et al., 1986). Green tomato fruit can even have a DM up to 90%, while ripe fruit has a DM of ~30% (Koch and Nevins, 1989). PME action does not result in complete de-esterification, as circa 20-30% of methyl esters cannot be removed (Versteeg, 1979; Kester et al., 1999), possibly because of the presence of acetylester groups (Alebeek et al., 2003). Numerous organisms, including plants, fungi, bacteria and nematodes, produce PMEs. PMEs may differ with respect to their pH optimum, pI values and requirement for calcium (Versteeg et al., 1978). In general, fungal PMEs have an optimal activity between pH 4 and 6, while plant and bacterial PMEs have an optimal activity between pH 6 and 8 (Alebeek et al., 2003). Another notable difference that has been reported is that fungal PMEs have been shown to hydrolyze the methyl esters in a random way, while plant and bacterial PMEs de-esterify in a 'block-wise' manner (Markovič and Kohn, 1984; Kester et al., 1999; Benen et al., 2002). However, recent studies have shown that the degree of methylation of pectin and the pH of the environment influence the PME activity. At a given pH, some isoforms are more effective than others on highly methylated pectin. Also some isoforms have the ability to act randomly at acidic pH and 'block-wise' at alkaline pH (Catoire et al., 1998; Denès et al., 2000; Micheli, 2001).

Molecular studies of PMEs have led to the characterization of several related genes in both plants and their pathogens. PME encoding genes have been characterized from *Ralstonia (Pseudomonas) solanacearum* (Spök et al., 1991), *Erwinia chrysanthemi* (Laurent et al., 1993) and *Botrytis cinerea* (Reignault et al., 1994). *R. solanacearum* produces one PME, which is required for growth on methylated pectin, but not for virulence (Tans-Kersten et al., 1998). However, mutants generated in *E. chrysanthemi* (*pemA*; Beaulieu et al., 1993; Boccara and Chatain, 1989) and *B. cinerea* (*Bcpme1*; Valette-Collet et al., 2003) showed reduced PME activity and showed a reduction in virulence on certain hosts. The residual virulence of the mutants was ascribed to the presence of at least one additional PME-encoding gene (*pemB* and *Bcpme2*, respectively; Shevchik et al.,1996; Valette-Collet et al., 2003).

It is generally considered that methylated pectin is a poor substrate for depolymerization by endopolygalacturonases (Koch and Nevins, 1989). Demethylation of pectin is presumably required prior to subsequent, efficient depolymerization. Previous studies have indicated the importance of endopolygalacturonases for virulence in *B. cinerea* (ten Have et al., 1998; Kars et al., 2005a), but it remained unclear whether PME action preceding pectin depolymerization is essential for virulence, as suggested by the phenotype of the *Bcpme1* mutant (Valette-Collet et al., 2003). It was our goal to extend these studies by functionally analyzing the two *Bcpme* genes individually and studying the concerted action between BcPMEs and BcPGs during infection. Gene replacement mutants were made for both the *Bcpme1* and the *Bcpme2* gene, individually. Furthermore, double mutants were generated lacking *Bcpme1* and *Bcpme2*, as well as mutants lacking *Bcpg1* and *Bcpme1*. All mutants were tested in virulence assays and in growth experiments on pectin with different degrees of methylation.

Results

Bcpme1 and Bcpme2 are expressed during tomato leaf colonization

The expression *in planta* of the *Botrytis cinerea* genes *Bcpme1* and *Bcpme2* was analyzed in a time-course experiment. Total RNA was extracted from each sample separately and RNA extracted from healthy tomato leaves served as a control. The northern blot was hybridized with the coding sequences of *Bcpme1* and *Bcpme2* (Figure 1). The transcripts of both genes were detected at 24 h post inoculation (hpi) and increased during the course of the infection process, similar to *Bcpg1* (ten Have et al., 1998) and the constitutively expressed *BcactA* (Figure 1), which served as a measure for fungal biomass (Benito et al., 1998).

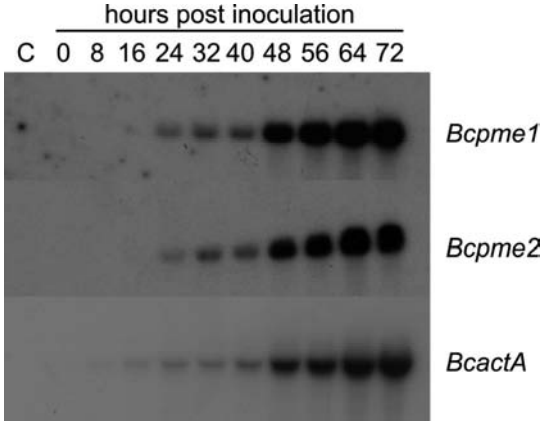

Figure 1. *Bcpme1* and *Bcpme2* expression during tomato leaf colonization by *Botrytis cinerea* with time. Sampling times are indicated above the lanes. Control RNA (C) extracted from an uninfected tomato leaf is in the left lane. Blots were probed with fragments of *Bcpme1*, *Bcpme2* and *BcactA* as indicated in the right margin.

Generation of Botrytis cinerea pectin methylesterase single and double mutants

B. cinerea mutants lacking the pectin methylesterase genes *Bcpme1* and *Bcpme2* were created by gene replacement (Figure 2). The technique used to perform gene-replacement is novel for filamentous fungi and requires no restriction or ligation steps. Instead, a single-step overlap-extension PCR was used to fuse fragments of the target gene to a selection marker (Figure 2, step 3). Consequently, a 3-4 kb PCR fragment was used to transform *B. cinerea* protoplasts instead of a linear plasmid (Figure 2, step 4). In case of homologous recombination, coding sequences of *Bcpme1* and *Bcpme2* were partly replaced by a cassette containing either the *hph* gene or *nat* gene, which confer resistance to hygromycin B and nourseothricin, respectively.

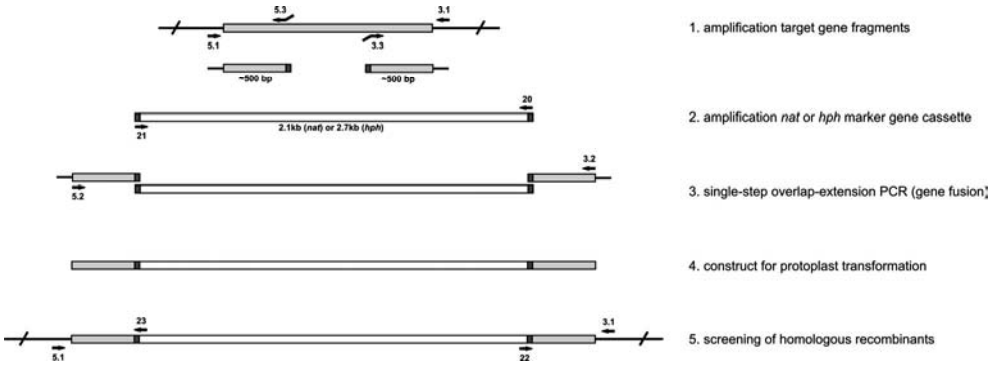

Figure 2. Schematic representation of the steps in the gene replacement strategy and screening method.

B. cinerea transformants resistant to hygromycin B and/or nourseothricin were screened for gene replacement by PCR (Figure 2, step 5). The screening method was similar for both *Bcpme1* and *Bcpme2*. In an amplification reaction with primers 5.1 and 23 (Table 1), a recombinant yielded a fragment of approximately 500 bp. Similarly, a fragment of approximately 500 bp was amplified with primers 3.1 and 22 (Table 1). In transformants with an ectopic integration, no fragment was amplified with either of these primer combinations. During the transformation procedure, *B. cinerea* may form heterokaryons, some of which contain mixtures of transformed and untransformed nuclei (van Kan et al., 1997). The correct recombinants were further analyzed to verify whether they were clean, homokaryotic mutants or heterokaryons. In case the wild type gene was absent, and consequently no product was amplified with primers 5.1 and 3.1 (Table 1), we presumed that a proper mutant was obtained. If recombinants were heterokaryotic and still contained some wild type nuclei, single spore plating on antibiotic was performed and antibiotic-resistant germlings were propagated for renewed DNA isolation and PCR analysis. Mutants were subsequently

validated in a Southern blot analysis. The screening by PCR strongly reduced the number of DNA isolations of transformants to be analyzed by Southern analysis.

Table 1. PCR primers used to amplify target gene fragments, gene-replacement constructs, and to check for correct homologous integration of gene-replacement construct in the transformants.

Target gene	Primer name	Primer sequence (5' > 3')
Bcpme1 (AJ309701)	pme1-5.1	GCGGCCACTCTTCTAGGAGC
	pme1-5.2	CTAGAGGCACGAGCGGTCTC
	pme1-5.3	GATGATTACTAACAGATATCAAGCTTGGCGAGGATAGTATCTTGGT
	pme1-3.1	GCTATCAACCCACCATTCGT
	pme1-3.2	AAGCACTTCCCAAAACGGTGG
	pme1-3.3	GGGTACCGAGCTCGAATTCCCGGAACTCAACTTTATGCTA
Bcpme2 (AJ428403)	pme2-5.1	CTCCCTAAGGCTGATCTCTTC
	pme2-5.2	CAACGCATCTTGAAGTCAAGC
	pme2-5.3	GATGATTACTAACAGATATCAAGCTTCGAGAACGGTGTCTTGGTAAC
	pme2-3.1	GAAGTGATAGTTCGACCGTTC
	pme2-3.2	CTCGCTAGTGTAGGATGTACC
	pme2-3.3	GGGTACCGAGCTCGAATTCCTTGATCGTCGGAGCCATTGA
Selection marker cassette	cassette-5 (20)	GAATTCGAGCTCGGTACCC
	cassette-3 (21)	AAGCTTGATATCTGTTAGTA
	screen-3 (22)	GGGTACCGAGCTCGAATTC
	screen-5 (23)	GATTACTAACAGATATCAAGCTT

The strategy, which yielded approximately 50 transformants per microgram of DNA, is described in detail in the Methods section. The recombination efficiency differed for each experiment and each target gene, but on average a gene replacement efficiency of 63% was achieved. Southern hybridization confirmed that in three independent *Bcpme1* mutants the 2.6-kb *Xba*I/*Bam*HI band of the wild type gene was replaced by a 4.4-kb band or a 3.5-kb band, when using a hygromycin or nourseothricin cassette, respectively (Figure 3A). In four independent mutants in the *Bcpme2* locus, the 3.5-kb *Cla*I/*Sal*I band of the wild type gene was replaced by two bands of 3.1 and 2.1 kb, respectively (Figure 3B). Antibiotic-resistant transformants resulting from random ectopic integration of the cassette gave rise to one or more bands of variable size additional to the wild type band (not shown).

Double mutants Δ*Bcpme1*/Δ*Bcpme2* were obtained by transforming mutant Δ*Bcpme1*-1B (*NAT*^{R}) with the *Bcpme2*/*HPH*^{R} cassette, whereas double mutants Δ*Bcpg1*/Δ*Bcpme1* were obtained by transforming mutant Δ*Bcpg1*-39 (*HPH*^{R}; ten Have et al., 1998) with the *Bcpme1*/*NAT*^{R} cassette. Screening of the transformants resulted in the identification of five independent Δ*Bcpme1*/Δ*Bcpme2* mutants (Figure 3C) and ten independent Δ*Bcpg1*/Δ*Bcpme1* mutants (Figure 3D), respectively. Correct replacement without any further detectable ectopic insertions of antibiotic marker cassettes was confirmed in all

mutants (summarized in Table 2) by Southern hybridization with gene-specific probes, as well as with selection marker probes (not shown).

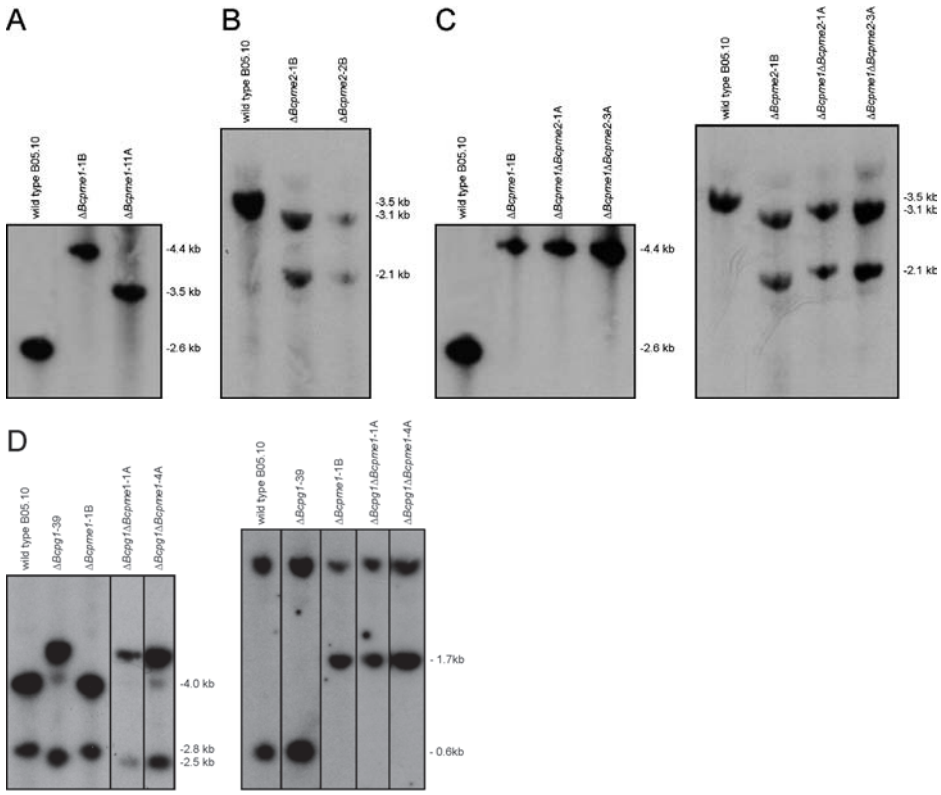

Figure 3. Southern hybridization with gene-specific probes to confirm gene replacement in the *Botrytis cinerea* mutants. Genomic DNA of the Δ*Bcpme1* mutants was digested with **(A)** *Xba*I/*Bam*HI and that of the Δ*Bcpme2* mutants was digested with **(B)** *Cla*I/*Sal*I. **(C)** Genomic DNA of the Δ*Bcpme1*Δ*Bcpme2* double mutants was digested with either *Xba*I/*Bam*HI (left panel hybridized with *Bcpme1*) or *Cla*I/*Sal*I (right panel hybridized with *Bcpme2*), and **(D)** that of the Δ*Bcpg1*Δ*Bcpme1* double mutants was digested with either *Xba*I (left panel hybridized with *Bcpg1*) or *Xba*I/*Bam*HI (right panel hybridized with *Bcpme1*). All Southern blots were also hybridized with the selection marker probes (not shown).

Table 2. Overview of independent *Botrytis cinerea* mutants obtained for each gene.

Target gene(s)	Background strain	# Independent mutants	Mutant names *
Bcpme1	B05.10	3	Δ*Bcpme1*-**1B** or 1C
			Δ*Bcpme1*-**11A** or 11B or 11C
			Δ*Bcpme1*-14A or 14C
Bcpme2	B05.10	3	Δ*Bcpme2*-1A or **1B** or 1C
			Δ*Bcpme2*-2A or **2B**
			Δ*Bcpme2*-3A or 3B or 3C
Bcpme2	Δ*Bcpme1*-1B	5	Δ*Bcpme1*Δ*Bcpme2*-**1A** or 1C
			Δ*Bcpme1*Δ*Bcpme2*-2A or 2B or 2C
			Δ*Bcpme1*Δ*Bcpme2*-**3A** or 3B or 3C
			Δ*Bcpme1*Δ*Bcpme2*-5A or 5B or 5C
			Δ*Bcpme1*Δ*Bcpme2*-7A or 7B or 7C
Bcpme1	Δ*Bcpg1*-39	10	Δ*Bcpg1*Δ*Bcpme1*-**1A** or 1C
	(ten Have et al., 1998)		Δ*Bcpg1*Δ*Bcpme1*-**4A**
			Δ*Bcpg1*Δ*Bcpme1*-6C
			Δ*Bcpg1*Δ*Bcpme1*-7A
			Δ*Bcpg1*Δ*Bcpme1*-8A or B
			Δ*Bcpg1*Δ*Bcpme1*-11A
			Δ*Bcpg1*Δ*Bcpme1*-12A
			Δ*Bcpg1*Δ*Bcpme1*-13A or B
			Δ*Bcpg1*Δ*Bcpme1*-16A
			Δ*Bcpg1*Δ*Bcpme1*-17B or C

* Independent mutants that have been used in virulence assays are indicated in bold and underlined

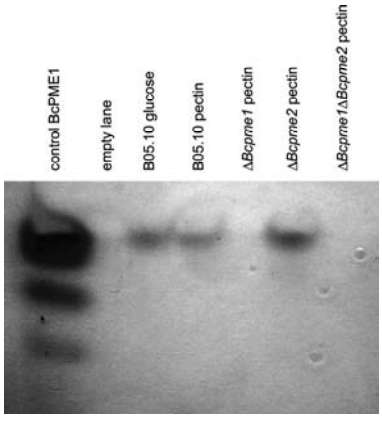

Figure 4. IEF analysis and zymogram of Δ*Bcpme1*, Δ*Bcpme2* and Δ*Bcpme1*Δ*Bcpme2* mutants. BcPME activity in 3-day-old *Botrytis cinerea* liquid cultures, either containing 1% (w/v) glucose or 1% (w/v) lemon pectin (DM 72%). Control BcPME1 was produced in *Pichia pastoris* cultures.

ΔBcpme1ΔBcpme2 mutants show no PME activity in vitro

B. cinerea wild type and mutant strains were grown in liquid cultures containing lemon pectin (DM 75%) as the sole carbon source. The enzymes, which were secreted in the culture filtrate, were analyzed using IEF PAGE and zymography for the presence of PME activity (Figure 4). As control, the culture filtrate of a *Pichia pastoris* transformant expressing *Bcpme*1 cDNA was co-electrophoresed (Figure 4, left lane). The zymogram shows a single band in strain B05.10, which co-migrates with the major band present in the BcPME1 sample expressed in *P. pastoris*. The mutant Δ*Bcpme1* and the double mutant Δ*Bcpme1*Δ*Bcpme2* showed no band of PME activity at this position, whereas a band was present in culture filtrates of the mutant Δ*Bcpme2*, confirming that the band visible in the zymogram represents BcPME1. No other BcPMEs were detected in any of the *B. cinerea* cultures.

BcPMEs are not required for virulence

Conidia of two Δ*Bcpme1*, two Δ*Bcpme2*, two Δ*Bcpme1*Δ*Bcpme2* and two Δ*Bcpg1*Δ*Bcpme1* mutants were used to inoculate tomato and grapevine leaves with 2 µl droplets. Fully expanded compound leaves of 6-week-old tomato plants were detached and inoculated on the adaxial surface, on the right-hand side of the main vein with a mutant strain and the recipient parent on the left-hand side, either strain B05.10 (wild type) or Δ*Bcpg1*-39. Experiments were conducted with two compound tomato leaves per mutant-wild type combination. Grapevine leaves were detached and inoculated with the mutant strains and the recipient parent, either strain B05.10 or Δ*Bcpg1*-39. Seventeen grapevine leaves were inoculated in one experiment. The percentage of primary lesions that expanded beyond the size of the inoculum droplet was > 92% on tomato and > 80% on grapevine for both wild type strain B05.10 and all Δ*Bcpme* mutant strains (Table 3). The Δ*Bcpg1* mutant showed a reduced proportion of expanding lesions as was previously described by ten Have et al. (1998). The Δ*Bcpg1*Δ*Bcpme1* double mutants, however, showed a proportion of expanding lesions similar to the wild type and Δ*Bcpme* mutant strains, for reasons that are not understood.

In total, 20 to 30 inoculation spots on tomato as well as 5 to 17 inoculation spots on grapevine leaves were analyzed per experiment per mutant-wild type combination. Expanding lesions were measured 3 days post inoculation and the average lesion size and standard deviation were calculated (Tables 4 and 5). All four single Δ*Bcpme* mutants (Δ*Bcpme1*-1B, Δ*Bcpme1*-11A, Δ*Bcpme2*-1B and Δ*Bcpme2*-2B) showed similar lesion sizes as the wild type strain on both tomato and grapevine (Table 4, Figure 5). Furthermore, the lesion sizes of both Δ*Bcpme1*Δ*Bcpme2* double mutants were similar to those of the wild type strain B05.10 (Table 4, Figure 5) and the lesion sizes of both Δ*Bcpg1*Δ*Bcpme1* mutants were similar to those of the Δ*Bcpg1* mutant (Table 5).

Table 3. Number of expanding lesions after grapevine and tomato leaf infection by *Botrytis cinerea*.

	# Expanding lesions / total number of inoculation spots*									
	Controls		Δ*Bcpme1*		Δ*Bcpme2*		Δ*Bcpme1*Δ*Bcpme2*		Δ*Bcpg1*Δ*Bcpme1*	
Plant	**B05.10**	**Δ*Bcpg1***	**1B**	**11A**	**1B**	**2B**	**1A**	**3A**	**1A**	**4A**
Tomato	176/180	5/30	30/30	30/30	29/30	30/30	30/30	30/30	18/20	20/20
Grapevine	34/34	5/5	17/17	17/17	17/17	16/17	17/17	17/17	4/5	5/5

* Lesions were classified as expanding when their diameter was larger than the inoculation droplet (2 mm). Results shown here are of one experiment. The results of additional experiments were similar.

Table 4. Average radial lesion size caused by Δ*Bcpme1*- and Δ*Bcpme2*-deficient strains on tomato and grapevine leaves.

	Radial lesion size +/- SD (mm)*						
	Control	ΔBcpme1		ΔBcpme2		ΔBcpme1ΔBcpme2	
Plant	**B05.10**	**1B**	**11A**	**1B**	**2B**	**1A**	**3A**
Tomato	13.5 ± 2.0 (n=176)	14.2 ± 1.7 (n=30)	13.3 ± 1.5 (n=30)	14.7 ± 1.3 (n=29)	13.5 ± 1.6 (n=30)	13.6 ± 1.8 (n=30)	13.4 ± 1.2 (n=30)
Grapevine	10.5 ± 3.3 (n=34)	10.6 ± 3.3 (n=17)	10.9 ± 3.2 (n=17)	11.4 ± 4.1 (n=17)	11.5 ± 3.9 (n=16)	11.7 ± 4.1 (n=17)	11.2 ± 3.7 (n=17)

* Data shown here are the results of one experiment. The results of a second experiment were similar. Lesions were measured 3 days post inoculation. n = total number of inoculation spots per strain. SD = standard deviation.

Table 5. Radial lesion size caused by the double mutants Δ*Bcpg1*Δ*Bcpme1*-1A and Δ*Bcpg1*Δ*Bcpme1*-4A on tomato and grapevine leaves.

	Radial lesion size +/- SD (mm)*			
	Control	Control	Double Mutants Δ*Bcpg1* Δ*Bcpme1*	
Plant	B05.10	**Δ*Bcpg1***	**1A**	**4A**
Tomato	-	10.3 ± 0.9 (n=5)	11.7 ± 1.9 (n=18)	13.7 ± 1.2 (n=20)
Grapevine	9.5 ± 3.4 (n=10)	4.1 ± 2.2 (n=5)	6.0 ± 2.3 (n=4)	5.3 ± 1.8 (n=5)

* Data shown here for tomato and grapevine are the results of one experiment. The results of a second experiment were similar. Both mutants and control strain(s) were inoculated on the same leaves. Lesions were measured 3 days post inoculation. n = total number of inoculation spots per strain. SD = standard deviation. - = not done.

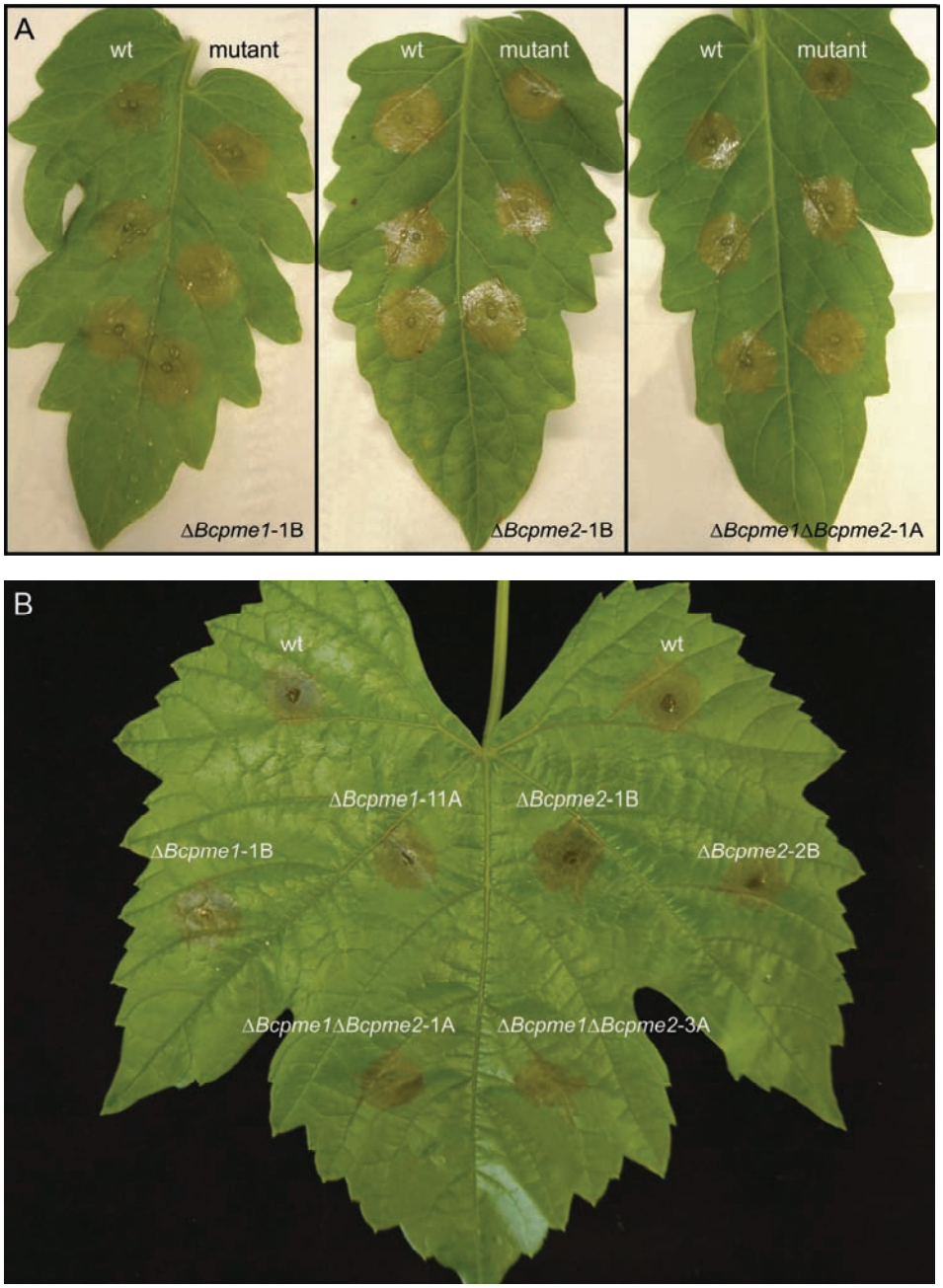

Figure 5. Virulence assays of Δ*Bcpme1*, Δ*Bcpme2* and Δ*Bcpme1*Δ*Bcpme2* mutants on **(A)** tomato leaf and **(B)** grapevine.

BcPMEs are not required for growth on highly methylated pectin

If pectin demethylation was important to permit its depolymerization by endopolygalacturonases, mutants with lower PME activity would presumably show a reduced ability to grow on highly methylated pectin. The growth of the *Bcpme* mutants on highly methylated pectin was compared to the wild type strain B05.10 (Figure 6). Mutants and wild type were grown on purified agar containing B5 medium with 10 mM glucose. Agar plugs were subsequently transferred to large plates containing as sole carbon source either glucose, non-methylated polygalacturonic acid (PGA), 7% methylated pectin or 75% methylated pectin. Four plates of each medium were used within one experiment. The radial growth of all strains was measured after 2 and 3 days. Plates containing B5 medium with glucose were used as controls for growth and morphology. The growth rates of all strains tested were very similar (not shown) and no obvious differences in morphology were observed between the mutants and B05.10 (Figure 6A). Also, no differences were found in radial growth between B05.10 and the different mutants on PGA, or on pectin with a low degree of methylation (Figure 6B, 6C). Even on medium containing 75% methylated pectin, the radial growth of the Δ*Bcpme* mutants was not different from that of the wild type strain B05.10 (Figure 6D). All strains grew better on media with glucose as sole carbon source compared to any supplemented with pectin. Unexpectedly, the strains tested all preferred 75% methylated pectin over 7% methylated pectin or PGA (Figure 6E). The experiment was performed twice with similar results.

Discussion

A PCR-based gene replacement strategy

We devised a PCR-based strategy for generating gene replacement constructs which is versatile, rapid and efficient. The principle of the method is the same as described by Kuwayama et al. (2002) for *Dictyostelium discoideum*, but it has not previously been applied in plant pathogenic fungi. Kämper (2004) described a similar strategy for the plant pathogen *Ustilago maydis*, but this procedure fuses the target gene fragments to the selection marker cassette by means of ligating rare restriction sites that are introduced via the PCR primer. The procedure we describe here circumvents the need for any cloning steps that were previously used for generating *Botrytis cinerea* gene replacement constructs (e.g. van Kan et al., 1997; ten Have et al., 1998) and is simpler than the method of Kämper (2004).

The target gene fragments and selection marker cassettes can be amplified in a first PCR amplification step and fused by overlap-extension in a second PCR amplification step. The use of primers 20 and 21 (Figure 2, step 2) permits universal use of all selection marker cassettes containing the same regulatory elements. The use of primers 22 and 23 (Figure 2,

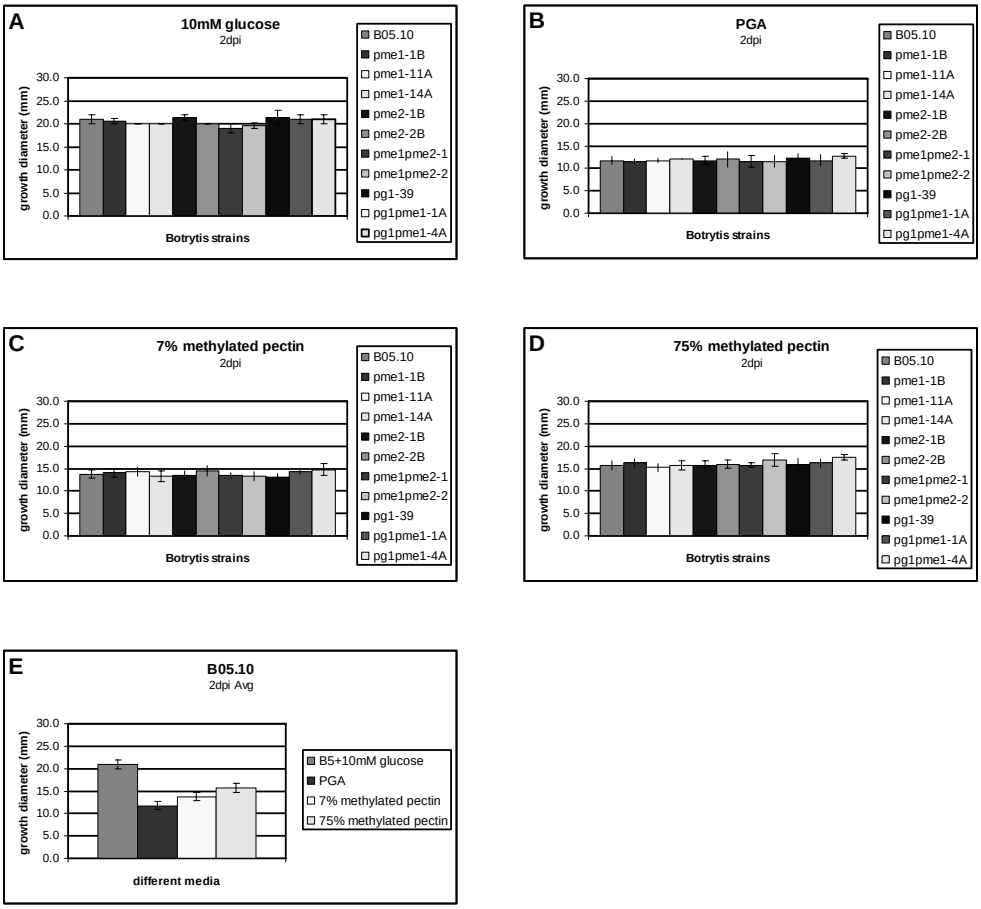

Figure 6. Average radial growth of *Bcpme* wild type and mutant strains on different carbon sources. *Botrytis cinerea* strains were grown on agar plates containing **(A)** glucose, **(B)** PGA, **(C)** 7% methylated pectin or **(D)** 75% methylated pectin as sole carbon source. The diameter of each colony was measured after 2 days. Four plates per experiment were used. Data shown here are the results of one experiment. **(E)** Chart comparing the growth of strain B05.10 on the four different carbon sources.

step 5) for identification of the correct recombinants among the transformants reduces the number of transformants that need to be taken through the laborious procedures of single spore isolation and DNA isolation for Southern analysis.

The method has successfully been applied for over 20 *B. cinerea* genes (unpublished). The efficiency of homologous recombination in these mutagenesis experiments has varied from 12% to well over 75% of the transformants. The bottleneck thus far has been the efficient amplification of the complete overlap extension PCR product (3.1 and 3.7 kb, respectively). The procedure might be further improved by shortening the promoter and

terminator fragments flanking the antibiotic resistance genes. The 844-bp *Aspergillus nidulans* oliC promoter fragment and the 756-bp *B. cinerea* β-tubulin terminator fragment could each possibly be shortened by several hundred base pairs, thereby improving the yield of the amplification products. It remains to be determined whether flanking regions of the target gene can be shortened without affecting the recombination efficiency. Ideally, the flanking regions chosen would be sufficiently short to be added as an extension to the primer used to amplify the selection marker cassette, as used in *Saccharomyces cerevisiae* (Baudin et al. 1993).

Diversity in PMEs between different Botrytis strains

Here we describe the functional analysis of two BcPME-encoding genes of *B. cinerea* strain B05.10. Previous studies on these genes (Reignault et al., 1994, 2000; Valette-Collet et al., 2003) were performed in different strains. *Bcpme1* (Genbank accession AJ309701) originates from strain T4, whereas *Bcpme2* (Genbank accession AJ428403) originates from strain Bd90.

Differences among strains have been reported for the biochemical characteristics of BcPME enzymes (Marcus and Schejter, 1983, 1988; Reignault et al., 1994). Two enzymes purified from *B. cinerea* strain Bd90 (Reignault et al., 1994) differed in their molecular mass and pI values (pI 7.0 and 7.4) from the two enzymes derived from an Israeli strain (pI 6.8, Marcus and Schejter, 1988). Reignault et al. (1994, 2000) detected even more than two bands in zymograms, which may either be different enzymes encoded by distinct genes, or isozymes encoded by the same gene. Site-directed mutagenesis of the *Bcpme*1 gene in *B. cinerea* strain Bd90 resulted in the disappearance of enzyme bands with pI values 7.4 and 6.5 in the zymogram, whereas an enzyme with pI value 7.1 remained detectable in cultures containing pectin (Valette-Collet et al., 2003). This indicates not only that multiple isozymes are encoded by *Bcpme1* but also that at least two truly distinct enzymes (BcPME1 and BcPME2) are produced by strain Bd90. Multiple isozymes with different pI values originating from the same gene could be caused by differences in glycosylation. However, site-directed mutagenesis of glycosylation sites of *A. niger* PMEs has shown that a difference in glycosylation does not affect PME activity on pectin (Warren et al., 2002).

In our experiments only one band was detected by IEF PAGE with overlay gel activity staining, in liquid cultures of *B. cinerea* strain B05.10 containing either glucose or lemon pectin (DM 75%) as sole carbon source. The band certainly represents BcPME1, as it was lacking in the Δ*Bcpme1* mutant and in the Δ*Bcpme1*Δ*Bcpme2* double mutant, but was still detectable in the Δ*Bcpme2* mutant. No activity of PMEs other than BcPME1 was detected in cultures containing lemon pectin. However, the expression or stability of other BcPME isozymes may be very low or they are inactive at the pH used in the zymogram.

BcPMEs are not required for virulence

Replacement of both *Bcpme1* and *Bcpme2* did not affect virulence of *B. cinerea*, when tested on tomato and grapevine (this study), and on pear fruits (A. Akagi and H. Stotz, Oregon State University, pers. comm.).

The assumption that pectin demethylation by PMEs is required prior to the (efficient) depolymerization by endopolygalacturonases, would predict that BcPMEs are important for growth on highly methylated pectin and for virulence. The phenotype of the *Bcpme1* mutant reported by Valette-Collet et al. (2003) did support this hypothesis. However, our results with the single and double *Bcpme* mutants do not support the hypothesis. One possible explanation could be that *B. cinerea* contains additional *Bcpme* genes that take over the function of *Bcpme1* and *Bcpme2* in the mutants. Indeed the full genome sequence of strain B05.10 that has recently been made public (http://www.broad.mit.edu/cgi-bin/annotation/fungi/botrytis_cinerea/download_license.cgi) contains one additional gene (denominated *Bcpme*3), which has all the typical features of a PME and is in many respects similar to BcPME1 and BcPME2 (not shown). The *Bcpme*3 gene displayed an expression pattern *in planta* that is very similar to the expression of *Bcpme*1 and *Bcpme*2, shown in Figure 1 (I. Kars, L. Wagemakers and J.A.L. van Kan, unpublished). Even when additional *Bcpme* genes are present in the *B. cinerea* genome, the only enzyme activity that was detected in the wild type grown in liquid cultures containing lemon pectin was apparently due to BcPME1. Single or double mutants lacking the *Bcpme*1 gene did not display any residual activity in the zymogram (Figure 4) unlike the situation in the Bd90 *Bcpme1* mutant (Valette-Collet et al., 2003). Could there be *Bcpme* genes that are exclusively expressed *in planta? Erwinia chrysanthemi* can produce two sets of pectate lyases; one set of enzymes is produced *in vitro* in minimal media containing pectate, while the second set of enzymes is exclusively expressed *in planta* (Kelemu and Collmer, 1993). If *B. cinerea* would possess additional *Bcpme* genes, these are unrelated in sequence to the three known *Bcpme* genes.

The wild type level of virulence of the *Bcpme* mutants would be fully explained if *B. cinerea* is able to depolymerize pectin efficiently without prior demethylation by BcPMEs. In this respect one should consider the possible role of pectin lyases, enzymes that may depolymerize methylated pectin (Chilosi and Magro, 1997). *B. cinerea* contains at least one gene encoding a pectin lyase, *Bcpnl*. However, a time-course infection assay on tomato showed that expression of *Bcpnl* was very low. The transcript was detectable at 24 hpi and did not increase with the fungal biomass (chapter 4). Moreover, pectin lyases are generally considered to have a basic pH optimum (Chilosi and Magro, 1997; 1998) and *B. cinerea* is renowned for acidifying its environment by means of oxalate (Germeier et al., 1994; Verhoeff et al., 1988). Lyases would likely not be very active in such an environment. Therefore, it seems doubtful that pectin lyases play a role in virulence. The only pectin depolymerizing enzymes that are active in acidic environment are endopolygalacturonases. Targeted

mutagenesis has shown that *Bcpg1* (ten Have et al, 1998) and *Bcpg2* (Kars et al., 2005a) are required for full virulence. Studies with a range of substrates with different DM have shown that the activity of purified BcPGs *in vitro* decreases with increasing DM (Kars et al., 2005a). The observation that the wild type *B. cinerea* and all mutants grow better on 75% methylated pectin than on PGA in fact suggests that pectin demethylation is not important for depolymerization *in vivo*.

How can we explain our results with those of Valette-Collet et al. (2003) who reported that a *Bcpme1* knockout mutant in a different strain, Bd90, is severely reduced in virulence and growth on pectin? Siewers et al. (2005) demonstrated that another virulence factor of *B. cinerea*, the phytotoxic metabolite botrydial, could also act in a strain-specific manner. *B. cinerea* is apparently a very versatile plant pathogen with multiple infection mechanisms with functional redundancy. Therefore, to validate virulence factors by targeted mutagenesis in future studies we suggest that several strains should be used, as also discussed by Tudzynski and Siewers (2004).

Experimental Procedures

Fungal strain and growth conditions

Botrytis cinerea strain B05.10 (van Kan et al., 1997) and mutant strains were grown and maintained as described (Wubben et al., 2000). For growth experiments, mutants and wild type were grown on purified agar containing Gamborg B5 (Duchefa, Haarlem, the Netherlands) with 10 mM glucose. Small agar plugs with a diameter of 2 mm were subsequently transferred to fresh agar plates (15 cm) containing 0.5 x B5 medium and as sole carbon source either 10mM glucose, 0.5% (w/v) PGA, 0.5% (w/v) lemon pectin (DM 7%) or 0.5% (w/v) lemon pectin (DM 75%). Four plates of each medium were used within one experiment. Strains were grown on these carbon sources in darkness at 20°C. Radial growth was measured daily. For IEF-PAGE analysis, *B. cinerea* strains B05.10, Δ*Bcpme1*, Δ*Bcpme2* and Δ*Bcpme1*Δ*Bcpme2* were grown for 3 days in liquid cultures containing either 1% (w/v) glucose or 1% (w/v) pectin (DM 75%). Mycelia were removed from the cultures by filtration over Mira Cloth membranes and the resulting filtrate was dialyzed and concentrated prior to IEF.

DNA and RNA analysis

Detached tomato leaves were spray-inoculated with *B. cinerea* strain B05.10 according to procedures described by Benito et al. (1998) and harvested at various time points after inoculation, with 8 h intervals. Isolation of total RNA from infected tomato leaves, northern blotting and hybridization were performed as described by Prins et al. (2000). Amplification products of *Bcpme1*, *Bcpme2* and *BcactA* were used as probes. *B. cinerea* genomic DNA

was isolated using either standard chlorophorm/isoamylalcohol DNA isolation method (Möller et al., 1992), or GenElute Plant Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis MO, USA).

Gene replacement strategy

Amplification of two target-gene fragments (400-650bp) was performed with primers (primers 5.1 or 3.1) chosen just outside the ORF and primers (primers 5.3 or 3.3, respectively) with an extension complementary to respectively 21 bp and 26 bp of the selection marker cassette (Figure 2). The amplification was carried out in a reaction volume of 50 µl using 1 U Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany) with appropriate PCR buffer, 1 µM of each primer, 0.2mM each dNTP and 10-50 ng DNA. The PCR conditions were as follows: heating at 95°C for 3 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min; followed by a final extension at 72°C for 5 min. Primers are listed in Table 1.

Two types of selection marker cassettes were used in the gene replacement procedure: a cassette (2.1 kb) carrying the *nat* gene for resistance to nourseothricin; the *hph* cassette (2.7 kb), carrying resistance to hygromycin B. In both cassettes, the resistance gene was flanked by the *Aspergillus nidulans* oliC promotor and the *B. cinerea* β-tubulin terminator. The plasmid carrying the *nat* cassette (pNR1) was a generous gift of Dr. Bettina Tudzynski (Muenster, Germany). For our use, the insert was cut out of this vector with restriction enzymes XbaI and HindIII and ligated into pUC18 to create pNR2. The plasmid carrying the *hph* cassette (pLOB1) was previously developed in our laboratory (Genbank accession AJ439603). The selection marker cassettes were amplified with primers 20 and 21 (Figure 2, Table 1). The amplification was carried out in a reaction volume of 100 µl using 2.25 U Expand High Fidelity polymerase (Roche Diagnostics, Mannheim, Germany) with appropriate PCR buffer, 0.4 µM of each primer, 0.2 mM dNTPs and 50-100 ng plasmid DNA. The PCR conditions were: heating at 95°C for 2 min; 10 cycles of 95°C for 15 s, 50°C for 45 s, 72°C for 3 min; 20 cycles of 95°C for 15 s, 50°C for 45 s, 72°C for 3 min increasing with 5 s/cycle; followed by a final extension at 72°C for 5 min.

Overlap-extension PCR was used to fuse the two target-gene fragments to the selection marker cassette in a single PCR amplification step using primers 5.2 and 3.2 (1µM each; Table1). The amplification was carried out in a reaction volume of 50 µl with 0.2mM of each dNTP, 1.5 U Expand High Fidelity polymerase (Roche Diagnostics, Mannheim, Germany) and the appropriate PCR buffer. The PCR conditions and other PCR ingredients differed depending on the selection marker cassette used. The *nat* gene has a much higher GC ratio than the *hph* gene, therefore, a longer denaturation time was used as well as a different ramp time. Also, different DNA template concentrations were used. For gene fusion to the hygromycin cassette equal amounts (20 ng each) of the three templates were used. If the

nourseothricin cassette was used, a PCR reaction with 120 ng DNA of the selection marker cassette and 15 ng of the gene fragments gave the best amplification products. PCR conditions used for amplification of the *hph* construct were: initial cycle of 94°C for 5 min, 55°C for 2 min, 72°C for 8 min; 30 cycles of 92°C for 30 s, ramp of 1.2°C/s to 55°C, 55°C for 2 min, ramp of 0.1°C/s to 72°C, 72°C for 7 min, ramp of 0.2°C/s to 92°C; followed by a final extension at 72°C for 8 min. Conditions for amplification of the *nat* construct were: initial cycle of 94°C for 5 min, 55°C for 2 min, 72°C for 8 min; 30 cycles of 94°C for 1 min, ramp of 1.2°C/s to 55°C, 55°C for 2 min, ramp of 0.1°C/s to 72°C, 72°C for 7 min, ramp of 0.2°C/s to 94°C], followed by a final extension at 72°C for 8 min. The amplified constructs were purified using Quickstep PCR purification spin columns (Edge BioSystems, Gaithersburg MD, USA) or purified from gel using the GFX PCR DNA and gel band purification kit (Amersham Biosciences, Little Chalfont, UK).

Botrytis cinerea transformation

B. cinerea Pers.:Fr. (teleomorph *Botrytis fuckeliana* (de Bary) Whetzel) strain B05.10 was used for transformation experiments. B05.10 was grown on potato dextrose agar (Oxoid, Basingstoke, UK) with additional homogenized tomato leaves (PDA-tom) at 20°C. Overnight treatment of the fungus with near UV light induced conidiation. Mature conidia were harvested and were germinated for 20 h in liquid culture (1% Malt Extract, Difco Laboratories, Detroit MI, USA). The young mycelium was treated with Glucanex (10 mg/ml) in order to produce protoplasts, which were transformed using 2-5 μg DNA and the PEG procedure (ten Have et al., 1998). Transformed protoplasts were plated on regeneration medium (SH) with sucrose, HEPES and ammonium phosphate. The next day, an overlay containing SH with the appropriate selection, 100 μg/ml hygromycin B (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) or 100 μg/ml nourseothricin (Jena Bioscience, Jena, Germany), was added to the plates. Resistant colonies were excised and grown on Malt Extract agar (MEA; Oxoid, Basingstoke, UK) with antibiotic selection. Hyphal tips were then excised and transferred to plates without selection medium. Subsequently, hyphal tips were for the second time transferred to plates with selection medium. After one round of single spore isolation, genomic DNA was isolated for PCR screening and Southern hybridization. Double mutants were made as described above with one adaptation: one mutant strain was used as recipient strain instead of B05.10.

Molecular analysis of the mutants

The initial screening of the *B. cinerea* transformants was done by PCR. Amplification was carried out using the same ingredients and conditions as described above for the target gene fragments (section Gene replacement strategy). Primer combinations used for the PCR screening were primers 5.1 and 23, 3.1 and 22, 5.1 and 3.1 (Table 1). Southern

hybridization was used to confirm the recombination, according to the protocol in ten Have et al. (1998).

Plant growth and virulence assays

Tomato plants (*Lycopersicum esculentum*) cv. Moneymaker were grown in potting soil for 6 weeks in the greenhouse at 23°C with a 16 h photoperiod. Tomato potting soil was supplemented with nutrients as described in ten Have et al. (1998). Grapevine plants cv. Albalonga were obtained at the vineyard "de Agthysen" (Zuidland, the Netherlands) and maintained in a climate chamber at 20°C with a 16 h photoperiod.

In virulence assays detached tomato and grapevine leaves were inoculated with *B. cinerea* spore suspensions harvested from 10-day-old cultures. Aliquots of 2 µl 10^6 conidia/ml Gamborg B5 medium (Duchefa, Haarlem, The Netherlands), supplemented with 10 mM glucose and 10 mM potassium phosphate pH 6.0 (Benito et al., 1998), were applied to the adaxial plant surface. Inoculated leaves were placed in closed containers in dark with high humidity (approx. 100%). Symptom development was followed and lesion diameter was usually measured after 3 days. Lesion sizes caused by the mutants were compared to those of the recipient strain. All virulence assays presented here were at least duplicated. The average lesion size and corresponding standard deviation were calculated for each strain.

IEF-PAGE analysis and activity staining

In order to detect PME production in *B. cinerea*, liquid cultures containing pectin were grown for 3 days and 20 µl samples of the culture filtrates were analyzed on an isoelectric focusing gel (IEF-PAGE). The experimental conditions used in this study were similar to those previously described by Valette-Collet et al. (2003). Focusing was performed in a pH gradient of 4-10 (Bio-Rad, Hercules, CA, USA) running for 2 h at 200 v. Following electrophoresis, the gels were washed extensively in a phosphate-citrate (McIlvain) buffer, pH 6.5, to re-activate the PMEs. PME activity was visualized in the IEF-PAGE gels by zymography (as described in Kars et al., 2005a) using agarose overlay gels containing 0.5 % (w/v) lemon pectin (DM 75%) as substrate. Following incubation, overlay gels were stained in 0.02% (w/v) ruthenium red for at least 30 min until red bands were visible representing de-methylation of pectin by the action of PMEs. PG activity was visible as a white band representing degradation of pectin and could mask PME activity in the culture filtrates. As a positive control, BcPME1 was produced in *Pichia pastoris* cultures, as described for BcPGs (Kars et al., 2005a). Filtrates from *P. pastoris* cultures, induced for heterologous gene expression with methanol, were concentrated, dialyzed and used without further purification for IEF.

Acknowledgements

We acknowledge Arjen ten Have for his ideas on the gene replacement strategy. Furthermore, we thank Geja Krooshof, for experimental help with IEF and PME overlay activity assay, Brian Williamson and Pierre de Wit for critical reading of the manuscript, Bert Essenstam and Pieter Kuiken (Unifarm) for excellent plant care. The research was funded by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (project WGC.5034).

Chapter 3

Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*

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This chapter is published as: Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A.E., and van Kan, J.A.L. (2005) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J., 43, 213-225.

Abstract

Five *Botrytis cinerea* endopolygalacturonases (BcPGs) were individually expressed in *Pichia pastoris*, purified to homogeneity and biochemically characterized. Although the pH optima of the five enzymes were similar (~pH 4.5) the maximum activity of individual enzymes differed significantly. For hydrolysis of polygalacturonic acid (PGA), the $V_{max,app}$ ranged from 10 to 900 U/mg, while the $K_{m,app}$ ranged from 0.16 to 0.6 mg/ml. BcPG1, BcPG2 and BcPG4 are single-attack enzymes as hydrolysis of PGA leads to the accumulation of oligomers with DP<7, whereas hydrolysis of PGA by BcPG3 and BcPG6 leads to the accumulation of monomers and dimers suggesting that they are multiple-attack (processive) enzymes. The necrotizing activity of all BcPGs was tested separately in tomato, broad bean and *Arabidopsis thaliana*. They showed different necrotizing activities on these plants. BcPG1 and BcPG2 possessed the strongest necrotizing activity as tissue collapse was observed within 10 minutes after infiltration of broad bean leaves. The amino acid D192A substitution in the active site of BcPG2 not only abolished enzyme activity but also the necrotizing activity, indicating that the necrotizing activity is dependent on enzyme activity. Furthermore, deletion of the *Bcpg*2 gene in *B. cinerea* resulted in a strong reduction in virulence on tomato and broad bean. Primary lesion formation was delayed by approximately 24 hours and the lesion expansion rate was 50 to 85% reduced. These data indicate that BcPG2 is an important virulence factor for *B. cinerea*.

Introduction

Plant cell walls give cells their structure, rigidity and protect them against abiotic and biotic factors including osmotic and drought stress and pathogens. Many bacterial and fungal plant pathogens are known to produce a spectrum of cell wall-degrading enzymes enabling them to degrade different types of cell wall polymers such as cellulose, hemi-cellulose and pectin (Walton, 1994). Especially enzymes that attack pectic substances are thought to play a major role in pathogenesis (Clark and Lorbeer, 1976; Cole et al., 1998; Collmer and Keen, 1986). Targeted mutagenesis of pectinase-encoding genes in several plant pathogenic fungi has provided functional evidence for the contribution of these enzymes to virulence (Isshiki et al., 2001; Oeser et al., 2002; Shieh et al., 1997; ten Have et al., 1998). A clear demonstration of damage caused by pectinases was provided by transient expression of a *Colletotrichum lindemuthianum* endopolygalacturonase (ClPG1) in tobacco. *Agrobacterium tumefaciens*-mediated expression of ClPG1 caused degradation of pectic substances both in junction zones and the middle lamellae of parenchyma tissue, leading to cell separation and necrosis (Boudart et al., 2003).

B. cinerea is an opportunistic plant pathogenic fungus able to cause rot in many plant tissues. Microscopic observations of *B. cinerea*-infected tissue have demonstrated that anticlinal host cell walls penetrated by hyphae swell by water absorption, which is indicative of pectin hydrolysis (Mansfield and Richardson, 1981). In later stages, the host cells adjacent to swollen walls die and collapse. Pectin hydrolysis not only weakens the cell wall to facilitate penetration and colonisation of the host, it also provides the fungus carbon sources for its growth, and this seems to be crucial in the infection process of *B. cinerea*. The fungus produces a variety of pectinases, including exo- and endo-polygalacturonases, pectin methylesterases, pectin and pectate lyases (reviewed by Kars and van Kan, 2004; ten Have et al., 2002). The *B. cinerea* genome contains at least six endopolygalacturonase-encoding (*Bcpg*) genes (Wubben et al., 1999), of which *Bcpg1* is required for full virulence (ten Have et al., 1998). Although the expression patterns of the *Bcpg* genes have been studied in liquid cultures (Wubben et al., 2000) and in infected plant tissues (ten Have et al., 2001), little is known about the biochemical properties of their products. Johnston and Williamson (1992) succeeded in purifying and partially characterising two endopoly-galacturonases from *B. cinerea* cultures, but it is not known which *Bcpg* genes encodes the proteins, as the protein sequences were not reported.

Based on the broad host range of *B. cinerea*, the existence of multiple gene family members and the different expression patterns of the individual *Bcpgs*, we hypothesised that the BcPGs would have distinct enzymatic properties and might have different biological activities and functions *in planta*. To test this hypothesis, we produced all five BcPGs individually in *P. pastoris*, characterized their biochemical properties and determined the necrotizing activity of each enzyme in a range of plant species. BcPG1 and BcPG2 showed

the highest necrotizing activities, inflicting rapid tissue collapse and subsequent necrosis. Therefore, a mutant of *B. cinerea* lacking the *Bcpg2* gene was made to study its role in pathogenesis.

Results

Production of Botrytis cinerea endopolygalacturonases in Pichia pastoris

The endopolygalacturonases cannot be purified individually to a high degree and in reasonable quantities from liquid cultures of *B. cinerea*. Not only are the production levels in *B. cinerea* low, the high viscosity due to the extensive production of fungal polysaccharides hampers the purification. Therefore, the methylotrophic yeast *P. pastoris* GS115 was used as heterologous host to produce significant amounts of pure individual BcPGs. Untransformed GS115 cells do not show polygalacturonase activity (not shown). Coding sequences of the genes *Bcpg*1, *Bcpg*2, *Bcpg*3, *Bcpg*4, *Bcpg*5 and *Bcpg*6 were cloned in the expression vector pPIC3.5. For each of the six gene constructs, several *P. pastoris* transformants were tested for enzyme activity in the culture supernatant after induction with methanol. Enzyme production was confirmed for BcPG1, BcPG2, BcPG3, BcPG4 and BcPG6, but was unsuccessful for BcPG5 for unknown reasons. BcPG-producing *P. pastoris* transformants were grown in a fermentor under controlled conditions. Maximum levels of active BcPG were reached after three to four days of induction. Production levels of BcPGs were reproducible for individual enzymes but varied significantly between the different enzymes, ranging from 5 mg/l for BcPG1 to 1000 mg/l for BcPG3 (Table 1). The production levels of BcPG1 were also low when produced in *Aspergillus* species (Benen et al., unpublished).

Characterization of recombinant BcPGs

Each recombinant BcPG was purified from clarified *P. pastoris* culture liquid by a two-step chromatography purification protocol as described in the Experimental procedures section. The overall yield of pure protein of the different BcPGs ranged from 0.5 to 500 mg per fermentation (Table 1). SDS-PAGE revealed a single protein band in the case of BcPG1 and BcPG3; multiple protein bands were detected in purified preparations of BcPG2, BcPG4 and BcPG6 (Figure 1A). N-terminal amino acid (aa) sequencing and mass-spectrometry confirmed the identity of the protein bands. Zymography following non-denaturing SDS-PAGE confirmed that all protein bands detected on the gel (Figure 1A) represented active polygalacturonases, as shown in Figure 1B for BcPG2 and BcPG6. Migration on the SDS-PAGE gel of BcPG1 and BcPG2 was in agreement with the molecular masses calculated on the basis of the aa sequences (Figure 1A, Table 1), whereas BcPG3, BcPG4 and BcPG6 migrated slower than predicted.

Table 1. Results of the purification and biochemical characterization of BcPGs produced in yeast.

BcPG	Mw (kDa) calc / app	pI_{calc}	pH optimum	$V_{max, app}$ (U/mg)	$K_{m, app}$ (mg/ml)	Production level (mg/l)	Yield pure enzyme (mg)
1	34 / 36	8.1	4.2	10	0.6	5	0.5
2	35 / 38	7.8	4.5	900	0.44	15	5
3	49 / 60	4.1	3.2 - 4.5	100	0.27	1000	500
4	36 / 50	4.2	4.9	20	0.33	100	10
6	36 / 60	4.8	4.5	150	0.16	500	100

Steady-state kinetic parameters were determined with PGA as substrate. Experiments were repeated at least two times with at least three replicates for each sample. Calc: calculated; app: apparent; $V_{max, app}$ in U/mg protein; $K_{m, app}$ in mg/ml substrate; production level in mg/l culture liquid.

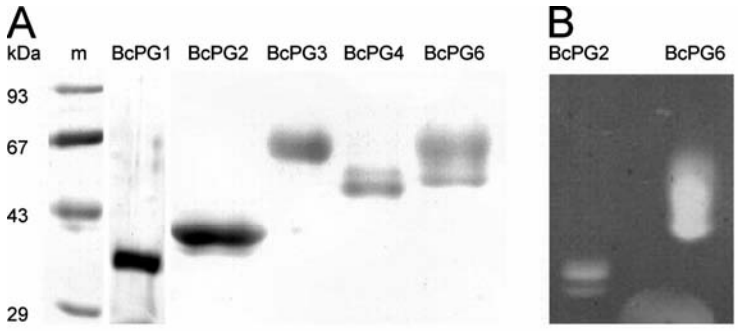

Figure 1. Purity and activity of recombinant *Botrytis cinerea* endopolygalacturonases. Lane 2-5 each contain approximately 10 µg BcPG. **(A)** SDS-PAGE showing silver-stained BcPG1 and coomassie brilliant blue R250-stained BcPG2, BcPG3, BcPG4, and BcPG6. m = molecular mass markers in kiloDaltons. **(B)** Zymogram of purified BcPG2 and BcPG6. The enzymes were separated by non-denaturing SDS-PAGE, renatured in sodium acetate, pH 4.2, and incubated with a 0.1% (w/v) PGA-agarose overlay gel. The overlay gel was stained with ruthenium red. A white halo is an indication of PGA hydrolysis.

Optimal enzyme activity using polygalacturonic acid (PGA) as substrate was observed at pH 4.2 for BcPG1, pH 4.9 for BcPG4, and pH 4.5 for BcPG2 and BcPG6. Purified BcPG3 displayed a broader pH optimum ranging from pH 3.2 to 4.5 (Table 1). The maximum activity of individual enzymes, however, differed markedly. For hydrolysis of PGA at pH 4.2 and 30°C, the $V_{max,app}$ values ranged from 10 to 900 U/mg, while the $K_{m,app}$ values ranged from 0.16 to 0.6 mg/ml (Table 1). The specific activities suggest that PGA is a poor substrate for BcPG1 and BcPG4 when compared to BcPG2, BcPG3 and BcPG6. The substrate preference of the enzymes was further tested using lemon pectin with a degree of methylation (DM), ranging from 7 to 75%. BcPG1, BcPG2 and BcPG4 preferred the non-methylated substrate PGA to pectin, whereas BcPG3 and BcPG6 showed highest activity on

7% and 22% methylated pectin, respectively. All BcPGs showed some residual activity on pectin with a DM of 75% (not shown).

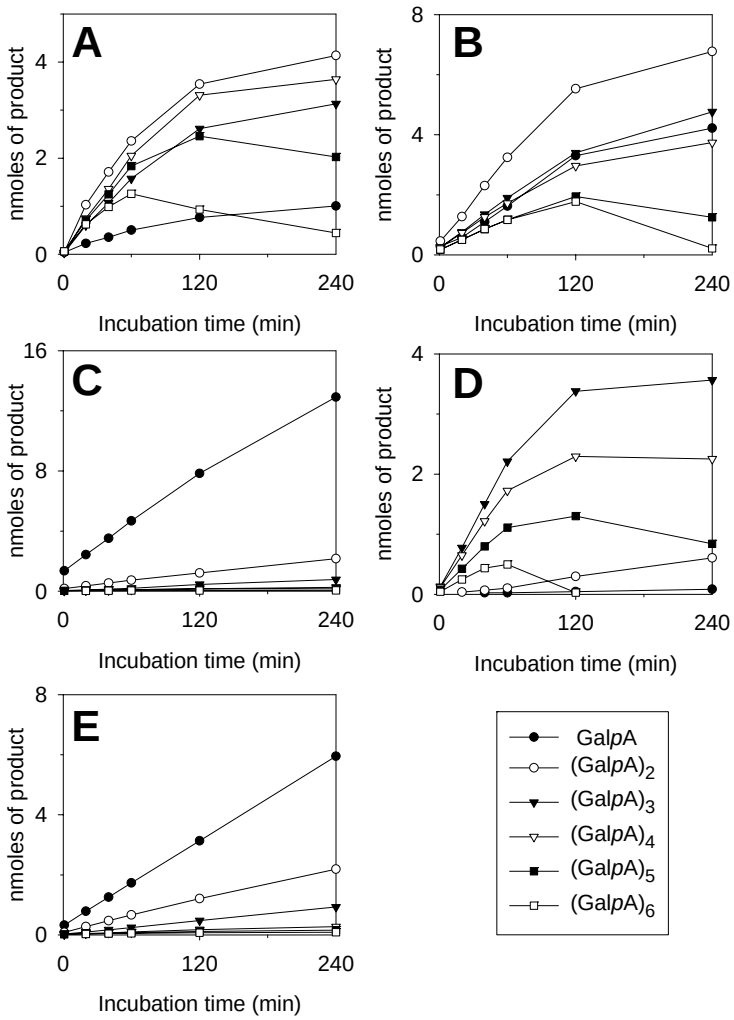

Figure 2. Transient accumulation of oligomers ((Gal*p*A)$_n$) released from PGA during incubation with different BcPGs. Hydrolysis of 0.25% (w/v) PGA was performed in 1ml 50mM sodium acetate, pH 4.5, at 30°C. **(A)** Incubation with 2µg BcPG1; **(B)** incubation with 22ng BcPG2; **(C)** incubation with 200ng BcPG3; **(D)** incubation with 1µg BcPG4; **(E)** incubation with 133ng BcPG6. Products were identified by HPAEC-PAD; symbols for the oligomers are indicated in the box right of panel E.

To obtain more insight in the mode of action of individual BcPGs, product formation was monitored during digestion of PGA in a time-course experiment. The product progression was typical for enzymes with a random endo-hydrolytic mode of action: the polymeric substrate PGA was rapidly cleaved into a broad range of long-chain oligogalacturonides (OGAs; oligomers) with a degree of polymerisation higher than 10 (DP>10), which were further converted into smaller oligomers. Figure 2 shows the product progression profiles of oligomers with DP<7 released from PGA by the pure BcPGs. The transient accumulation of these oligomers is clearly visible in the product progression profiles of BcPG1, BcPG2 and BcPG4 (Figure 2A, B, D). In the final stages of the reaction, predominantly monomers and dimers had accumulated for BcPG1 and BcPG2, whereas mainly trimers had accumulated for BcPG4 (not shown). This profile is typical of single-attack enzymes, which cleave a substrate molecule only once during an encounter. In contrast, digestion of PGA with BcPG3 or BcPG6 resulted in a less explicit transient accumulation of oligomers with DP<7. Monomers and dimers accumulated from the start of the reaction (Figure 2C, E). This indicates that BcPG3 and BcPG6 are so-called processive or multiple-attack enzymes, which attack a polymer molecule numerous times during one encounter.

For a more detailed understanding of the cleavage patterns of the recombinant BcPGs, OGAs of defined length (DP 2 to 6) were used as substrates. Table 2 summarises the hydrolysis rates and bond cleavage frequencies during the initial phase of the reactions. Substrate degradation rates increased with increasing chain length of the substrates as can be expected for enzymes with substrate-binding sites comprising multiple subsites. BcPG3 and BcPG6 appeared to be the only enzymes able to hydrolyse dimers. All BcPGs were able to hydrolyse trimers and tetramers, in which case cleavage occurred at the first glycosidic bond from the reducing end. However, when pentamers were used, BcPG1, BcPG2 and BcPG4 displayed a preference for cleaving the second glycosidic bond instead of the first. With hexamers, BcPG1 and BcPG2 still preferred the second glycosidic bond from the reducing end, but BcPG4 showed preference for the third bond, demonstrating that all the enzymes differ with respect to their subsite-architecture. Bond cleavage frequencies could not be determined for BcPG3 and BcPG6 using pentamers and hexamers, since the products deviated from stoichiometry as a result of the processive behaviour of these enzymes on these oligomeric substrates.

Active BcPGs cause tissue collapse and necrosis

Leaf segments of tomato, broad bean and *A. thaliana* were infiltrated with the pure solution of BcPG2, BcPG3, BcPG4 or BcPG6. BcPG1 was infiltrated in broad bean only. Due to the relatively low production levels, loss during purification and low specific activity of pure BcPG1 (Table 1), there was insufficient enzyme available for all infiltration experiments as were carried out with the other BcPGs. Infiltrations were carried out in a protein concentration series ranging from 1 to 10 ng/µl and an enzyme activity series ranging from 1

to 10 U/ml. Symptoms (chlorosis, tissue collapse or necrosis) were monitored over several days. Necrosis is defined as dying tissue, which becomes brown and brittle within a few days. Water, 0.001% (w/v) azide, 10 mM sodium acetate buffer, pH 4.2, culture filtrate of wild type *P. pastoris* and 320 µg/ml bovine serum albumin (BSA), used as controls, did not cause any symptoms when infiltrated in leaves of the different plant species.

The individual purified BcPGs showed different necrotizing activities (NAs) on different plants (Figure 3, Table 3). BcPG1 and BcPG2 showed the highest NA as the tissue collapsed within 10 minutes after infiltration of 3 U/ml (300 ng/µl) BcPG1 or 10 U/ml (11 ng/µl) BcPG2 into broad bean leaves. The mesophyll tissue between veins collapsed within 10 minutes, whereas the veins and the palisade parenchyma remained intact for a few hours. After 48 to 72 hours, the collapsed tissue became necrotic (Figure 3A). In most cases, a chlorotic halo was observed around the edges of the infiltrated sector. The NA of BcPG2 in *A. thaliana* and tomato was also very significant (Figure 3B, C). BcPG3, BcPG4 and BcPG6 showed either lower or no NA when compared to BcPG2, depending on the plant species tested (Table 3).

Table 3. Symptoms in various plant species after infiltration of individual BcPGs.

Enzyme	Activity [a] (U/ml)	Tomato	Broad bean	*A. thaliana* Lip-0 [c]	*A. thaliana* Est-0	*A. thaliana* Oy-0
BcPG1	10	n.d.[b]	n.d.	n.d.	n.d.	n.d.
	3	n.d.	necrosis	n.d.	n.d.	n.d.
	1	n.d.	necrosis	n.d.	n.d.	n.d.
BcPG2	10	necrosis	necrosis	necrosis	chlorosis	discolouration
	3	necrosis	necrosis	necrosis	no symptoms	no symptoms
	1	necrosis	necrosis	necrosis	no symptoms	no symptoms
BcPG3	10	no symptoms	discolouration	necrosis	necrosis	necrosis
	3	no symptoms	no symptoms	necrosis	no symptoms	chlorosis
	1	no symptoms	no symptoms	necrosis	no symptoms	no symptoms
BcPG4	10	necrosis	necrosis	necrosis	chlorosis	necrosis
	3	necrosis	no symptoms	necrosis	chlorosis	chlorosis
	1	no symptoms	no symptoms	necrosis	chlorosis	chlorosis
BcPG6	10	necrosis	no symptoms	necrosis	no symptoms	n.d.
	3	no symptoms	no symptoms	necrosis	no symptoms	chlorosis
	1	no symptoms	no symptoms	necrosis	no symptoms	no symptoms

Symptoms were observed 4 days post infiltration. [a] Enzyme activity was determined with PGA as substrate prior to and after infiltration. [b] n.d. = not done. [c] Results obtained with *Arabidopsis thaliana* accessions Col-0 and Kas-0 were essentially similar to those obtained with Lip-0. In each experiment, at least four infiltrations were performed per enzyme-plant combination. Experiments were performed at least twice with independently produced and purified batches of enzymes, with essentially similar results.

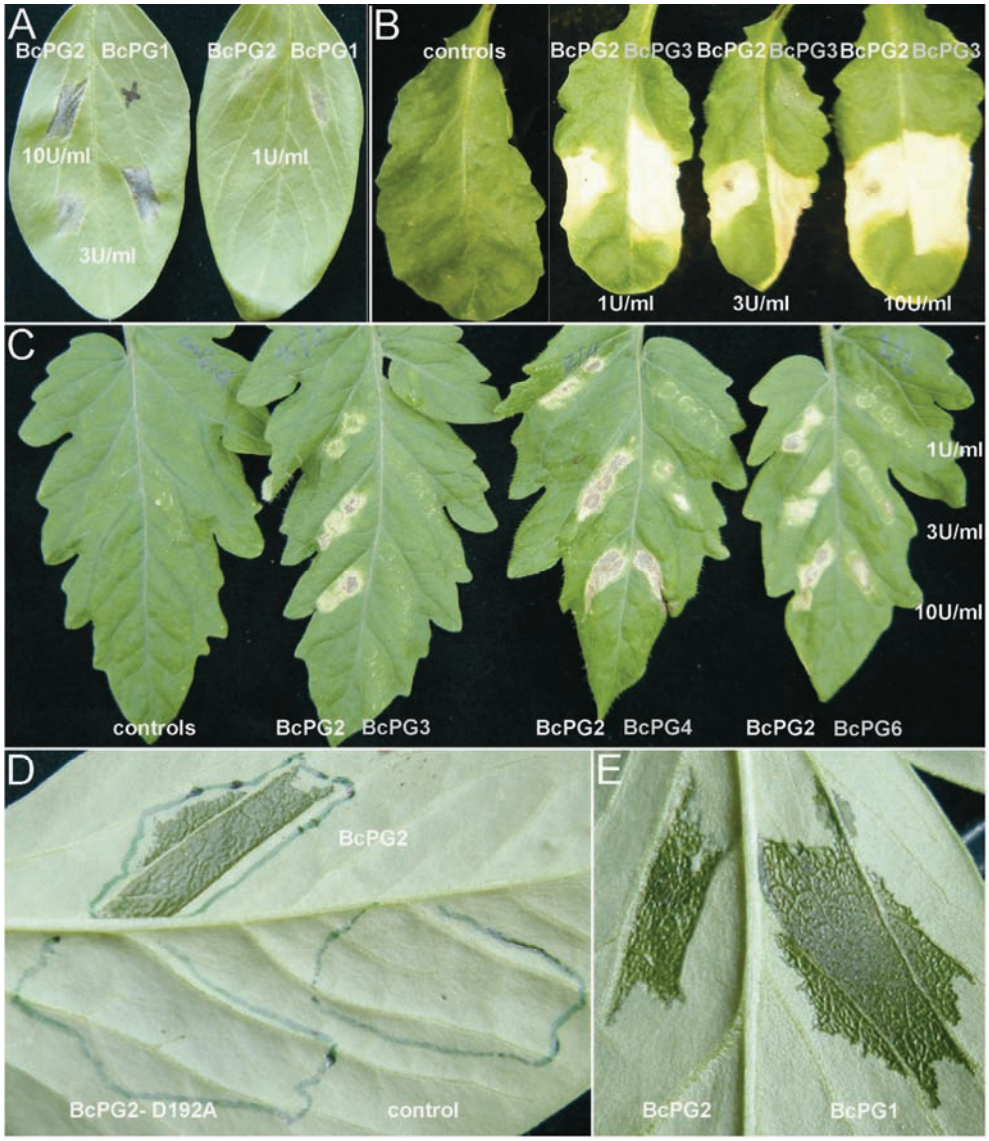

Figure 3. Symptoms caused upon infiltration of different BcPGs in broad bean, *Arabidopsis thaliana* and tomato leaves. All BcPGs were infiltrated in a range of 1, 3 and 10 U/ml (as indicated). **(A)** Broad bean leaves infiltrated with BcPG1 and BcPG2. **(B)** *A. thaliana* accession Lip-0 infiltrated with BcPG2 and BcPG3. **(C)** Tomato leaves infiltrated with BcPG2, BcPG3, BcPG4 and BcPG6. **(D)** Broad bean infiltrated with active BcPG2 (10 U/ml, 40 ng/µL) and mutant protein BcPG2-D192A (400 ng/µl). The infiltrated sectors are marked. **(E)** Close-up of collapsed broad bean tissue 10 minutes after infiltration with BcPG1 and BcPG2. Water and 10 mM sodium acetate, pH 4.2, were used as controls.

In tomato leaves, BcPG2 showed NA at all concentrations tested, whereas BcPG4 and BcPG6 only showed NA at 3 and 10 U/ml, respectively (Figure 3C). BcPG3 did not show NA in tomato leaves at any concentration tested. In broad bean leaves BcPG2 showed NA at all concentrations tested and BcPG4 at 10 U/ml only. No NA was observed for BcPG3 and BcPG6. BcPG3 caused some tissue discoloration scattered over the infiltrated sector, but this never developed into dry necrosis as observed for BcPG2 and BcPG4 (Table 3). In *A. thaliana* accessions Lip-0, Col-0 and Kas-0, BcPG2, BcPG3, BcPG4 and BcPG6 showed NA at all tested concentrations. In contrast, in *A. thaliana* accessions Est-0 and Oy-0, BcPG2, BcPG3, BcPG4 and BcPG6 caused only some or no chlorosis (Table 3).

In order to distinguish whether symptom development was caused by the enzymatic activity of BcPG2 or solely by protein recognition, an inactive mutant of BcPG2 was generated by changing the active-site residue Aspartate (D) 192 into Alanine (A), a mutation that blocks enzyme activity but does not affect protein structure (Armand et al., 2000). The BcPG2-D192A mutant enzyme was expressed in *P. pastoris* and purified to homogeneity as described for BcPG2. The BcPG2-D192A mutant enzyme did not hydrolyse PGA (not shown), neither did it show any symptoms when infiltrated in broad bean and tomato leaves in concentrations up to 400ng/µl (Figure 3D), indicating that the enzyme activity of BcPG2 is essential to cause necrosis. Figure 3E shows a close-up of collapsed broad bean tissue upon infiltration of BcPG1 and BcPG2.

Bcpg2 mutants of Botrytis cinerea are strongly reduced in virulence

In order to study the role of BcPG2 in pathogenesis of *B. cinerea*, targeted mutagenesis of the *Bcpg*2 gene was performed. Four independent mutants were obtained, in which the *Bcpg*2 gene had been replaced by either the antibiotic marker gene hygromycin or nourseothricin. Conidia of two *B. cinerea* mutant strains Δ*Bcpg2*-1B and Δ*Bcpg2*-14B were used to inoculate detached leaves of tomato and broad bean, whereas wild type strain B05.10 was used as a control. The lesion development was monitored daily and the diameter of expanding lesions was measured 3, 4 and 5 days post inoculation. For both mutant and wild type the percentage of lesions that expanded beyond the size of the inoculum droplet was similar: >65% on tomato and 100% on broad bean. The mutant strains formed primary lesions approximately 24 hours later than the wild type strain (Figure 4A, B). Also, the lesion expansion rate of the mutants was 50 to 85% lower than that of the wild type (Table 4). These results indicate a significant role for *Bcpg2* in the early stages of infection both during primary lesion formation and lesion expansion.

Discussion

The role of endopolygalacturonases in pathogenesis of Botrytis cinerea

The host range of *B. cinerea* is generally considered to be restricted to dicots and corolliferous monocots, plants with relatively high pectin contents in their cell walls. Monocots with low pectin contents (e.g. Poaceae) are poor hosts for *B. cinerea*. In *B. cinerea*-infected tomato stems, hyphal growth preferably occurs in pectin-rich regions around the vascular bundles and less prominently in regions low in pectin (ten Have and van Kan, unpublished). These observations suggest that *B. cinerea* is particularly adapted for colonising pectin-containing plant tissues. The availability of a set of endopolygalacturonases with slightly different characteristics might enable the pathogen to hydrolyse a larger spectrum of pectin types, originating from different host species. Two criteria are crucial for *B. cinerea* to perform as a necrotrophic pathogen with a broad host range. First, it should to be able to kill cells of a wide range of plant species. For this purpose, molecules with a broad phytotoxic activity are more effective than specialised molecules such as host-specific toxins (Walton, 1996). Secondly, *B. cinerea* needs to convert host tissue into fungal biomass; endopolygalacturonases are useful tools in the decomposition process.

*Bcpg*1 and *Bcpg*2 are among the earliest expressed genes during infection of tomato leaves (chapter 4; Kars et al., 2005a; ten Have et al., 2001) implicating that the gene products have a role in early stages of the infection process. The rapid tissue collapse caused by BcPG1 or BcPG2 indicates that these enzymes play a very important role during infection and colonisation of pectin-rich plant species. Indeed, *Bcpg*1 mutants (ten Have et al., 1998) and *Bcpg*2 mutants (this study) show a severe reduction in virulence. Whereas the mutants in the *Bcpg*1 gene showed an approximate reduction of 25% in lesion expansion rate on various hosts, the *Bcpg*2 mutants showed a dual effect: the appearance of primary necrotic lesions was delayed by one day and the subsequent growth rate of expanding lesions was at least 50% reduced compared to the wild type recipient strain.

BcPGs differ in biochemical properties

The biochemical properties of the purified endopolygalacturonases studied using model substrates revealed that the family of BcPGs is as diverse as those of the saprophytic fungus *A. niger* (Benen et al., 1999; Pařenicová et al., 1998, 2000a, b) and the plant pathogenic fungus *Sclerotinia sclerotiorum* (Cotton et al., 2002; Martel et al., 1996). All latter enzymes display a low pH optimum, which reflects the adaptation to the acidic environment that is created by these fungi (Germeier et al., 1994; Maxwell and Lumsden, 1970; Verhoeff et al., 1988). BcPG3 is the only enzyme that displayed a broader pH optimum and is active between pH 3.2 and 4.5. Reports on *Bcpg3* gene expression in *in vitro* cultures at low pH (Wubben et al., 2000) and during infection of apple tissue that is acidic by nature (ten Have et al., 2001), are in agreement with this low pH optimum.

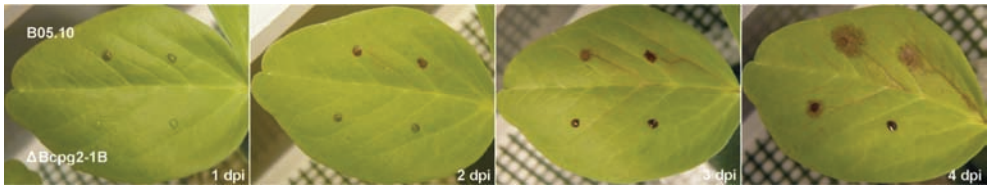

Figure 4. Virulence assay of the *Botrytis cinerea* Δ*Bcpg2* mutant in broad bean. Four droplets of inoculum were placed on each leaf: two droplets of the Δ*Bcpg2* mutant and two droplets of the wild type strain B05.10. Lesion development was scored at 1, 2, 3 and 4 days post inoculation (dpi). Primary lesions (black dots) appeared beneath the inoculation droplets of B05.10 and Δ*Bcpg2* at 1 and 2 dpi, respectively. Subsequently, the primary lesions of B05.10 and Δ*Bcpg2* start expanding beyond the inoculation droplet at 3 and 4 dpi, respectively. Similar results were obtained in virulence assays performed with tomato leaves.

Table 4. Lesion diameters caused by mutant Δ*Bcpg2*-1B and wild type recipient strain B05.10 during infection of tomato and broad bean leaves.

		ΔBcpg2-1B				Wild type			
Plant	n	Lesion diameter +/- SD (mm)			Avg growth rate (mm/day)	Lesion diameter +/- SD (mm)			Avg growth rate (mm/day)
		3dpi	4dpi	5dpi		3dpi	4dpi	5dpi	
Broad bean	48	3.0±0.5	3.5±0.6	4.4±1.2	0.7	4.3±1.3	9.0±4.7	13.2±7.8	4.5
Tomato	90	4.4±1.4	8.0±2.2	13.1±3.0	4.4	9.9±2.1	18.1±3.7	27.4±4.4	8.8

Lesion diameters were measured 3, 4 and 5 days post inoculation (dpi). n = number of inoculation droplets per strain. SD = standard deviation. Data shown here are results of one experiment. Similar results were obtained in repeated experiments and with an additional, independent mutant strain (Δ*Bcpg2*-14B).

The diversity among fungal endopolygalacturonases is further reflected by the large variation observed in specific activity, ranging from a few U/mg for some *S. sclerotiorum* enzymes to 4000 U/mg for PGII of *A. niger* (Benen et al., 1999; Cotton et al., 2002). Furthermore, processive behaviour of endopolygalacturonases was demonstrated for BcPG3, BcPG6, AnPGI, AnPGA, AnPGC, and AnPGD, whereas BcPG1, BcPG2, BcPG4, AnPGII, AnPGB, and AnPGE are non-processive (Benen et al., 1999; Pařenicová et al., 1998, 2000a, b). The processive behaviour of BcPG3 and BcPG6 becomes evident upon hydrolysis of pentamers and hexamers, where the products do not accumulate in stoichiometric amounts. Typically, processive AnPGD, BcPG3 and BcPG6 are able to hydrolyse OGA dimers, a property not displayed by other well-characterized endopolygalacturonases. BcPG3, BcPG6 and AnPGD form a small phylogenetic group also comprising FmPGA of *Fusarium moniliforme* and FoPG1 of *F. oxysporum f. sp. lycopersici* (Markovič and Janeček, 2001). However, FmPGA does not hydrolyse dimers and is a non-processive enzyme (Bonnin et al., 2001), demonstrating that these rather unique properties

are not shared among all members of this phylogenetic group. Indeed, for AnPGI and AnPGII it was shown that non-processive and processive behaviour could be interchanged by a single aa substitution (Pagès et al., 2000), making it unlikely that such a property is retained in groups of proteins that are united on the basis of global rather than site-specific aa conservation. Comparison of the bond cleavage frequencies reveals that BcPG1, BcPG2 and BcPG4 show similar preferences for hydrolysing a particular bond. However, the rates of hydrolysing model substrates differ nearly 100-fold (Table 2).

BcPGs strongly differ in necrotizing activity

The availability of purified *B. cinerea* endopolygalacturonases enabled us to study the role of BcPGs both as destructive enzymes and as elicitors of cell death. Infiltration of BcPGs into plant tissue caused chlorosis or necrosis to different extents depending on the BcPG and infiltrated plant species. It is intriguing that BcPG1 and BcPG2 show much higher NAs than the other BcPGs, albeit that these two enzymes show a 90-fold difference in specific activity on PGA. The specific activity of BcPG2 is most similar to BcPG3 and the products released from PGA by BcPG2 are most similar to those generated by BcPG1 and BcPG4. Furthermore, BcPG1 and BcPG2 are among the enzymes that prefer non-methylated substrates, when tested *in vitro*. Unfortunately, there is (to our knowledge) no detailed information available on the degree of methylation of pectin in leaves of the plant species tested in our study. We propose that BcPG1 and BcPG2 hydrolyse a specific, yet unidentified linkage in the pectin backbone that is crucial for maintaining the integrity of the pectic matrix in the cell wall. The other BcPGs might, for unknown reasons, not be able to hydrolyse these specific bonds.

Do the pectic fragments released by endopolygalacturonases play a role in the tissue collapse and necrosis induction that we observed? The enzymes display differences in product progression profiles, which could lead to (temporary) accumulation of different types of oligomers. We have infiltrated a number of linear OGAs of defined lengths (DP 2 to 10), either pure or in mixtures, in a concentration range up to 1 mM. None of the OGAs caused any visible damage to leaf tissue. This excludes that linear non-methylated OGAs play a detectable role in BcPG-mediated necrosis.

Plant defense to BcPGs

The extent of damage caused upon infiltration of endopolygalacturonases strongly varies, depending on the enzyme-plant combination. It is difficult to provide an explanation for this observation for several reasons. The plant cell wall is a heterogeneous and dynamic structure. The pectin composition differs between plant species, tissues, cell types and even in different regions of the wall surrounding an individual cell (e.g. Freshour et al., 1996). There may be differences in the degrees of methylation and/or acetylation, in the extent of

cross-linking and the embedding in other polymers. Recently, Vincken et al. (2003) proposed an entirely novel pectin structure model in which homogalacturonan is proposed to be a side chain of rhamnogalacturonan that connects neighbouring cells. The latter model predicts that cleavage of homogalacturonan would disturb the connection between plant cells and lead to cell separation.

Plants contain cell wall-associated proteins that inhibit fungal endopolygalacturonases (PGIPs; reviewed in De Lorenzo et al., 2001). PGIPs may influence the BcPG activity and hence the degree of tissue damage inflicted by a particular enzyme. PGIPs are selective in affinity for different endopolygalacturonases (De Lorenzo et al., 2001). However, in most plant species *pgip* genes are barely expressed in non-stressed leaf tissue (Li et al., 2003; Powell et al., 2000; Stotz et al., 1993, 1994). Even if wounding due to infiltration would induce PGIP expression, time would be limited to accumulate sufficient amounts of PGIP to have a significant inhibiting effect, because the tissue collapse caused by some BcPGs is achieved within 10 minutes. The constitutive expression of PGIPs in leaves, however, reduces *B. cinerea* lesion growth to some extent (Ferrari et al., 2003; Powell et al., 2000). We have studied the correlation between *B. cinerea* resistance and decreased sensitivity to BcPG infiltrated in transgenic tomato lines expressing pear-PGIP (Powell et al., 2000). However, as the non-transgenic control plants already displayed symptoms after infiltration of negative controls no conclusion could be drawn (Kars et al., unpublished).

Poinssot et al. (2003) recently proposed that recognition of the BcPG1 protein by grapevine suspension cells leads to activation of defense responses, even when the enzyme activity was reduced by heat-treatment. The only way to test whether BcPGs are indeed elicitors is to block enzyme activity by mutagenesis. For example, Boudart et al. (2003) transiently expressed a mutant ClPG1 protein lacking catalytic activity and concluded that enzyme activity of ClPG1 was essential for its necrotizing ability in tobacco. Therefore, we conducted experiments in which BcPG1 and the inactive mutant protein BcPG1-D203A were transiently expressed in *Nicotiana benthamiana* using *A. tumefaciens*. This approach did not yield any detectable levels of wild type or mutant protein (chapter 5; Joubert et al., 2007). Moreover, we did not succeed in producing inactive mutant BcPG1-D203A protein in *P. pastoris*. Data obtained with the inactive mutant protein BcPG2-D192A produced by *P. pastoris* clearly showed that the protein molecule is not recognised and therefore enzyme activity is required to inflict necrosis *in planta*. In any case, our observation that BcPG1 and BcPG2 cause mesophyll tissue of broad bean to collapse within as little as 10 minutes suggests that elicitor activity of BcPG1 probably is not of biological relevance.

All data presented here point to the conclusion that the symptoms caused upon BcPG infiltration do not result from recognition of the protein or the OGAs, but are the consequence of BcPG action causing loss of cell wall integrity, culminating in tissue collapse and subsequent cell death. Host defense responses, such as induction of PGIP, are likely to

be secondary to pectin hydrolysis and subsequent cell collapse inflicted by BcPGs. The identification of *A. thaliana* ecotypes that display differential sensitivity to BcPGs (Table 3) opens perspectives to study underlying mechanisms by genetic analysis in a segregating population (chapter 6).

Experimental procedures

Strains, plasmids and culture media

Escherichia coli DH5α cells were used for cloning purposes. *P. pastoris* GS115 (*his4)* and expression vector pPIC3.5, both purchased from Invitrogen (Carlsbad, USA), were used to produce endopolygalacturonases from *B. cinerea* strain B05.10. Bacterial and yeast cultures and the selection of recombinants were carried out following the supplier's instructions. Basal salts medium supplemented with PMT4 trace elements was used for fermentation, whereas the fermentor preculture was grown in FM22 medium supplemented with PMT4 trace elements (Stratton et al., 1998). *B. cinerea* strain B05.10 (van Kan et al., 1997) and mutant strains were grown and maintained as described (Wubben et al., 2000).

Plasmid construction for expression in Pichia pastoris

Standard molecular DNA techniques were carried out as described by Sambrook et al. (1989). Plasmids were isolated using the Wizard Plus SV miniprep kit (Promega, Madison, USA). DNA fragments were purified from agarose gels with the GFX PCR DNA and Gel Band Purification kit from Amersham Biosciences (Uppsala, Sweden). The *Bcpg*1 gene, which does not contain introns, was amplified by high-fidelity PCR with Pwo polymerase (Roche, Mannheim, Germany) using *B. cinerea* strain B05.10 genomic DNA as a template and primers listed in Table 5. cDNAs of *Bcpg*2, *Bcpg3*, *Bcpg4*, *Bcpg5* and *Bcpg*6 were generated using the Superscript one-step RT-PCR kit (Invitrogen) according to the manufacturer's protocol, together with appropriate primers (Table 5) and total RNA isolated from *B. cinerea* B05.10-infected tomato leaves (ten Have et al., 2001) as template. The primers (Amersham Biosciences Europe GmbH, Freiburg, Germany) were designed on the genomic sequences of the *Bcpg* genes from *B. cinerea* strain SAS56 (GenBank accession numbers U68715, U68716, U68717, U68719, U68721, and U68722). The PCR products were cloned behind the *AOX1* promoter in pPIC3.5 using the appropriate restriction enzymes (Table 5). Insert sequences were verified by DNA sequence analysis (BaseClear, Leiden, the Netherlands) and compared with the published sequences of *B. cinerea* strain SAS56. Nucleotide (nt) differences were observed between strains B05.10 and SAS56 in the ORFs of genes encoding BcPG1, BcPG2, BcPG4 and BcPG6. In *Bcpg1* 44 different nt were found, leading to 14 different aa, whereas in *Bcpg2* 13 different nt were found, leading to 7 different aa. *Bcpg4* and *Bcpg6* contained 1 and 3 silent nt substitutions, respectively. As

these differences were found in several independent cDNA clones, as well as in PCR products generated on genomic DNA, they thus represent true sequence polymorphisms between the two strains. Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, USA) according to the procedure recommended by the manufacturer. The forward and reverse mutagenic primers used to substitute aspartate (D) 192 by alanine (A) in the active centre of BcPG2 were 5'-CTTGGCCACAACACC**GCT**GCTTTTGACATCAAC-3' and 5'-GTTGATGTCAAAAGC**AGC** GGTGTTGTGGCCAAG-3', respectively (codon of aa 192 in bold). The cDNA of *Bcpg2*, ligated in the pCR4-TOPO vector (Invitrogen) was used as template for mutagenesis. The complete mutated *Bcpg*2 gene was sequenced to confirm the mutation and subsequently cloned in pPIC3.5 as described above.

Table 5. Forward (F) and reverse primers (R) used to amplify the *Bcpg* coding sequences for expression in *Pichia pastoris*.

Bcpg	Primer name	Primer sequence (5'>3')[a]	Restriction site	PCR product (bp)
1	Bcpg1-Fbgl	CCGCCAGATCTAAT**ATG**GTTCAACTTCTCTCAATG	BglII	1174
	Bcpg1-Reco	GCCGGGAATTC**TTA**ACACTTGACACCAGATGGG	EcoRI	
2	Bcpg2-Fbam	CGCCGGATCCAAA**ATG**GTTCATATCACAAGCC	BamHI	1149
	Bcpg2-Reco	GCCGGGAATTC**TTA**GCAAGAAGCTCCGGTAGG	EcoRI	
3	Bcpg3-Fbgl	CCGCCAGATCTAAT**ATG**CGTTCTGCGATCATCCTCGG	BglII	1570
	Bcpg3-Reco	GCCGGGAATTC**TTA**TGATGGGCATCCAGTAGATGG	EcoRI	
4	Bcpg4-FbglA	CCGCCAGATCTATC**ATG**CCTTCCACCAAGTCC	BglII	1219
	Bcpg4-Reco	GCCGGGAATTC**TTA**AGAGCAAGAACCAAC	EcoRI	
5	Bcpg5-Feco	CCGGGAATTCAAG**ATG**GTTAAGTTTTCTGCCTGTCTCC	EcoRI	1168
	Bcpg5-Rnot	GCCGGGCGGCCG**C**T**A**CAAGGAACAAGAGACACC	NotI	
6	Bcpg6-Fbam	CGCCGGATCCATC**ATG**CCTAAGAACTCTCAGATCCC	BamHI	1137
	Bcpg6-Reco	GCCGGGAATTC**TTA**TGCGGGACAGCCAGTTGC	EcoRI	

[a] Restriction sites introduced for cloning purposes are underlined; the start and stop codons are indicated in bold, respectively.

Expression and purification of recombinant BcPGs in Pichia pastoris

After digestion with *Stu*I (pPIC3.5-BcPG4) or *Sal*I (all others), linearised pPIC3.5-BcPG (5 μg) was used to transform *P. pastoris* GS115 by electroporation. His^+ transformants were tested for Mut^+ phenotype and expression of BcPG in liquid buffered methanol-complex medium at 30°C was monitored in flasks in a rotary shaker at 250 rpm. Methanol was added every 24 hours to a final concentration of 1% (v/v). After four days, expression of BcPG was determined by measuring polygalacturonase activity in the culture supernatant using a qualitative plate assay. The *P. pastoris* transformant showing the highest activity was used for large-scale BcPG expression in fermentors. *P. pastoris* fermentation was performed in 3-l jacketed vessels (Applikon, Schiedam, the Netherlands) containing 1.5 l medium. The temperature was kept at 30°C, agitation at 1200 rpm, and aeration rate at 2 vvm (air volume

per liquid medium volume per minute). The pH was maintained at 5.0 using a 25% (v/v) ammonium hydroxide solution, which also served as nitrogen source. Antifoam 204 (Sigma, A6426) was added manually as required throughout the fermentation. The fermentor medium was inoculated to a start optical density (OD_{600}) of 5, using 150 ml of preculture grown in flasks for 2 days. The batch culture in the fermentor was grown until glycerol was consumed. Subsequently, a glycerol fed-batch was initiated by feeding 50% (v/v) glycerol supplemented with 4 ml/l PMT4 trace elements under control of the dissolved oxygen (DO) level. The DO set point was 35%, above which glycerol was fed to the culture. When the wet cell weight reached approximately 180 g/l, the glycerol feed was switched to a 100% methanol feed containing 4 ml/l PMT4 trace elements to start the fed-batch induction phase. Similarly, methanol was fed at DO levels above 35% saturation. The culture was harvested after three days of induction, which contained a wet cell weight of 300 g/l. The cell-free supernatant was stored at 4°C or at -20°C until further use. Sodium azide was added to a final concentration of 0.02% (w/v) to prevent microbial growth. The supernatant was concentrated from 1.5 l to 200 ml using a Pellicon XL-10PLCGC membrane in a Labscale TFF system (Millipore, Bedford, MA, USA). The concentrated solution was dialysed extensively against buffer X. Buffer X represented 20 mM sodium acetate, pH 4.2, in the case of BcPG1, BcPG2 and BcPG2-D192A; 10 mM piperazine/HCl, pH 5.0, for BcPG4; and 10 mM sodium phosphate, pH 7.5, for BcPG3 and BcPG6. All column materials were obtained from Amersham Bioscience and equilibrated with the appropriate buffer. The dialysed crude material was first applied to a DEAE-Sepharose FF column (60 ml). Protein was eluted using a linear gradient of 0 to 0.35 mM NaCl in buffer X. Fractions containing enzyme activity were pooled, diluted five times with buffer X and further purified using a Source 30Q column and a linear gradient of 0 to 0.35 mM NaCl in buffer X for protein elution. BcPG1, BcPG2 and BcPG2-D192A, which both have a high pI, did not bind to the DEAE Sepharose column at pH 4.2. The DEAE flow-through was subsequently loaded onto a Source 30S cation exchange column, and BcPG1, BcPG2 or BcPG2-D192A was eluted with a linear gradient of 0-0.35 M NaCl in buffer X. Finally, fractions containing pure BcPG were pooled, dialysed extensively against 10 mM sodium acetate, pH 4.2, and stored at 4°C after addition of sodium azide (0.02% w/v) to prevent microbial growth. Pure proteins were kept at -20°C for prolonged storage without the addition of sodium azide.

Endopolygalacturonase activity measurements

Endopolygalacturonase activity was determined qualitatively by spotting a droplet of culture medium on agar plates containing 0.1 % (w/v) PGA, pH 4.2 (Sigma, P3850). After incubation at 30°C for at least 30 min, the plate was flooded with 4 M HCl. Enzyme activity was observed as a white halo in a turbid background. Alternatively, after incubation the plates were stained with ruthenium red. Enzyme activity appeared as a white spot in a red background.

The catalytic activity of endopolygalacturonases was determined quantitatively by colorimetric detection of release of reducing sugars (Pařenicová et al., 1998). One unit of enzyme activity was defined as the amount of enzyme that catalyses the production of 1 μmol of reducing sugars per minute. To determine steady-state parameters, initial reaction rates were measured with increasing PGA concentrations. $V_{max,app}$ and $K_{m,app}$ values were calculated from the production rates by non-linear regression analysis using the Michaelis-Menten equation. Phosphate-citrate (McIlvain) buffers ranging from pH 2 to 8 were used for determination of the pH optimum. The effect of the degree of pectin methylation on specific enzyme activity was determined using 0.25 % (w/v) methylated pectin (7, 22, 45, 60 or 75% methylated; Copenhagen Pectin, Lille Skensved, Denmark) as substrate in the standard enzyme assay.

Bond-cleavage frequencies and reaction kinetics on OGAs of defined length were determined as described by Pařenicová et al. (1998). OGAs were prepared and quantified according to Kester and Visser (1990). Reaction products were analysed and quantified on a BioLC HPLC system (Dionex Co., Sunnyvale, USA) using a CarboPac PA-100 anion-exchange column (HPAEC) in combination with pulsed amperometric detection, as described by Pařenicová et al. (1998).

Analytical methods

The concentration of purified enzyme was measured by its absorbance at 280 nm using the following absorbance coefficients as calculated using Protean v4.02 (DNAStar Inc., Madison, USA): ε_{280} = 3.27 x 10^4 for BcPG1, 3.34 x 10^4 for BcPG2 and BcPG2-D192A, 2.87 x 10^4 for BcPG3, 3.78 x 10^4 for BcPG4, and 2.51 x 10^4 $M^{-1}cm^{-1}$ for BcPG6. The purity of the isolated enzymes was analysed by SDS-PAGE, loaded with 10 μg protein per well. The molecular mass was estimated using the low molecular weight calibration kit 4 (Serva, Heidelberg, Germany). Proteins were visualised by staining with coomassie brilliant blue R250 or by staining with the Silverstain Plus kit from Bio-Rad (Hercules, CA, USA). Endopolygalacturonase activity was detected in SDS-PAGE gels by zymography as follows. Agarose overlay gels contained 0.1 % (w/v) PGA and 1 % (w/v) agarose in 50 mM sodium acetate, pH 4.2 and were poured onto Gelbond film (Pharmacia Biotech, Uppsala, Sweden). In order to detect activity after SDS-PAGE, non-boiled protein samples (10 μg) were loaded

in sample buffer lacking β-mercaptoethanol and SDS. Following electrophoresis, SDS-PAGE gels were washed extensively in 50 mM sodium acetate, pH 4.2, to re-activate the PGs and then covered with the agarose gel. Following incubation, overlay gels were stained in 0.02% (w/v) ruthenium red for 30 minutes and destained in water until clear bands were visible.

For peptide mapping, coomassie brilliant blue-stained protein bands of interest were excised and subjected to trypsin digestion (sequence-grade; Promega, Madison, WI) according to the supplier's manual. The tryptic peptides were analysed by nano-LC-MS (Fischer et al., 2002). For N-terminal aa sequencing, protein was blotted onto Immobilon P transfer membrane (Millipore) in an LKB MultiphorII Electrophoresis unit. Transfer buffer was 10 mM CAPS buffer, pH 11.0 with 10% (v/v) methanol. Blots were stained with coomassie brilliant blue and bands were cut out for N-terminal protein sequencing (Sequentie centrum Utrecht, The Netherlands) to confirm the identity of the BcPGs.

Plant material and growth conditions

Lycopersicum esculentum cv. Moneymaker plants were grown in potting soil for six weeks in the greenhouse at 23°C with a photoperiod of 16 hours. Tomato potting soil was supplemented with nutrients as described by ten Have et al. (1998). Broad bean (*Vicia fabae*) plants were grown in potting soil for 3 to 4 weeks at the same greenhouse conditions as described for tomato. *A. thaliana* accessions Lip-0 (CS6780), Ler-0 (NW20), Col-0 (N907), Est-0 (CS6700), Oy-0 (CS6824) and Kas-0 (CS903) were obtained from Maarten Koornneef, Wageningen University, The Netherlands. After a cold period of 4 days to break seed dormancy, *A. thaliana* plants were grown in a climate chamber at 20°C with a photoperiod of 8 hours and 70% relative humidity. Six-to eight-week-old plants were used for infiltration with different purified enzymes.

Infiltration of plants with BcPGs

Infiltration occurred with a needle-less 1-ml or 2-ml syringe placed against the lower side of the leaf. The intercellular spaces of the leaf were filled with sample fluid by pressing the syringe. Water, 0.001% (w/v) azide, 10 mM sodium acetate, pH 4.2, culture filtrate of untransformed *P. pastoris*, and 320 µg/ml BSA were used as negative controls. BcPG1, BcPG2, BcPG3, BcPG4 and BcPG6 were applied in 10 mM sodium acetate, pH 4.2, in different concentrations. Enzyme activity was quantified prior to infiltration and verified afterwards with remnants of the samples, using 0.25% (w/v) PGA as substrate as described above. In every experiment, each sample was infiltrated in two sections per leaf, two leaves per plant, and assays were performed with at least two plants. Experiments were performed at least twice with fresh batches of enzymes.

Gene replacement and transformation of Botrytis cinerea

B. cinerea genomic DNA was isolated using either the standard chlorophorm/ isoamylalcohol DNA isolation method (Möller et al., 1992) or GenElute Plant Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis MO, USA). *B. cinerea* mutants lacking *Bcpg2* were created by gene replacement. Two target-gene fragments of ~500 bp were amplified using Pwo DNA polymerase (Roche Diagnostics, Mannheim, Germany) with a primer chosen just outside the ORF and a primer with a 21 bp extension complementary to the selection marker cassette. A single-step overlap-extension PCR procedure was used to fuse the two fragments of the target gene to a selection marker using Expand High Fidelity polymerase according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Either a cassette from pNR1 (Malonek et al., 2004) conferring resistance to nourseothricin or a cassette from pLOB1 (Genbank accession number AJ439603) conferring resistance to hygromycin was used. The amplified constructs were purified using Quickstep PCR purification spin columns (Edge BioSystems, Gaithersburg MD, USA) and used to transform *B. cinerea* protoplast as described by ten Have et al. (1998). Recombination was confirmed by Southern analysis as described (van der Vlugt-Bergmans et al., 1997).

Virulence assays

Detached tomato- and broad bean leaves were inoculated with conidial suspensions of *B. cinerea* harvested from 10-day-old cultures of the wild type strain B05.10 and mutant strain Δ*Bcpg2*, as described by Benito et al. (1998). On each leaf both the mutant and wild type strain were inoculated (at least) twice, on separate leaf halves. Inoculated leaves were placed in closed containers at approximately 100% relative humidity in the dark. Symptom development was monitored and the lesion diameter was measured on three successive days. The virulence assays were repeated twice for both tomato and broad bean. The average lesion sizes and corresponding standard deviations were calculated for each strain. The data were analysed statistically using SPSS.

Sequence data

Sequence data from the genes presented in this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY665552 (*Bcpg*1), AY665553 (*Bcpg*2), AY665554 (*Bcpg*3), AY665555 (*Bcpg*4), AY665556 (*Bcpg*5) and AY665557 (*Bcpg*6).

Acknowledgements

We are grateful to Pierre de Wit for his advice and critical reading of the manuscript, Henk Schols for helpful comments on the manuscript, and Kim Burgers and Harry Kester for experimental help. We thank Bert Essenstam and Casper Pillen (Unifarm) for excellent plant care, Dr. Maarten Koornneef for providing *A. thaliana* accessions Lip-0 (CS6780), Ler-0 (NW20), Col-0 (N907), Est-0 (CS6700), Oy-0 (CS6824) and Kas-0 (CS903) and Dr. Bettina Tudzynski (University of Münster, Germany) for providing pNR1. This research was funded by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (project WGC.5034).

Chapter 4

Functional analysis of *Botrytis cinerea* endopolygalacturonases *Bcpg3-6*

Ilona Kars, Lia Wagemakers, Melysia McCalman and Jan A.L. van Kan

Abstract

In order to degrade plant cell walls *Botrytis cinerea* produces several pectinolytic enzymes during infection, among which several endopolygalacturonases (BcPGs). Previously, we showed that strains mutated in genes *Bcpg1* (ten Have et al., 1998) and *Bcpg2* (Kars et al., 2005a) are reduced in virulence, which indicates that pectin degradation is important for successful infection. In this chapter, we report the functional analysis of four additional *Bcpg* genes, previously identified by Wubben et al. (1999), namely *Bcpg3*, *Bcpg4*, *Bcpg5* and *Bcpg6.* Gene replacement mutants Δ*Bcpg3*, Δ*Bcpg4*, Δ*Bcpg5* and Δ*Bcpg6 of B. cinerea* showed no detectable reduction in virulence on tomato or broad bean leaves. The timing of primary lesion formation and the lesion expansion rate by all mutants were similar to those of the wild type recipient strain B05.10. Northern blot analysis showed that the *Bcpg*4 and *Bcpg*6 genes are predominantly expressed during lesion expansion, whereas transcripts of the *Bcpg*3 and *Bcpg*5 genes remained undetected in this assay. In addition to the *Bcpg* genes, we studied the expression of pectate lyase, *Bcpel*, and pectin lyase, *Bcpnl*, genes. The *Bcpel* expression pattern is similar to *Bcpg6* indicating expression during lesion expansion, whereas *Bcpnl* expression is similar to that of *Bcpg2*, which suggests that *Bcpnl* plays a role at an early phase of the infection process.

Introduction

Many fungal plant pathogens produce a range of cell wall-degrading enzymes (CWDEs) during infection and colonization of plants. The role of fungal CWDEs in pathogenesis has been studied in several plant-pathogen interactions (reviewed by Walton, 1996; ten Have et al., 2002). Many of these studies concentrated on enzymes that degrade pectin or pectate, major components of the plant cell wall, and include exo- and endopolygalacturonases, lyases as well as pectin methylesterases. Earlier studies on the function of these enzymes have focused on the production of enzymes *in vitro* and *in vivo* using PAGE-gels and zymograms (Bucheli et al., 1989; Caprari et al., 1993; Johnson and Williamson, 1992; Leone et al., 1989; Reignault et al., 1994, 2000). Since the late 1980s, progress in the development of molecular genetic techniques provided new ways to analyse the function of these enzymes and their encoding genes in filamentous fungi. The role of a particular gene product in pathogenesis can now be analysed using DNA-mediated transformation, which allows the re-introduction of altered copies of cloned genes (Yoder, 1988; Fincham, 1989). From that time, gene disruption by targeted mutagenesis became the method of choice to elucidate the function of genes in fungus-plant interactions. In most cases, inactivation of genes encoding CWDEs had no apparent effect on virulence (reviewed in Tudzynski and Tudzynski, 1998, 2000). Up to the turn of the century, only two cases had been reported where targeted mutagenesis of genes encoding CWDEs resulted in a detectable phenotype. Shieh et al. (1997) reported involvement of endopolygalacturonase P2c in the invasion and spread of *Aspergillus flavus* in cotton balls, and ten Have et al. (1998) showed that the *Botrytis cinerea* endopolygalacturonase 1 gene (*Bcpg1*) was required for full virulence. Since then, however, many more studies have been reported in which targeted inactivation of genes encoding CWDEs affected virulence (Issiki et al., 2001; Kars et al., 2005a; Oeser et al., 2002; Rogers et al., 2000; Valette-Collet et al., 2003; Yakoby et al., 2001). All of these cases refer to enzymes that act on pectin. More recently, a *B. cinerea* endo-1,4-xylanase was the first hemicellulase of a plant pathogen shown to contribute to virulence (Brito et al., 2006).

B. cinerea is a necrotrophic fungal plant pathogen that uses pectinolytic enzymes to penetrate and degrade healthy plant tissue, which facilitates colonization. Genes were isolated that encode enzymes with a putative role in pectinolysis, including pectin and pectate lyases (van Kan et al., unpublished), six endopolygalacturonases (Bcpg1-6; Wubben et al., 1999) and pectin methylesterases (Kars et al, 2005b). The *B. cinerea* genes encoding pectinases are expressed *in planta* at different stages of infection (ten Have et al., 2001; Kars et al., 2005a, b). Transcripts of *Bcpg1* and *Bcpg2* were detected in the early stages of infection of tomato, whereas the other *Bcpg* genes were either expressed at a later stage of infection or their transcripts could not be detected by northern blot analysis (ten Have et al., 2001). Both BcPG1 and BcPG2 have been shown to possess necrotizing activity (Kars et

al., 2005a) and targeted mutagenesis of *Bcpg1* (ten Have et al., 1998) and *Bcpg2* (Kars et al., 2005a) showed that these genes are important for colonization of the host tissue by *B. cinerea*.

In this chapter we studied the role of *Bcpg3*, *Bcpg4*, *Bcpg5* and *Bcpg6* in virulence by gene replacement studies and report on the *in planta* expression patterns of all cloned *B. cinerea* endopolygalacturonases.

Results

Gene replacement and characterization of ΔBcpg mutants

In order to study the role of endopolygalacturonases BcPG3, BcPG4, BcPG5 and BcPG6 in pathogenesis of *Botrytis cinerea,* mutants were generated by targeted mutagenesis using the gene replacement method previously described by Kars et al. (2005b). Gene fragments of *Bcpg3*, *Bcpg4*, *Bcpg5* or *Bcpg6* were amplified using the primers listed in Table 1. By a single-step overlap-extension PCR the fragments of each target gene were fused to either of the available selection marker cassettes, conferring resistance to hygromycin or nourseothricin. The amplified fusion product was used to transform *B. cinerea* protoplasts. When integration occurred by homologous recombination, the coding sequences of the target genes were partly replaced by the selection marker cassette. In order to detect homologous recombinants, the transformants growing on selective medium were screened by PCR as described by Kars et al. (2005b) (not shown). Once identified, the recombinants were studied in more detail by Southern blot analysis.

Five independent mutants were obtained, in which the *Bcpg3* gene had been replaced by the hygromycin resistance cassette and two independent mutants were obtained for *Bcpg4.* Three independent mutants were obtained for *Bcpg5* and *Bcpg6* carrying the nourseothricin and hygromycin resistance cassette, respectively (Table 2). Mutants in the *Bcpg2* gene used in this study have been described previously by Kars et al. (2005a).

Southern blot analysis confirmed that in the *Bcpg3* mutants, the 0.6-kb *Kpn*I band characteristic for the wild type gene was no longer present (Figure 1A). In *Bcpg4* mutants, the 0.5-kb *Bam*HI band characteristic for the wild type gene was replaced by a band of 0.9 kb (Figure 1B). In *Bcpg5* mutants, the 1.3-kb *Hind*III band characteristic for the wild type gene was replaced by bands of 0.7 kb and 2.4 kb, respectively (Fig. 1C). In the *Bcpg6* mutants, the 0.9-kb *Pst*I band characteristic for the wild type gene was replaced by a band of approximately 2.0 kb (Fig. 1D). Antibiotic-resistant transformants resulting from random ectopic integration of the selection marker cassette gave, in addition to the wild type band, rise to one or more bands of variable size (not shown). Correct replacement without any detectable ectopic insertion of antibiotic resistance cassettes was confirmed by Southern blot analysis with both gene-specific probes, and the selection marker probes (not shown) in all the mutants listed in Table 2.

Several experiments were performed to generate double mutants lacking *Bcpg1* and *Bcpg2*, as well as double mutants lacking *Bcpg3* and *Bcpg5*. However, none of the attempts resulted in recovery of correct double mutants.

Table 1. PCR primers used to amplify target gene fragments, gene-replacement constructs, and to check for correct homologous integration of gene-replacement construct in the transformants.

Target gene	Primer name	Primer sequence (5' > 3')
Bcpg2 (AY665553)	Bcpg2-5.1	ACCTCTATGTGGGAGCTCAAG
	Bcpg2-5.2	CGCTCATCAATCAGCACAG
	Bcpg2-5.3	GATGATTACTAACAGATATCAAGCTTCAGTGATACTGGAAGATCCA
	Bcpg2-3.1	CCGGTAGGAATACCAGCAC
	Bcpg2-3.2	CCAGCCTGAGCAGTTTGCAC
	Bcpg2-3.3	GGGTACCGAGCTCGAATTC GATAACTCTGCTGGAGATGC
Bcpg3 (AY665554)	Bcpg3-5.1	GCATGCAAGTGCATGAGATTC
	Bcpg3-5.2	CCAGCGGTAGCAATCGAGC
	Bcpg3-5.3	GATGATTACTAACAGATATCAAGCTT GAGATACTTACGGCAACAGT
	Bcpg3-3.1	CCATGCCTCAGTTCACCAC
	Bcpg3-3.2	CGGTGAATGTGATATCGGAGC
	Bcpg3-3.3	GGGTACCGAGCTCGAATTC GCCGCTCACAACAGTGATG
Bcpg4 (AY665555)	Bcpg4-5.1	GTACCCAGGAGTACTCCTGAA
	Bcpg4-5.2	CTTACTCTCATCTTGGCTATTC
	Bcpg4-5.3	GATGATTACTAACAGATATCAAGCTTGAATCAGTCAAGTCGTGGGC
	Bcpg4-3.1	GATCGCTTAAGAGCAAGAACC
	Bcpg4-3.2	GTCTTACCACCAGTGACAGCA
	Bcpg4-3.3	GGGTACCGAGCTCGAATTCGAATGGTTACTGCTCAGGTGG
Bcpg5 (AY665556)	Bcpg5-5.1	GCTCCAACGGCTGCTCCAG
	Bcpg5-5.2	CATCAGCAAGCAAGTCCAAG
	Bcpg5-5.3	GATGATTACTAACAGATATCAAGCTT CAGTGTCACCATCCTGTGC
	Bcpg5-3.1	GGGACGTTAAGACAAGAGGA
	Bcpg5-3.2	CACGGAAACATCGGTCCATG
	Bcpg5-3.3	GGGTACCGAGCTCGAATTC GATCTGTCGGCGGTAGAGATG
Bcpg6 (AY665557)	Bcpg6-5.1	GCGGAGACCCAATGTACATAG
	Bcpg6-5.2	CTATGCTTGGCCTCTAGGACT
	Bcpg6-5.3	GATGATTACTAACAGATATCAAGCTTGCTCAGCAGTAACAGTGATT
	Bcpg6-3.1	CCCACTTGTCCTCAATCATGC
	Bcpg6-3.2	GCTGGGTAGTTACAGGAGCTT
	Bcpg6-3.3	GGGTACCGAGCTCGAATTCCAATCAGGATGATTGCGTCGC
Selection marker	cassette-5 (20)	GAATTCGAGCTCGGTACCC
	cassette-3 (21)	AAGCTTGATATCTGTTAGTA
	screen-3 (22)	GGGTACCGAGCTCGAATTC
	screen-5 (23)	GATTACTAACAGATATCAAGCTT

Table 2. Overview of the independently obtained *Botrytis cinerea* endopolygalacturonase replacement mutants (Δ*Bcpg*).

Target gene	# Independent mutants	Mutant codes *	Selection marker gene
Bcpg2	4	Δ*Bcpg2*-1B, 1C	Nat
		Δ*Bcpg2*-7B	Hph
		Δ*Bcpg2*-9C	Hph
		Δ*Bcpg2*-14B	Hph
Bcpg3	5	Δ*Bcpg3*-10A, 10B, 10C	Hph
		Δ*Bcpg3*-11A, 11B, 11C	Hph
		Δ*Bcpg3*-14B	Hph
		Δ*Bcpg3*-20A, 20B, 20C	Hph
		Δ*Bcpg3*-23B	Hph
Bcpg4	2	Δ*Bcpg4*-8B	Hph
		Δ*Bcpg4*-16C	Hph
Bcpg5	3	Δ*Bcpg5*-6B	Nat
		Δ*Bcpg5*-8A	Nat
		Δ*Bcpg5*-12A	Nat
Bcpg6	3	Δ*Bcpg6*-3A	Hph
		Δ*Bcpg6*-5A, 5C	Hph
		Δ*Bcpg6*-6A	Hph

* Independent mutants that have been analyzed in virulence assays are underlined.

Effect of deletion of Bcpg genes on virulence

Conidia obtained from several *B. cinerea Bcpg* replacement mutants (underlined in Table 2) were used to inoculate detached leaves of tomato and broad bean. Wild type strain B05.10 was used as a control. Lesion development was monitored daily and the diameter of the lesions was measured three or four days post inoculation depending on the plant species. For all *Bcpg* mutants and the wild type strain, the proportion of lesions that expanded beyond the size of the inoculum droplet was high: 99% on tomato and >91% on broad bean. The gene replacement mutants Δ*Bcpg3*, Δ*Bcpg4*, Δ*Bcpg5* and Δ*Bcpg6* formed primary lesions at the same time as the wild type strain and these lesions expanded at similar rates (not shown). On both tomato and broad bean, the diameter of the lesions caused by the Δ*Bcpg3*, Δ*Bcpg4*, Δ*Bcpg5* and Δ*Bcpg6* mutants were not significantly different from those caused by wild type strain B05.10 in multiple independent experiments (Table 3), whereas the *Bcpg2* mutants repeatedly showed a reduced virulence as previously reported (Kars et al., 2005a).

Table 3. Lesion diameter caused by the *Botrytis cinerea* endopolygalacturonase mutants (Δ*Bcpg*) and control strain B05.10 on tomato and broad bean leaves at three and four days post inoculation, respectively.

	Lesion diameter +/- SD (mm)*										
	Control	Mutants Δ*Bcpg2*		Mutants Δ*Bcpg3*		Mutants Δ*Bcpg4*		Mutants Δ*Bcpg5*		Mutants Δ*Bcpg6*	
Plant	B05.10	1B	14B	10A	11C	8B	16C	6B	8A	3A	5A
Tomato	18.1± 3.7 (n=90)	8.0 ± 2.2 (n=90)	n.d.								
	11.4 ± 1.1 (n=58)			11.1± 0.9 (n=58)							
	10.9 ± 1.6 (n=60)				10.0 ± 1.6 (n=58)						
	11.3 ± 1.3 (n=57)							10.5 ±1.3 (n=59)			
	10.4 ± 1.1 (n=59)								10.4 ±1.4 (n=60)		
	12.7 ± 0.8 (n=30)					12.8 ± 1.6 (n=18)	13.6 ± 0.9 (n=18)				
	13.3 ± 1.3 (n=29)									13.8 ± 1.3 (n=19)	13.3 ± 1.8 (n=18)
Broad bean	9.0 ± 4.7 (n=48)	3.5 ± 0.6 (n=48)									
	12.0 ± 2.2 (n=24)		8.7 ± 1.4 (n=24)								
	13.4 ± 2.1 (n=24)			12.7 ± 1.9 (n=24)							
	11.1 ± 2.4 (n=24)				9.4 ± 2.5 (n=24)						
	12.7 ±3.8 (n=24)							12.6 ±3.7 (n=23)			
	12.0 ± 3.5 (n=24)								11.3 ± 2.2 (n=24)		
	8.0 ± 3.5 (n=48)					8.8 ± 4.4 (n=47)	11.3 ± 4.5 (n=48)			9.6 ± 4.6 (n=44)	9.5 ± 4.2 (n=48)

* Data shown for each plant are results of one experiment. Mutants and control strains were inoculated on the same leaves on separate halves. Repeated experiments showed essentially the same results. n = total number of inoculation spots per strain. SD = standard deviation. N.d. = not done.

Expression studies

The expression *in planta* of the six *Bcpg* genes was analyzed in a time-course experiment as previously described by ten Have et al. (2001), but with shorter sampling intervals. *B. cinerea* strain B05.10 was used to inoculate tomato leaves and samples were harvested with 8-h intervals (up to 144 hours post inoculation, hpi). Total RNA was extracted from each sample and subjected to northern blot analysis. RNA extracted from healthy tomato leaves served as control. The results obtained by hybridization with the coding sequences of genes *Bcpg1*, *Bcpg2*, *Bcpg4*, *Bcpg6* and *BcactA* are shown in Figure 2A. Transcripts of *Bcpg1* were detected at 16 hpi and their level increased during the course of the infection process, similar to the constitutively expressed *BcactA* gene (Figure 2A), which served as a measure for fungal biomass (Benito et al., 1998). Transcripts of *Bcpg2* were detected at 16 hpi and remained more or less at a constant level between 24 and 120 hpi, whereas fungal biomass continued to increase. Low transcript levels of *Bcpg4* were detected from 48 hpi onwards and they increased at 96, 120 and 144 hpi, when the tomato leaves were fully colonized by *B. cinerea*. Low levels of transcripts of *Bcpg6* were detected at 40, 48 and 56 hpi, and they decreased at later times. Transcripts of *Bcpg3* and *Bcpg5* were undetectable in this experiment (not shown).

Following hybridization with the *Bcpg* genes, the blots were de-probed and subsequently hybridized with probes of genes encoding a pectate lyase gene (*Bcpel*) and a pectin lyase gene (*Bcpnl*), as shown in Figure 2B. Transcripts of *Bcpel* were detected between 24 and 72 hpi with the highest levels at 40, 48 and 56 hpi. Transcripts of *Bcpnl* were detected from 24 hpi onwards and largely remained at similar levels with a slight increase at 96 and 120 hpi.

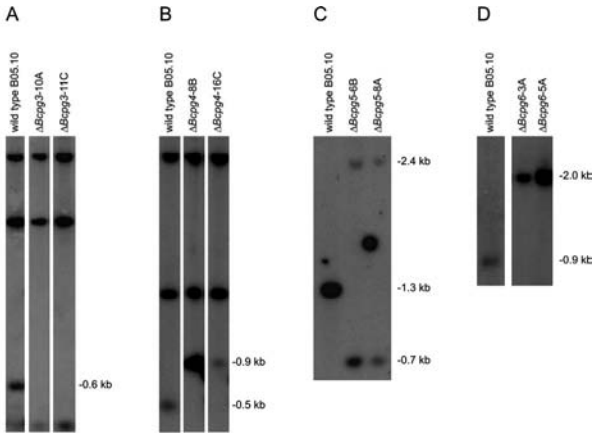

Figure 1. Southern hybridization with gene-specific probes to confirm gene replacement in the *Botrytis cinerea* endopolygalacturonase mutants (Δ*Bcpg*). Genomic DNA of the Δ*Bcpg3* (A), Δ*Bcpg4* (B), Δ*Bcpg5* (C) and Δ*Bcpg6* (D) mutants was digested with *Kpn*I, *Bam*HI, *Hind*III and *Pst*I, respectively. DNA of the wild type strain, B05.10, was used as control. All blots were hybridized with gene-specific

probes, except for the selection marker probes (not shown). The black spots between 1.3 and 2.4 kb (C) are hybridization artifacts and should be ignored.

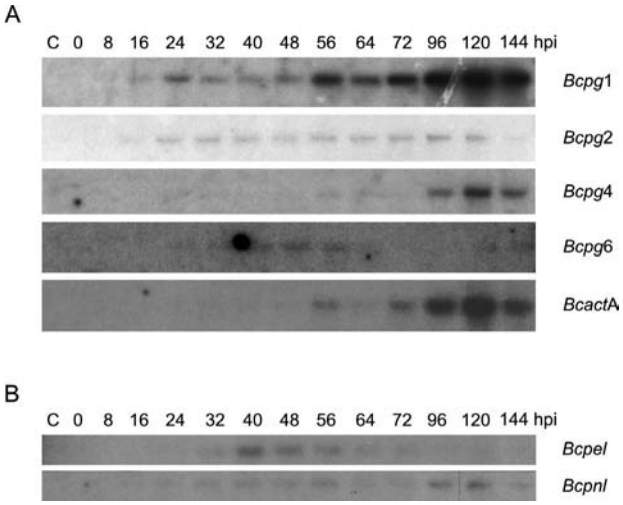

Figure 2. Expression of the *Botrytis cinerea* endopolygalacturonase genes *Bcpg1*, *Bcpg2*, *Bcpg3*, *Bcpg4*, *Bcpg5*, *Bcpg6*, pectin lyase gene, *Bcpnl*, and the pectate lyase gene, *Bcpel*, at 15°C in light at different time points after inoculation of tomato leaves. Blots were probed with fragments of respective polygalacturonase genes **(A)** and lyase genes **(B)** as indicated in the right margin. Sampling times are indicated above the lanes. Control RNA (C) extracted from a mock-inoculated tomato leaf is shown in the first lane. Blots hybridized with *Bcpg3* and *Bcpg5* did not give a hybridization signal (not shown). The black spot between 32 and 40 hpi (*Bcpg6*) is a hybridization artifact and should be ignored.

Discussion

In this chapter we describe the role of various different pectinolytic genes during infection and colonization of two host plants by *Botrytis cinerea*. The infection protocol was adapted to obtain a high percentage of primary lesion formation, consistent growth of lesions and tissue maceration, which enabled a more detailed examination of their role at different time points in the infection process.

Ten Have et al. (2001) previously studied the transcript levels of the *Bcpg* gene family on detached tomato leaves by spray-inoculation with a conidial suspension of *B. cinerea*. This study was repeated with shorter time intervals and under slightly different incubation conditions, i.e. 15°C in indirect light. Ten Have et al. (2001) incubated inoculated leaves at 20°C in full light or at 4°C in darkness, as initially described by Benito et al. (1998). We considered these conditions less favourable for the following reasons. Incubation at 20°C in full light caused primary necrotic lesions at 16 hpi, but only a proportion of the primary

lesions expanded following a quiescent period of 24-48 hours after appearance of the primary lesion (Benito et al., 1998). Expanding lesions had a dry necrotic appearance under these incubation conditions. Incubation at 4°C in darkness caused primary lesions with a "soft rot" appearance, which expanded consistently at a fairly uniform, albeit slow rate.
Incubation at 15°C in indirect light gave more consistent and reproducible infections. It increased the frequency of expanding secondary lesions as compared to incubation at 20°C in full light, yet the lesion growth rate was faster than in incubations at 4°C in darkness. *B. cinerea* lesions expanding at 15°C in indirect light typically had a "soft rot" appearance, indicative of maceration presumably by pectinolysis. Therefore, we considered the latter incubation conditions appropriate for studying the expression of pectinolytic genes and the effect of mutations on virulence.

The expression patterns of the *Bcpg* genes suggest that most of these genes play a role in certain stages of colonization and breakdown of plant tissues. Transcripts of *Bcpg2* were detected very early in the infection (from 16 hpi onwards) and their levels remained more or less constant during colonisation, even when fungal biomass increased substantially over time. Such a pattern would be consistent with the expression of *Bcpg2* predominantly occurring in the hyphal tips at the edge of the expanding lesion, and decreasing in toward the centre of the lesion. Whether the *Bcpg2* gene is indeed predominantly expressed transiently at the hyphal tips remains to be studied in more detail by promoter–reporter gene fusions. The *Bcpg*4 and *Bcpg*6 genes are predominantly expressed during lesion expansion and might contribute to the complete decomposition of the primary pectin hydrolysis products, generated by BcPG1 and BcPG2, into smaller fragments that can be taken up and metabolized by *B. cinerea.* The expression of *Bcpg*3 and *Bcpg*5 remained undetected, suggesting that these enzymes might not contribute to pathogenesis on tomato leaves under given conditions. We cannot exclude, however, that *Bcpg*3 and *Bcpg*5 might still play a role in the colonization process when they are differentially regulated in time or space. In addition to the endopolygalacturonase expression patterns, we also studied the expression levels of pectin methylesterases (Kars et al., 2005b), pectin lyase and pectate lyase. Pectate lyase (Figure 2B) shows an expression pattern similar to *Bcpg6*, suggesting that it contributes to further breakdown of pectin hydrolysis products into smaller fragments at later stages of infection. The pectin lyase (Figure 2B) expression pattern, however, is similar to that of *Bcpg2*, which suggests that *Bcpnl* plays a role at an early phase of the infection process. It should, however, be considered that lyases are presumably only active at pH>6 (Yakoby et al., 2000). In view of the observation that *B. cinerea* acidifies its environment by producing substantial amounts of oxalate (Gentile, 1954; Kunz et al., 2006), it is unlikely that lyase enzymes are active during infection even when their encoding genes are expressed. Targeted mutagenesis of the *Bcpnl* gene needs to be done in order to evaluate its role in infection.

The reduced virulence of the endopolygalacturonase mutants *Bcpg1* (ten Have et al., 1998) and *Bcpg2* (Kars et al., 2005b) strongly supports the importance of pectin degradation for successful colonization of the host tissue by *B. cinerea*. In contrast to Δ*Bcpg1* and Δ*Bcpg2* mutants, *B. cinerea* mutants Δ*Bcpg3*, Δ*Bcpg4*, Δ*Bcpg5* and Δ*Bcpg6* showed no detectable reduction in virulence. The primary lesions produced by these mutants appeared at the same time and expanded in a similar way as those caused by the wild type recipient strain B05.10.

Our targeted mutation studies have indicated that none of the single *Bcpg* genes is essential for penetration and host colonization (ten Have et al. 1998; this work). This might be due to overlapping activities of the individual BcPG enzymes, causing significant functional redundancy. In order to experimentally confirm functional overlap between BcPG isozymes, we intended to make targeted mutants lacking two *Bcpg* genes. Unfortunately, all attempts to generate Δ*Bcpg1*/Δ*Bcpg2* and Δ*Bcpg3*/Δ*Bcpg5* double mutants failed for unknown reasons.

Experimental procedures

Gene replacement

The gene replacement strategy used to generate *Botrytis cinerea* endopolygalacturonase (*Bcpg*) mutants was described by Kars et al. (2005b). Primers used for the amplification of gene replacement fragments are listed in Table 1. Two types of selection marker cassettes were used to replace part of the target gene: the hygromycin *(hph)* cassette and the nourseothricin *(nat)* cassette. Both cassettes were previously described in detail (Kars et al., 2005b). The cassette carrying the *hph* resistance gene was used for *Bcpg3*, *Bcpg4* and *Bcpg6* replacement, whereas the cassette carrying the *nat* resistance gene was used for *Bcpg5*.

Molecular analysis of the transformants

Fungal genomic DNA was isolated using either standard chlorophorm/ isoamylalcohol extraction method (Möller et al., 1992), or GenElute Plant Genomic DNA miniprep kit (Sigma-Aldrich, St. Louis MO, USA). The initial screening of the *B. cinerea* transformants was performed by PCR. PCR amplification was carried out using the ingredients and conditions described by Kars et al. (2005b) for the target gene fragments. Primer combinations used for the PCR screening were primers 5.1 and 23, 3.1 and 22, 5.1 and 3.1 (Table 1). Southern hybridization was used to confirm the recombination, as described by ten Have et al. (1998).

Botrytis cinerea transformation

Protocol for *B. cinerea* protoplast transformation was adapted from ten Have et al. (1998) and described by Kars et al. (2005b). *B. cinerea* strain B05.10 and mutant strains were grown and maintained as described by Wubben et al. (2000).

Plant growth and virulence assays

Tomato plants (*Lycopersicum esculentum*) cv. Moneymaker were grown in potting soil for six weeks in the greenhouse at 23°C with a 16 h photoperiod. Tomato potting soil was supplemented with nutrients as described by ten Have *et al.* (1998). Broad bean (*Vicia faba*) plants were grown in potting soil for four weeks at the same greenhouse conditions as described for tomato.

In virulence assays detached tomato and bean leaves were inoculated with *B*. *cinerea* conidial suspensions harvested from 10-day-old cultures. Aliquots of 2-5 µl 10^6 conidia/ml Gamborg B5 medium (Duchefa, Haarlem, The Netherlands), supplemented with 10 mM glucose and 10 mM potassium phosphate, pH 6.0 (Benito et al., 1998), were applied to the adaxial plant surface. Inoculated leaves were placed in closed transparent containers with high humidity (approximately 100%) in growth chambers at 15-20°C in indirect light. Symptom development was monitored daily and lesion size diameter was measured three (tomato) or four (broad bean) days after inoculation. Lesion sizes caused by the mutants were compared to those caused by wild type strain B05.10. All virulence assays presented here were at least repeated once. The average lesion size and corresponding standard deviation were calculated for each strain tested.

RNA analysis

Detached tomato leaves were spray-inoculated with *B. cinerea* strain B05.10 according to procedures described by Benito et al. (1998). Inoculated leaves were placed in closed transparent containers approximately 100% relative humidity at approximately 15°C and harvested at various time points after inoculation, with 8 h intervals. Isolation of total RNA from infected tomato leaves, northern blotting, and hybridization were performed as described by Prins et al. (2000). Amplification products of *Bcpg1*, *Bcpg2*, *Bcpg3*, *Bcpg4*, *Bcpg5*, *Bcpg6*, *Bcpnl*, *Bcpel* and *BcactA* were used as probes.

Ackowledgements

We thank Bert Essenstam and Pieter Kuiken (Unifarm) for excellent plant care and Pierre de Wit for critical reading of the manuscript. The research was funded by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (project WGC.5034).

Chapter 5

A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for *in vitro* interaction

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This chapter is published as: Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and van Kan J.A.L. (2007) A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Niicotiana benthamiana* leaves without any evidence for *in vitro* interaction. Mol. Plant-Microbe Interact., 20, 392-402.

Abstract

Six endopolygalacturonases from *Botrytis cinerea* (BcPG1 to BcPG6) as well as mutated forms of BcPG1 and BcPG2 were transiently expressed in leaves of *Nicotiana benthamiana* using agroinfiltration. Expression of BcPG1, BcPG2, BcPG4, BcPG5 and mutant BcPG1-D203A caused symptoms whereas BcPG3, BcPG6 and mutant BcPG2-D192A caused no symptoms. Expression of BcPG2 caused the most severe symptoms, including wilting and necrosis. BcPG2 has previously been shown to be essential for *B. cinerea* virulence. The *in vivo* effect of this enzyme and the inhibition by a polygalacturonase inhibiting protein (PGIP) was examined by co-expressing *Bcpg2* and the *Vvpgip1* gene from *Vitis vinifera* in *N. benthamiana*. Co-infiltration resulted in a substantial reduction of the symptoms inflicted by the activity of BcPG2 *in planta*, as evidenced by quantifying the variable chlorophyll fluorescence yield. *In vitro,* however, no interaction between pure VvPGIP1 and pure BcPG2 was detected. Specifically, VvPGIP1 neither inhibited BcPG2 activity nor altered the degradation profile of polygalacturonic acid by BcPG2. Furthermore, using surface plasmon resonance technology, no physical interaction between VvPGIP1 and BcPG2 was detected *in vitro*. The data suggest that the *in planta* environment provided a context to support the interaction between BcPG2 and VvPGIP1, leading to a reduction in symptom development, whereas neither of the *in vitro* assays detected any interaction between these proteins.

Introduction

Pectin is an important component in plant cell walls, and it is especially prevalent in primary cell walls and the middle lamella (Chapple and Carpita, 1998). The importance of pectin for plant tissue architecture and cell integrity was illustrated by expressing pectinolytic enzymes of fungal origin in plants (Boudart et al., 2003; Capodicasa et al., 2004). Stable transgenic tobacco plants expressing an endopolygalacturonase (PG) from *Aspergillus niger* were stunted and showed anatomical alterations. Only transgenic plants containing low levels of enzyme activity could be recovered, presumably because high expression is lethal (Capodicasa et al., 2004). Transient *Agrobacterium tumefaciens*-mediated expression of a *Colletotrichum lindemuthianum* PG in tobacco resulted in tissue collapse and induction of necrosis (Boudart et al., 2003), confirming the usefulness of agro-infiltration to study *in planta* effects of PGs.

A wide variety of biotrophic and necrotrophic plant-pathogenic fungi produce pectinolytic enzymes during infection of their hosts (ten Have et al., 2002). Production of these enzymes facilitates the hydrolysis of pectin, paving the way for the pathogen to colonize the host tissue, while at the same time providing products of pectin degradation which serve as nutrients. One of the pathogenic fungi for which the role of pectinases has been studied is *Botrytis cinerea*, the cause of grey mould on a wide range of plants (Kars and van Kan, 2004). The fungus possesses at least six BcPG encoding genes (Wubben et al., 1999), which are differentially expressed *in planta*, depending on host tissue and incubation conditions (ten Have et al., 2001). Functional analysis has demonstrated that at least BcPG1 and BcPG2 are very important for virulence (Kars et al., 2005a; ten Have et al., 1998). These two enzymes, produced in pure form in a heterologous system, are able to cause rapid and massive maceration and tissue collapse when infiltrated in leaves of several plant species (Kars et al., 2005a).

Plants have developed a mechanism to counteract the action of PGs by expressing proteins known as polygalacturonase-inhibiting proteins (PGIPs) that inhibit the activity of fungal PGs. These proteins are considered to contribute to resistance against pectinase-producing pathogenic fungi (reviewed by De Lorenzo et al., 2001). PGIPs are typically cell wall bound, tissue-specific, developmentally regulated and inducible by various stimuli, including pathogen attack, wounding, salicylic acid, jasmonic acid, oligogalacturonides (OGAs) and cold treatment (Bergmann et al., 1994; De Lorenzo et al., 2001; Desiderio et al., 1997; Devoto et al., 1998; Ferrari et al., 2003; Komjanc et al., 1999; Mahalingam et al., 1999; Stotz et al., 1994). The functional importance of PGIPs in plants is corroborated by the observation that PGIP genes are under positive evolutionary selection (Bishop, 2005, Stotz et al., 2000). PGs and PGIPs interact in a stoichiometric 1:1 ratio. Most of the interaction and inhibition data available for PG:PGIP pairs has been generated from *in vitro* assays. A commonly accepted model for PGIP-mediated defence suggests a dual function for PGIPs

(De Lorenzo et al., 2001). PGIP may inhibit the action of fungal PGs by either binding to the active cleft of the PG, thereby preventing binding of the ligand to the enzyme (Federici et al., 2001) or by binding to the opposite site of the PG molecule, preventing the conformational changes necessary for ligand binding (King et al., 2002). *In vitro* evidence also suggests that the inhibition of PGs results in the prolonged existence of medium-chained length OGAs that can subsequently activate plant defence responses (Cervone et al., 1989; Reymond et al., 1995).

Thus, the inhibition of fungal PGs by PGIP may slow down infection by limiting cell wall hydrolysis and maceration, and in doing so, allow time to activate multiple defence responses to counteract the pathogen. Both these properties have generated an interest in exploiting PGIPs as tools for enhancing plant resistance. Several papers have reported the over-expression of various PGIP encoding genes, mostly in heterologous systems. These studies include the over-expression of pear *pgip* in tomato (Powell et al., 2000) and *Vitis vinifera* (Agüero et al., 2005), bean *pgip* in tobacco (De Lorenzo and Ferrari, 2002), the Arabidopsis *pgip* genes in Arabidopsis (Ferrari et al., 2003), as well as the grapevine *pgip1* gene in tobacco (Joubert et al., 2006). In all cases, a reduction in disease symptoms was reported when such transgenic plants were infected with *B. cinerea*.

We examined the damage that individual BcPGs are able to inflict when transiently expressed by agro-infiltration. BcPG2 was the most destructive enzyme and was therefore studied in more detail by co-expressing a PGIP gene (*Vvpgip1*) from *V. vinifera*. In this study we show that VvPGIP1 quantitatively reduces the symptoms caused by BcPG2 expression *in planta*. Surprisingly, *in vitro* studies could not provide any indication that the two proteins in pure form could interact under the conditions tested. The results suggest a complex *in vivo* interaction between the protein pair tested.

Results

Individually transiently expressed Botrytis cinerea endopolygalacturonases cause different symptoms in planta

Botrytis cinerea endopolygalacturonases BcPG1, BcPG2, BcPG3, BcPG4, BcPG5 and BcPG6 were transiently expressed in *Nicotiana benthamiana. Agrobacterium tumefaciens* cultures containing any of the six *Bcpg* gene constructs, either containing the native fungal signal peptide or a signal peptide from the tobacco PR1a protein, were infiltrated into *N. benthamiana* and symptom development was monitored. Intercellular washing fluid was collected from infiltrated leaves and endopolygalacturonase (PG) activity was tested both in a qualitative polygalacturonic acid (PGA) plate assay and a quantitative reducing sugar assay.

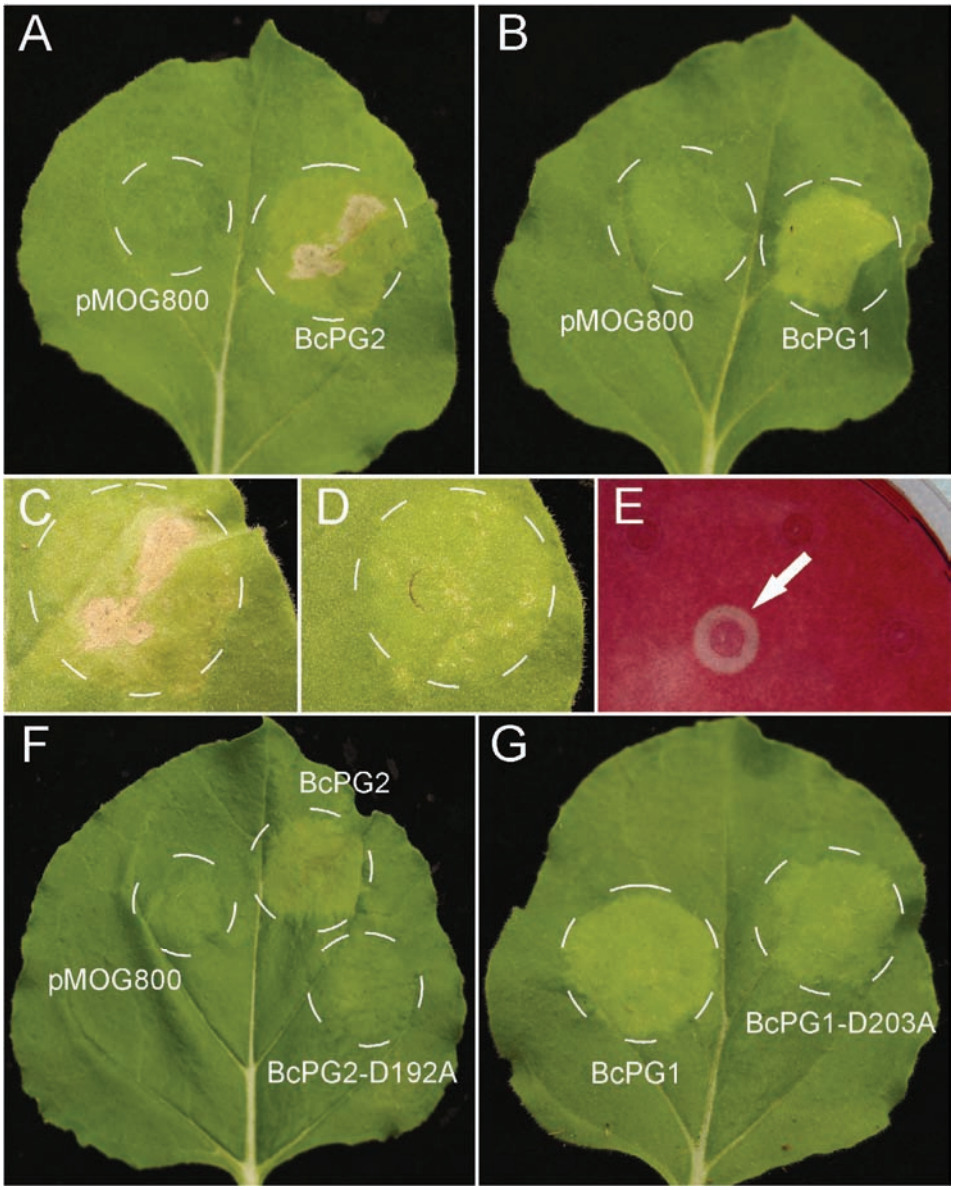

Figure 1. Symptoms caused by transient expression of BcPGs in *Nicotiana benthamiana.* Zones where *Agrobacterium tumefaciens* cells were infiltrated are marked with a dotted white circle. **(A)** BcPG2 caused necrosis, while empty vector pMOG800 caused no symptoms. **(B)** BcPG1 caused chlorosis. **(C)** Close-up of tissue collapse caused by BcPG2 expression. **(D)** Close-up of yellow/brown tissue patches caused by BcPG1 expression. **(E)** BcPG2 activity in intercellular fluid (arrow), visualized on PGA stained with ruthenium red **(F)** Inactive mutant BcPG2-D192A caused symptoms similar to the empty vector control. **(G)** Inactive mutant BcPG1-D203A caused symptoms similar to the wild type BcPG1.

Of the constructs carrying the full length BcPG ORF including its own fungal signal peptide, only the BcPG2 construct caused symptoms, visible as chlorotic and necrotic responses with some tissue collapse. These symptoms typically appeared only at 120 - 144 h post infiltration (hpi).

With constructs carrying the tobacco PR1a signal peptide, the first symptoms were generally observed at 24 to 36 h after infiltration of the *A. tumefaciens* cells, and they progressed in subsequent days. Three types of responses were observed (Figure 1; Table 1). BcPG2 caused necrosis and tissue collapse (Figure 1A, close-up in Figure 1C), whereas BcPG1 caused chlorosis (Figure 1B) and the development of yellow/brown patches scattered over the infiltrated area of the leaf (close-up in Figure 1D). BcPG4 and BcPG5 caused symptoms similar to BcPG1 (not shown), whereas the empty vector pMOG800 (Figure 1A, B), BcPG3 and BcPG6 (not shown) caused no symptoms or at most mild chlorosis. PG activity was detected in the intercellular fluid extracted from BcPG2 expressing leaves (Figure 1E) from one up to five days after infiltration. PG activity in the intercellular fluid extracted from leaves infiltrated with *A. tumefaciens* carrying the other *Bcpg* constructs remained below detection level (not shown).

Transient expression experiments were also carried out with all BcPG constructs in *N. tabacum* and tomato. Transient expression of BcPGs in *N. tabacum* cultivars caused a mild chlorosis 5 to 7 days after infiltration, but results were inconsistent between experiments. Tomato leaves showed significant chlorotic symptoms upon infiltration with *A. tumefaciens* cells carrying the empty vector, and were thus excluded from further experiments.

Enzyme activity is required for symptom development by BcPG2, but not BcPG1

In order to determine whether symptoms inflicted by transient expression of BcPG1 and BcPG2 require enzyme activity, constructs were generated encoding enzymatically inactive forms of BcPG1 and BcPG2 by introducing a mutation in their active site (D203A and D192A, respectively). The BcPG2-D192A mutant protein, produced in *Pichia pastoris*, lacks PG activity and is unable to cause symptoms in plant tissue upon infiltration (Kars et al., 2005a). The mutation D203A in the BcPG1 protein is in the analogous position in the catalytic site. The wild type and mutant constructs were transiently expressed in *N. benthamiana* as described above, and the symptoms and PG activity in the intercellular fluid were monitored. The mutant form BcPG2-D192A neither caused symptoms (Figure 1F, Table 1) nor showed detectable PG activity in the IF (not shown) in agreement with results of Kars et al. (2005a). The mutant BcPG1-D203A construct caused chlorotic symptoms with scattered yellow/brown patches to similar extent as the wild type BcPG1 (Figure 1G, Table 1). PG activity in the intercellular fluid of plants infiltrated with the BcPG1 and BcPG1-D203A constructs was in both cases below the detection level.

Table 1. Symptoms on leaves of *Nicotiana benthamiana* observed after infiltration of transformed *Agrobacterium tumefaciens* cells. All constructs contain a tobacco signal peptide unless otherwise indicated.

Expression construct	Symptoms[1]
BcPG1	Chlorosis; scattered yellow/brown spots
BcPG2	Necrosis and tissue collapse
BcPG2 (native signal peptide)	Delayed necrosis, yellowing and tissue collapse (only from 144 h onwards)
BcPG3	None
BcPG4	Chlorosis; scattered spots
BcPG5	Chlorosis; scattered spots
BcPG6	None
BcPG2-D192A	None
BcPG1-D203A	Chlorosis; scattered spots
pMOG800 without BcPG	None
MMA[2] buffer only	None

[1] Symptom development observed 48 hours post infiltration, unless otherwise specified

[2] 10 mmol.L-1 MgCl2, 10 mmol.L-1 MES pH 5.6

Assessment of tissue damage by chlorophyll fluorescence measurements

The macroscopic tissue damage in *N. benthamiana* leaves was most prominent upon expression of BcPG2. The damage caused by this enzyme was further assessed by measuring the reduction in chlorophyll fluorescence yield following infiltration with *A. tumefaciens* cultures containing the *Bcpg2* construct carrying the PR1A protein signal peptide. Leaves were infiltrated at different cell densities ranging from OD_{600}=0.6 to OD_{600}=0.06. The $^{Fv}/_{Fm}$ ratio, representing the efficiency of energy capture of PSII, were measured before infiltration as well as 24 h after infiltration and the $\Delta^{Fv}/_{Fm}$ ($^{Fv}/_{Fm}$ pre-infiltration – $^{Fv}/_{Fm}$ post-infiltration) was determined (Figure 2). The greater the $\Delta^{Fv}/_{Fm}$, the larger is the reduction in efficiency of non-photochemical quenching due to damage to PSII, providing an *in planta* quantitative measurement of symptom development. The $\Delta^{Fv}/_{Fm}$ increased when higher cell densities of *A. tumefaciens*, containing the *Bcpg2* construct, were infiltrated into the leaves (Figure 2). This dose-dependent reaction confirms that chlorophyll measurement provides a useful quantitative assessment for the damage caused by PG activity *in vivo*.

Co-expression of a Vitis vinifera PGIP reduces damage inflicted by BcPG2

BcPG2 was the only enzyme for which substantial levels of enzyme activity were detected upon transient expression (Figure 1E). The *Vitis vinifera* polygalacturonase-inhibiting protein VvPGIP1 has been shown to differentially inhibit PGs from *B. cinerea* (Joubert et al., 2006). Experiments were conducted to determine whether VvPGIP1 could affect the damage caused by BcPG2 *in planta*. As controls, we included in the experiments the inactive mutant

BcPG2-D192A construct as well as a construct encoding the apoplastic protein VvAMP1 from *V. vinifera* (A. de Beer and M.A. Vivier, unpublished), which has no known interaction with either PGs or PGIPs. *N. benthamiana* leaves were infiltrated separately with the *Bcpg2, Bcpg2-D192A*, *Vvpgip1* or *Vvamp1* constructs, or co-infiltrated with various combinations of constructs in a 1:1 ratio. Northern analysis was performed, PG and PGIP activity was monitored in extracts from infiltrated leaves and symptom development was scored 24 hpi (Figure 3). Infiltration with *A. tumefaciens* cells containing the Bcpg2 construct, as well as co-infiltration of leaves with the *A. tumefaciens* cells containing Bcpg2 and the *Bcpg2-D192A* or *Vvamp1* constructs caused extensive wilting at 24 hpi. In contrast, leaves co-infiltrated with *Bcpg2* and *Vvpgip1* displayed substantially less wilting (Figure 3A). Leaves infiltrated with empty vectors, VvPGIP1, VvAMP1, or *Bcpg2-D192A* alone or in combination (not shown) did not show any symptoms 24 hpi.

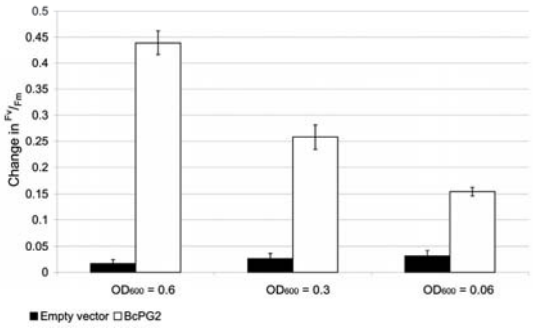

Figure 2. Dosage-dependent effect of BcPG2 expression on the variable chlorophyll fluorescence ratio ($^{Fv}/_{Fm}$) in leaves of *Nicotiana benthamiana*. *Agrobacterium tumefaciens* containing the *Bcpg2* construct carrying the PR1a signal peptide were infiltrated at different OD_{600} densities (0.6, 0.3 and 0.06, as indicated) and $^{Fv}/_{Fm}$ was monitored after 24 h. Closed bars represent the $\Delta^{Fv}/_{Fm}$ in control leaves infiltrated with empty vectors (pART27 and pMOG800), while the open bars represent leaves infiltrated with *Bcpg2* constructs.

At 24 hpi, *Bcpg2*, *Bcpg2-D192A*, *Vvamp1* and *Vvpgip1* mRNAs were expressed in the infiltrated leaves (Figure 3B). PG activity was detected in crude protein extracts from all leaves infiltrated with a functional *Bcpg2* construct and PG levels did not significantly differ between single and co-infiltration experiments (Figure 3C). Inhibitory activity against *Aspergillus niger* AnPG-I could be detected in all leaves infiltrated with *Vvpgip1* alone, or in combination with *Vvamp1* and did not differ significantly (Figure 3D). PGIP activity against AnPG-I in leaves co-infiltrated with the *Vvpgip1* and *Bcpg2* constructs could not be determined due to the presence of BcPG2 activity in the extract. VvAMP1 activity in leaves infiltrated with the *Vvamp1* construct was similar for single- and co-infiltration experiments

(not shown). Co-infiltration of *A. tumefaciens* cells containing the *Bcpg2* construct, with cells containing a *pgip* encoding gene isolated from the grapevine rootstock cultivar Ramsey, encoding a protein with 6 polymorphic residues, gave similar results as with the *Vvpgip1* gene (not shown).

The $\Delta^{Fv}/_{Fm}$ values for leaves infiltrated with the single *Bcpg2* construct as well as leaves co-infiltrated with the *Bcpg2* and *Vvamp1* or *Bcpg2-D192A* constructs were significantly higher than the $\Delta^{Fv}/_{Fm}$ values of leaves co-infiltrated with the *Bcpg2* and *Vvpgip1* constructs (Figure 3E). Leaves infiltrated with empty vectors, *Vvpgip*1, *Vvamp*1, *Bcpg2-D192A* alone or in any combination thereof did not show any symptoms and $\Delta^{Fv}/_{Fm}$ values were below 0.05 (Figure 3E).

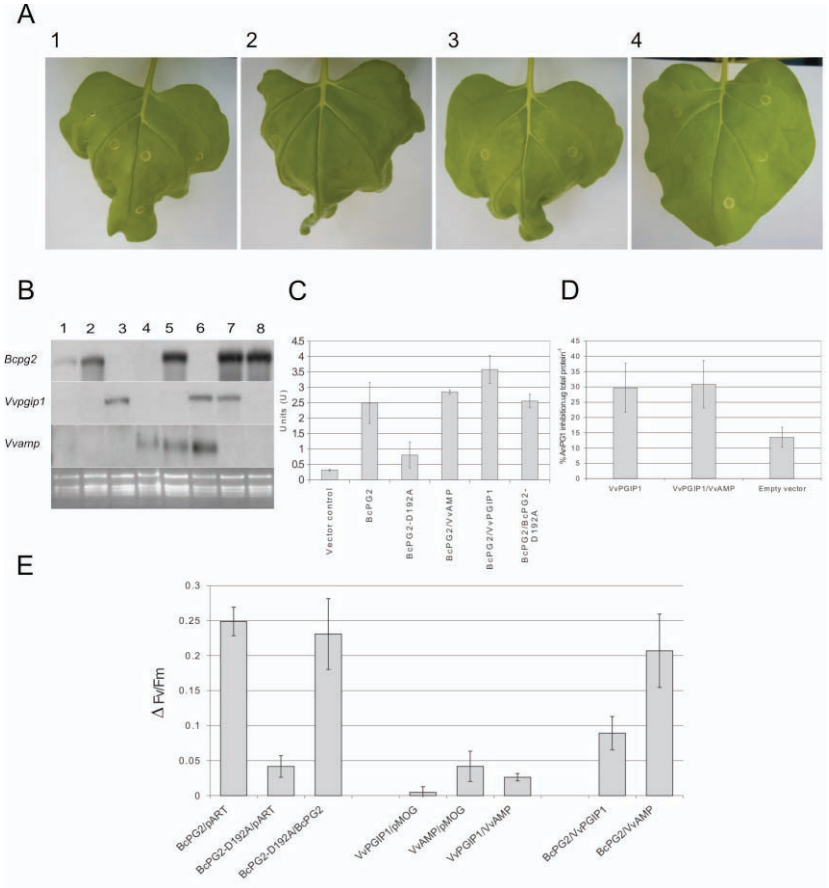

Figure 3. Co-expression of *Bcpg2* with *Bcpg2-D192A, Vvamp1* or *Vvpgip1* in *Nicotiana benthamiana.* Leaves were infiltrated with *Agrobacterium tumefaciens* strains carrying the *Vvpgip1, Vvamp1, Bcpg2-D192A* as well as the *Bcpg2* constructs containing the PR1a signal peptide in a 1:1 ratio. **(A)** Symptoms in leaves infiltrated with 1. *Bcpg2* + pART27 (empty vector control); 2. *Bcpg2* + *Bcpg2-D192A*; 3. *Bcpg2* + *Vvamp1* and 4. *Bcpg2* + *Vvpgip1* at 24 hpi. **(B)** Northern blot analysis of *Bcpg2*, *Bcpg2-D192A,*

Vvpgip1 and Vvamp1 expression in whole infiltrated leaves at 24 hpi. Blots were hybridized with probes specific for *Bcpg2* (top panel), *Vvpgip1* (middle panel) and *Vvamp1* (bottom panel), respectively. Lane 1. *Bcpg2*; lane 2. *Bcpg2-D192A*; lane 3, *Vvpgip1*; lane 4. *Vvamp1*; lane 5. *Bcpg2* + *Vvamp1*; lane 6. *Vvpgip1* + *Vvamp1*; lane 7. *Bcpg2* + *Vvpgip1*; lane 8. *Bcpg2* + *Bcpg2-D192A*. **(C)** PG activity in crude protein extracts from leaves infiltrated with single constructs or combinations (as indicated below the bars) at 24 hpi, determined by agarose diffusion assay. **(D)** Inhibition of AnPG1 by crude protein extracts isolated from leaves infiltrated with *Vvpgip1*, *Vvpgip1* + *Vvamp1* and empty vector, determined by agarose diffusion assay. **(E)** Change in variable chlorophyll fluorescence yield ($\Delta^{Fv}/_{Fm}$) in *N.benthamiana* leaves infiltrated with combinations of *Bcpg2*, *Bcpg2-D192A, Vvamp1, Vvpgip1* and empty vector (pART or pMOG), mixed in 1:1 ratios as indicated below the bars.

If VvPGIP1 and BcPG2 physically interact in a 1:1 ratio, one may predict that increasing the relative amount of BcPG2 would aggravate the symptoms, whereas increasing the relative amount of VvPGIP1 would not affect the symptoms. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells containing the respective genes mixed in different *Bcpg2:Vvpgip1* ratios, ranging from 10:1 (10X PG excess) to 1:10 (10X PGIP excess). After 24 h, wilting was observed in leaves infiltrated with *Bcpg2* and *Vvpgip1* in ratios of 10:1 and 5:1, whereas leaves infiltrated with ratios of 1:1, 1:5 and 1:10 appeared phenotypically normal at this point in time (not shown). Chlorophyll fluorescence ($\Delta^{Fv}/_{Fm}$) values were determined to assess the damage inflicted by PG action (Figure 4). The $\Delta^{Fv}/_{Fm}$ increased with higher levels of BcPG2; leaves infiltrated with a 10-fold BcPG2 excess to VvPGIP1 showed a higher $\Delta^{Fv}/_{Fm}$ compared to leaves infiltrated with a 5-fold BcPG2 excess and BcPG2:VvPGIP1 in a 1:1 ratio at 24 hpi. The $\Delta^{Fv}/_{Fm}$ values of leaves infiltrated with a 1:1 ratio and a 5-fold or 10-fold VvPGIP1 excess to BcPG2 did not differ significantly between them, nor did they differ from the empty vector controls (Figure 4).

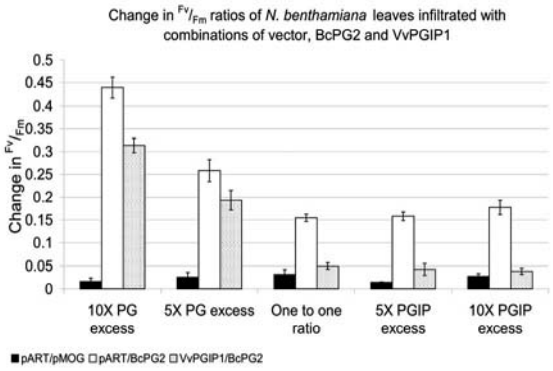

Figure 4. The effect on $^{Fv}/_{Fm}$ of over-expressing BcPG2 alone or in combination with VvPGIP1 in *Nicotiana benthamiana*. Leaves were infiltrated with different ratios (as specified in Table 3) of two empty vectors (closed bars), one empty vector + *Bcpg2* (open bars) and *Bcpg2* + *Vvpgip1* (stippled bars) and $\Delta^{Fv}/_{Fm}$ in infiltrated leaves were measured after 24 hpi.

VvPGIP1 does not inhibit or physically interact with BcPG2 in vitro

The interaction between VvPGIP1 and BcPG2 *in vitro* was studied by analysing PG inhibition (reducing sugar assays), physical interaction (by plasmon resonance spectroscopy), as well as substrate profiling (anion exchange chromatography with amperometric detection). For these experiments, VvPGIP1 was used that was purified from transgenic tobacco over-expressing the *Vvpgip1* gene (Joubert et al., 2006). Tobacco leaves contain no detectable endogenous PGIP activity (not shown). The heterologously expressed VvPGIP1 showed no detectable differences in glycoslylation patterns or inhibition spectra compared to that of a PGIP purified from grapevine berries (Joubert et al., 2006). In all experiments, the *A. niger* PGI (AnPGI) was used as a positive control to verify that VvPGIP1 was active and the experimental conditions allowed for interaction. In reducing sugar assays using purified BcPG2 and VvPGIP1, no inhibition of BcPG2 activity was observed at any of the pH values tested (Figure 5A) whereas AnPGI was readily inhibited by VvPGIP1 (Figure 5B).

In order to test for the occurrence of physical interaction by plasmon resonance, purified VvPGIP1 was immobilised on a sensor chip and purified BcPG2 was applied to the chip at three pH values ranging from 4.2 to 5.0. No interaction between VvPGIP1 and BcPG2 could be detected, whereas VvPGIP1 interacted with AnPGI at pH 4.75 (Figure 6). Results at pH 4.2 and 5.0 were essentially the same for both protein pairs (not shown).

PGIPs were reported to influence the product profiles of OGAs released from PGA by PG action (Cervone et al., 1987; Cook et al., 1999; Kemp et al., 2004). Degradation of PGA by BcPG2, either in the absence or presence of VvPGIP1 was investigated. No differences in OGA profiles were observed between PGA digested with BcPG2 in the absence or presence of VvPGIP1 (data not shown).

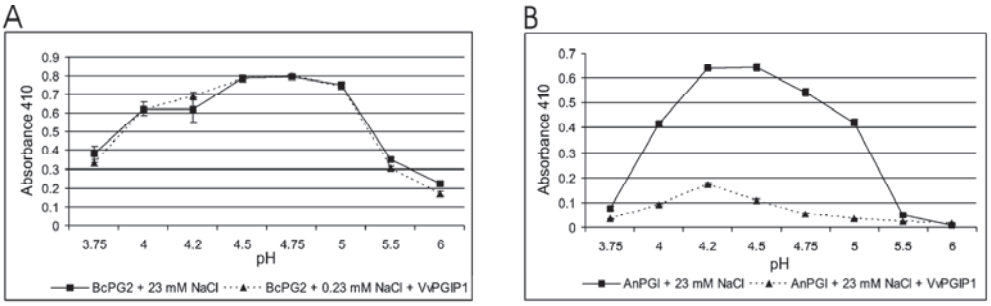

Figure 5. Quantitation of PG inhibition using colorimetric reducing sugar assays. *Botrytis cinerea* BcPG2 (~50 ng) (A) or *Aspergillus niger* AnPGI (B) was incubated with PGA at a pH ranging from 3.75 to 6.0. The activity of AnPGI and BcPG2 alone is indicated by a solid line and squared data points. Activity of both enzymes in the presence of VvPGIP1 (~100 ng) is indicated by a dotted line and triangular data points.

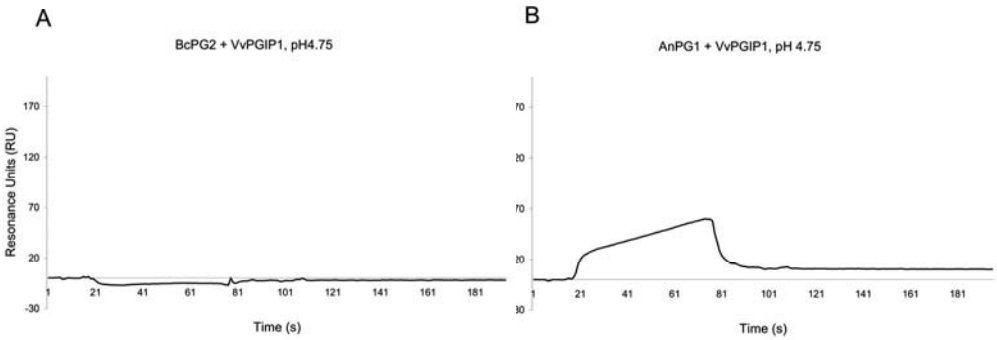

Figure 6. The physical interaction at pH 4.75 between pure VvPGIP1 and pure BcPG2 **(A)** or pure *Aspergillus niger* AnPGI **(B)**, measured by plasmon resonance. Association, dissociation, and regeneration phases were followed in real time and observed as a change in response units (RU). Binding was quantified by subtracting the flow cell sensorgram from the control flow cell sensorgram. 1000 RU represent approximately 1 ng.mm-2 of protein bound to the sensor chip surface. Results at pH 4.2 and 5.0 were essentially the same for both protein pairs.

Discussion

Agrobacterium infiltration is suitable to analyse the in planta effects of individual Botrytis cinerea PGs and PG-mutants

The transient expression of *Botrytis cinerea* endopolygalacturonases (BcPGs) by *Agrobacterium tumefaciens*-mediated infiltration of leaf material, first described for *Colletotrichum lindemuthianum* ClPG1 (Boudart et al., 2003), enabled us to examine the extent of damage that individual BcPGs are able to cause *in planta*. As in the case of the ClPG1 (Boudart et al., 2003), a plant signal peptide was much more effective than the fungal signal peptide to obtain PG enzyme activity and symptoms. The PR1a signal peptide constructs consistently caused symptoms within 48 h, whereas constructs with the native fungal signal peptide only caused symptoms several days later, if any. The fungal signal peptide presumably confers only low secreted protein production levels in *Nicotiana benthamiana*, consequently leading to less severe pectin degradation and delay of symptoms.

Transient expression enabled us to monitor the effects of BcPGs that could not be produced *in vitro* in *Pichia pastoris*, such as BcPG5 (Kars et al., 2005a). The latter enzyme was able to cause symptoms comparable in severity to BcPG1 and BcPG4. The method also allowed an examination of the effects of point mutations in individual enzymes, specifically mutations in the catalytic site that render the enzymes inactive without affecting their tertiary structure. The use of such mutants allows differentiation between plant tissue damage caused directly by enzymatic activity and damage resulting from defence responses

following elicitor recognition, as best exemplified for BcPG1. The transient expression of BcPG1 *in planta* led to the development of chlorotic symptoms with yellow/brown patches of tissue, even when enzyme activity could not be detected in intercellular fluid. This may be due to the specific activity of BcPG1, which is substantially lower than that of other BcPGs (up to 90-fold lower than BcPG2; Kars et al., 2005a). The observation that expression of the inactive mutant protein BcPG1-D203A led to comparable symptom development in *N. benthamiana*, suggests that BcPG1 can be recognised by the plant as an elicitor as previously concluded by Poinssot et al., (2003). It should, however, be noted that the symptoms inflicted by BcPG1 were moderate and delayed as compared to BcPG2. The latter enzyme was expressed to high activity levels in early stages and its expression resulted in massive cell collapse and tissue disintegration (Figure 1A and C). Leaf tissue that expressed BcPG2 became brittle and easily fractured upon handling, possibly as a consequence of the rigidity of the cellulose network from which pectin had been removed. Expression of the inactive mutant protein BcPG2-D192A caused no symptoms, proving that enzyme activity is required for symptom development, as was also described for the *C. lindemuthianum* ClPG1 (Boudart et al., 2003). The dramatic effect of BcPG2 action on tissue integrity probably overrules any elicitor responses that might be caused by recognition of BcPG1 or other BcPGs. The lack of symptoms triggered by infiltration with *A. tumefaciens* carrying *Bcpg*3 or *Bcpg*6 constructs is probably related to the fact that these enzymes do not cause any detectable damage in plant tissue, even when high activity levels are infiltrated in pure form into leaves (Kars et al., 2005a).

The physiological effect of BcPG2 on leaves was quantified using a non-destructive and non-invasive assay technique. Chlorophyll fluorescence measurements demonstrated that the extent of damage in leaves expressing *Bcpg2* increased with higher *A. tumefaciens* cell densities. The chlorophyll fluorescence measurement was therefore considered a suitable method to quantify tissue damage.

***In planta* interaction of an individual BcPG and a PGIP upon transient co-expression**

In spite of over twenty years of investigation, the role of PGIPs in plant defense responses is not fully understood. PGIPs differentially inhibit fungal PGs *in vitro*, but little evidence is available to support the assumption that a successful *in vitro* interaction between a given PGIP - PG pair actually reflects the potential of this particular PGIP to increase resistance when expressed *in planta*. Over-expressing PGIPs in transgenic plants was in several cases shown to reduce *B. cinerea* disease symptoms (Agüero et al., 2005; De Lorenzo and Ferrari, 2002; Ferrari et al., 2003; Powell et al., 2000). Also VvPGIP1 reduced the susceptibility to *B. cinerea* when over-expressed in *N. tabacum* (Joubert et al., 2006). In view of the important role of BcPG2 in virulence of *B. cinerea* (Kars et al., 2005a) and the observed increased resistance in PGIP-expressing plants, one would be tempted to ascribe this resistance (at

least partially) to the inhibition of BcPG2 action by the PGIP. Co-expression of VvPGIP1 with BcPG2 indeed led to substantial symptom reduction, both visually and by chlorophyll fluorescence measurement whereas no symptom reduction was observed when BcPG2 was co-expressed with a different secreted protein, such as BcPG2-D192A or VvAMP1. The extent of tissue damage in leaves expressing *Bcpg2* increased with higher *A. tumefaciens* cell densities (Figure 2), but the damage was significantly reduced or even negated by co-expression of *Vvpgip1* (Figures 3A, 3E). This result was corroborated by expressing various ratios of enzyme versus inhibitor (Figure 4) and the results of this experiment provided confidence regarding the suitability of the test system to assess true interaction. Specifically, no appreciable differences in $\Delta^{Fv}/_{Fm}$ values were observed in leaves infiltrated with 1:1 , 1:5 or 1:10 *Bcpg2:Vvpgip1* ratios, strongly suggesting that a 1:1 stoichiometric interaction indeed occurs between VvPGIP1 and BcPG2, as is generally observed for the interaction of PGIP and PG *in vitro* (King et al., 2002).

In vitro analyses detect no interaction between BcPG2 and VvPGIP1

Surprisingly, no physical interaction could be detected *in vitro* between VvPGIP1 and BcPG2, and VvPGIP1 did not alter the oligogalacturonide profile generated from PGA by BcPG2. Comprehensive methods were employed that are normally used to evaluate PG-PGIP interactions *in vitro*. The test systems all performed well as established with appropriate controls, yet the interaction between BcPG2 and VvPGIP1 that was observed *in planta* could not be demonstrated *in vitro*. BcPG2 appears to be a rather unusual enzyme, because it has thus far not been possible to detect any *in vitro* inhibitors. Crude PGIP-containing cell wall extracts from eight other plants (lemon, leek, pepper, apple, bean, onion, cucumber and pear), as well as purified PGIPs from bean, tomato and pear, were all unable to inhibit pure BcPG2 *in vitro* to any detectable level under any experimental condition tested, even though these extracts did inhibit several other pure BcPG enzymes (G.H. Krooshof, C. Bergmann, R. Joosten, J.A.E. Benen and J.A.L. van Kan, unpublished).

The present models for the interaction between PGIPs and PGs postulate that their interaction depends on structural requirements in both proteins (Bishop, 2005; Federici et al., 2001; Leckie et al., 1999). It has always been considered that the most potent *in vitro* inhibitors would be the most attractive proteins to express in plants for achieving optimal resistance to fungal pathogens in biotechnology approaches. The results of the present study, however, suggest that the current working hypothesis for PGs and PGIPs may require refinement by considering the possibility for the occurrence of an *in vivo* interaction in the absence of any detectable *in vitro* interaction between the protein pair. The transient co-expression system would be a suitable tool to examine whether similar observations can be made for different PG:PGIP protein pairs.

Recent evidence was presented that bean PGIPs are able to interact with pectin (Spadoni et al., 2006). It remains to be established whether VvPGIP1 can also bind to pectin. If so, rather than truly directly inhibiting BcPG2, VvPGIP1 might in fact operate by shielding the most exposed and vulnerable positions in the pectin, thereby making the substrate less accessible to BcPG2. Such a substrate shielding mechanism was proposed for the AVR4 protein from the fungal pathogen *Cladosporium fulvum*, which has chitin-binding properties and can protect the fungal cell wall from attack by plant chitinases, presumably by preventing the hydrolase from getting access to the substrate (van der Burg et al., 2006). Future surface plasmon resonance experiments might unravel whether VvPGIP1 can indeed interact with pectin and thereby protect the substrate from hydrolysis by BcPG2.

Experimental procedures

Transient expression of Botrytis cinerea endopolygalacturonases in N. benthamiana

Agrobacterium tumefaciens strain GV3101 was transformed by electroporation. Recombinant *A. tumefaciens* containing the binary vector pMOG800 (Honée et al., 1998) was used for transient expression of the *Botrytis cinerea* endopolygalacturonase genes *Bcpg1*, *Bcpg2*, *Bcpg3*, *Bcpg4*, *Bcpg5* and *Bcpg6*. For each gene two types of constructs were made: one containing the full length ORF (including the native *B. cinerea* signal peptide), and one containing the sequence encoding the mature protein, preceded by a functional plant signal peptide sequence. The target genes were amplified with primers containing specific restriction sites for cloning purposes (Table 2). The amplified products were digested with the appropriate restriction enzymes and subsequently cloned into pAT1 or pAT2 (derivates of pFM10; Honée et al., 1998). The sequences of the mature proteins were cloned in pAT2 in frame with the signal sequence of tobacco PR1a using *SalI* and *SacII*. The resulting construct was subsequently cloned into pMOG800 with *XbaI* and *EcoRI*. All inserts were verified by DNA sequence analysis (BaseClear, Leiden, The Netherlands). The empty pMOG800 vector was used as control. Transformed *A. tumefaciens* cells were grown as described in van der Hoorn et al. (2000), except that they were suspended in MMA (10 mmol.L^{-1} $MgCl_2$, 10 mmol.L^{-1} MES pH 5.6), to final OD_{600} 1.0 prior to infiltration into *N. benthamiana*, using disposable syringes without needle.

Co-infiltration of BcPG2 with VvPGIP1, BcPG2D192A or VvAMP1 in N. benthamiana

Vvpgip1 was amplified with primers containing *Sal*I and *Xba*I sites (Table 2), purified and cloned into the *Xho*I and *Xba*I sites of the pGEM9Zf derivative, pART7 (Gleave et al., 1992) in between the 35SCaMV promoter and nopaline synthase terminator elements. The *Vvpgip1* expression cassette was subsequently excised from pART7 with *NotI* and cloned

Table 2. Primers (forward and reverse) used to generate expression constructs.

Vector	Insert	Primer name	Primer sequence (5'-3')[1]	Restriction site
pAT1	Bcpg1	00-12	AGAGAGCCATGGTCAACTTCTCTCAATGG	NcoI
		00-14	AGAGAGCCGCGGTTAACACTTGACACCAGATGGG	SacII
	Bcpg2	00-1	CACTCACCATGGTTCATATCACAAGCC	NcoI
		00-2	GAGAGCCGCGGTTAGCAAGAAGCTCCGGT	SacII
	Bcpg3	00-9	GAAGAGCCATGGCTTCTGCGATCATCCTCGGA	NcoI
		00-11	AGAGAGCCGCGGTTATGATGGGCATCCAG	SacII
	Bcpg4	00-15	AGAGAGCCATGGCTTCCACCAAGTCTATGGCAGCT	NcoI
		00-17	AGAGAGCCGCGGTTAAGAGCAAGAACCAAC	SacII
	Bcpg5	00-18	AGAGAGCCATGGTTAAGTTTTCTGCCTG	NcoI
		00-20	AGAGAGCCGCGGCTACAAGGAACAAGAGAC	SacII
	Bcpg6	00-21	GAGAGGCCATGGCTAAGAACTCTCAGATCTC	NcoI
		00-23	GAGGAGCCGCGGTATGCGGGACAGCCAG	SacII
pAT2	Bcpg1	00-13	GAGAGAGTCGACGCGTGGTACCGCCTGTACCT	SalI
		00-14	AGAGAGCCGCGGTTAACACTTGACACCAGATGGG	SacII
	Bcpg2	00-3	GAGAGGTCGACGCGTGCTGGTTGCACATTC	SalI
		00-2	GAGAGCCGCGGTTAGCAAGAAGCTCCGGT	SacII
	Bcpg3	00-10	GAGAGAGTCGACCTGCGACAACCCAGACCATGA	SalI
		00-11	AGAGAGCCGCGGTTATGATGGGCATCCAG	SacII
	Bcpg4	00-16	AGAGAGCTCGAGGCGTGCCGCAACCTGT	XhoI
		00-17	AGAGAGCCGCGGTTAAGAGCAAGAACCAAC	SacII
	Bcpg5	00-19	AGAGAGGTCGACGCGAGCAACTACCTGCACT	SalI
		00-20	AGAGAGCCGCGGCTACAAGGAACAAGAGAC	SacII
	Bcpg6	00-22	GAGAGAGTCGACGCAAACAGCTTGTACTGCC	SalI
		00-23	GAGGAGCCGCGGTATGCGGGACAGCCAG	SacII
pART7	Vvpgip1	Pgips	GTCGACATGGAGACTTCAAAAC	SalI
		Pgipas	TCTAGAACTTGCAGCTCTGGAGTGGAG	XbaI

[1] Restriction sites introduced for cloning purposes are underlined.

Table 3. *Agrobacterium tumefaciens* cell densities used for agro-infiltrations of *Vvpgip1* and *Bcpg2* carrying the PR1a signal peptide.

pART:pMOG; pART:BcPG2 and VvPGIP1:BcPG2	OD_{600}
1:10	0.06:0.6
1:5	0.06:0.3
1:1	0.06:0.06
5:1	0.3:0.06
10:1	0.6:0.06

into the binary vector pART27 (derived from pMON530; Rogers et al., 1987; Gleave et al., 1992).

A full-length cDNA of an antifungal peptide from *Vitis vinifera* (VvAMP1) that was identified in an EST database (TC69032 - http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) and cloned into pART27 was obtained from Abre de Beer (Institute for Wine Biotechnology, Stellenbosch University, South Africa). The VvAMP1 protein has been shown to be secreted to the apoplastic space when expressed in transgenic tobacco (A. de Beer, pers. comm.).

N. benthamiana plants were grown from seeds in a peatmoss mixture (Jiffy) and maintained in a greenhouse at 26ºC and 70% humidity under natural light. Eight-week-old *N. benthamiana* plants were used for infiltration experiments. *A. tumefaciens* infiltrations were done according to Wroblewski et al. (2005). The *A. tumefaciens* strain EHA105 (Hood et al., 1993) was used for all co-infiltrations and was transformed with the appropriate constructs by electroporation. Unless otherwise stated, for co-infiltrations based on a 1:1 ratio, the cell densities of Agrobacterium cells carrying each construct were adjusted to OD_{600} 0.3, therefore allowing for the total cell density to = OD_{600} 0.6. For the co-infiltration experiment where the effect of different ratios of VvPGIP1 to BcPG2 was compared (Figure 4), the Agrobacterium cell densities were mixed in the ratios described in Table 3. All infiltrations were done in triplicate using three separate leaves on the same plant. Infiltrated plants were maintained in a greenhouse at 26ºC and 70% humidity under natural light until chlorophyll fluorescence measurements were taken or tissue collected for subsequent analysis.

Activity assays

Intercellular fluids (IF) were isolated from *N. benthamiana* leaves at 1 to 5 days after infiltration with *A. tumefaciens* cells, as described by de Wit and Spikman (1982). PG activity in IF was examined qualitatively by spotting a 2 μL droplet of IF on agar plates containing 0.1 % (w/v) PGA, pH 4.2 (Sigma). After incubation at 30°C for 30 min the plates were stained with ruthenium red (Fluka). Enzyme activity appeared as a white spot in a red background.

Crude protein extracts from *N. benthamiana* leaves were isolated by freezing the leaves in liquid nitrogen and grinding the tissue with a mortar and pestle to a fine powder. Extraction buffer (20 mM sodium acetate, pH 5.2 for crude PG, 100 mM phosphate buffer, pH 7.0 for AMP isolations, and 20 mM sodium acetate, 1 M NaCl, pH 5.2 for crude PGIP isolations) was added to a final ratio of 2 mL buffer per 1 g starting tissue. Tissue was further ground in extraction buffer to a fine homogenate and incubated on ice for one hour with gentle shaking. The mixture was centrifuged at 15 000 x g for 25 min at 4°C and the supernatant filtered through one layer of miracloth, aliquoted and stored at –20°C. Protein

concentration was determined according to Bradford (1976) using a Biorad protein assay kit (Biorad) and bovine serum albumin (BSA) as a standard. PG activity from crude protein extracts was measured using an agarose diffusion assay as described by Taylor and Secor (1988) using 0.5% polygalacturonic acid (PGA) as substrate at pH 4.2. One enzyme unit (U) is defined as the amount of enzyme necessary to increase the zone size by one mm^2 over a period of 16 h.

To determine VvPGIP1 activity in crude protein isolates, 1 µg of total protein was incubated with 10 ng of purified AnPG1 for 16 h at 30°C using an agarose diffusion assay as described. VvPGIP1 activity was expressed as the % reduction in clearing zone sizes effected by AnPG1 with crude VvPGIP1 compared to clearing zones sizes effected by AnPG1 alone.

VvPGIP1 was purified from transgenic tobacco over-expressing the *Vvpgip1* gene essentially as described by Favaron et al., (1994). The inhibition of AnPGI and BcPG2 by VvPGIP1 was assayed using reducing sugar assays with purified VvPGIP1. VvPGIP1 was incubated with AnPGI or BcPG2 and 0.025% PGA (Sigma), buffered in 50 mM sodium acetate with a pH ranging from 3.75 to 6.0 for 1 h at room temperature. The inhibition of AnPGI and BcPG2 by VvPGIP1 was quantified following incubation by spectrophotometrically (410 nm) measuring the decrease in the release of reducing sugars, using the PAHBAH (p-hydroxybenzoic acid hydrazide) procedure (York et al., 1985). PGA was included as control. In order to compensate for the possible influence of chromatography buffer gradient on the enzyme activity, equivalent amounts of salt were added to all assays. All assays were done in triplicate.

RNA extraction and northern blot analysis

Whole infiltrated leaves without the petiole were excised, the tissue ground and subsequently used for both RNA extractions, according to Joubert et al., (2006). RNA was electrophoresed, blotted and blots were hybridised with probes containing the full length ORFs of *Vvamp1*, *Bcpg2* and *Vvpgip1* respectively. The probes were labelled and northern blotting and detection was done using the DIG system according to the DIG Application Manual for Filter Hybridization (Roche Diagnostics GmbH, Mannheim, Germany).

Chlorophyll fluorescence parameters

The maximal photochemical yield of photosystem II was measured as variable chlorophyll fluorescent yield of dark adapted leaves (overnight) at room temperature with the following formula $^{Fv}/_{Fm} = {}^{(Fm-Fo)}/_{Fm}$ (Krause and Weis, 1991). A Hansatech Plant Efficiency Analyzer was used. All measurements were performed in the dark by exposing leaves for 5 s to a maximum light intensity of 3000 $\mu mol.m^{-2}.s^{-1}$. $\Delta^{Fv}/_{Fm}$ values were determined by deducting the $^{Fv}/_{Fm}$ value of a leaf 24 h post-infiltration from the pre-infiltration $^{Fv}/_{Fm}$ value of the same

leaf. All chlorophyll fluorescence measurements were done in triplicate for each infiltrated leaf. Three leaves were infiltrated for each experiment, bringing the total number of chlorophyll fluorescence measurements to nine for each experiment. Abbreviations: Fv = variable fluorescence; Fm = maximal fluorescence, F0 = initial fluorescence

Statistical analysis

All statistical analysis was done by one-way Analysis of Variance (ANOVA) using the STATISTICA 7 (StatSoft Inc, Tulsa, OK, USA) software package at 95% confidence intervals.

Plasmon resonance experiments

Immobilisation of VvPGIP on Sensor Chip

A single flow cell of a CM5 sensor chip was equilibrated at 25^{o}C with HBS buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% P20, pH 7.4) followed by the automated immobilisation of purified VvPGIP1. During the automated immobilisation, equal volumes of 0.1 M NHS (N-hydroxy succinimide) and 0.1 M EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) were mixed by the BIAcore system and eluted over the surface of the sensor chip to activate the carboxymethylated dextran. Subsequently, 65 µL of VvPGIP (20 µg.µL^{-1}) was injected over the activated surface at a flow rate of 5 µL.min^{-1} and the remaining active carboxyl groups were deactivated with an injection of 1 M ethanolamine. This was followed by a 1 h wash with HBS buffer to ensure a stable base line. As a control, this procedure was repeated in a separate flow cell without the immobilisation of VvPGIP1. Approximately 5400 resonance units (RU) of VvPGIP1 were coupled.

Binding Assays on the BIAcore

All experiments were carried out at 25°C with a constant flow rate of 10 µL.min^{-1} running buffer (10 mM sodium acetate, 150 mM NH_4Cl, 0.005% P_2O, pH range: 4.2, 4.75, 5.0). Purified ligand (AnPGI and BcPG2) at a final concentration of about 10 ug.ml^{-1} in running buffer was injected over the immobilised VvPGIP and binding was qualitatively evaluated. Association, dissociation, and regeneration phases were followed in real time as a change in signal expressed in RU. The resulting sensorgrams used for binding analysis were obtained by subtracting the flow cell sensorgram from the control flow cell sensorgram. All steps described were fully automated and carried out by the BIAcore system's robotics. 1000 RU represents approximately 1 ng.mm^{-2} of protein bound to the sensor chip surface. All BIAcore binding experiments were repeated with an independently constructed sensor chip.

High performance anion exchange chromatography with amperometric detection

The oligogalacturonide (OGA) profile generated by BcPG2 from homogalacturon was obtained by anion exchange chromatography using a Metrohm Peak HPLC with a Bioscan (model 817) pulsed amperometric detector and a Dionex CarboPAC PA1 column coupled to a Dionex amperometric detector as described by Cook et al. (1999). PGA (0.025%) was used as substrate and the reaction was allowed to continue for one hour at room temperature in 20 mM sodium acetate, pH 4.0.

Acknowledgements

The authors would like to thank A. Jones for excellent technical assistance and A. de Beer for providing the VvAMP1 construct. A. ten Have is acknowledged for design and construction of the expression vectors pAT1 and pAT2. This work was supported by grants from the South African Wine Industry (Winetech), the National research foundation of South Africa (NRF), by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (project WGC.5034), the US Department of Energy (DOE) (DE-FG02-96ER20221) and the DOE-funded Center for Plant and Microbial Complex Carbohydrates (DE-FG02-93ER20097).

Chapter 6

The quantitative trait locus RBPG1 controls the response to *Botrytis cinerea* endopolygalacturonases in *Arabidopsis thaliana*

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This chapter is to be submitted.

Abstract

The necrotrophic fungal plant pathogen *Botrytis cinerea* causes grey mould on a broad range of host species. During infection *B. cinerea* secretes six endopolygalacturonases (BcPGs) that are able to degrade pectin present in the cell walls of plants. The natural variation in the response of *Arabidopsis thaliana* to four *B. cinerea* BcPGs has been investigated by infiltration of leaves of different *A. thaliana* accessions. Considerable variation in responses was observed, ranging from no visible symptoms to full necrosis of the entire infiltrated leaf area. Of the 47 *A. thaliana* accessions tested, Br-0 and Est-0 showed a significantly lower response to the infiltrated BcPGs than any of the other accessions tested. Quantitative trait locus (QTL) mapping was performed in two segregating populations, i.e. an existing RIL population L*er*0 x Kas-2, and a F_2 population originating from a cross between Col-0 and Br-0. A single QTL controlling the response to BcPGs was detected in the progeny of the cross between accessions Col-0 and Br-0. This QTL explains 60 to 81% of the observed variation in the response to BcPG3, BcPG4 and BcPG6. The Br-0 allele at this QTL is recessive and significantly diminishes the response to the BcPGs. The QTL was positioned on chromosome 3 of the *A. thaliana* genome and was designated RBPG1. The QTL is a starting point to identify the gene(s) involved in response to BcPGs and its role in resistance to *B. cinerea*. Identification of the gene(s) will allow to study the mechanism involved in the response to BcPGs in more detail.

Introduction

Botrytis cinerea is a necrotrophic pathogenic fungus that produces non-selective phytotoxins and extracellular enzymes enabling it to infect a broad range of host species (van Kan, 2006), resulting in severe pre- and post-harvest losses. Plants possess a range of incomplete natural defense mechanisms in response to *Botrytis* attack, including structural barriers and the production of antifungal proteins or secondary metabolites (Van Baarlen et al., 2004a). So far, resistance genes that completely restrict the growth of the pathogen have not been reported in any crop species. Recently, a number of quantitative trait loci (QTL) has been identified that originate from a wild relative of tomato, *Solanum habrochaites* LYC 4, and confer partial resistance to *B. cinerea* in cultivated tomato, *Solanum lycopersicum* (Finkers et al., 2007a, b). The genes and mechanisms underlying the partial resistance conferred by these loci remain to be unraveled.

B. cinerea causes disease symptoms on *Arabidopsis thaliana* (Hammond-Kosack and Parker, 2003; Thomma et al., 1998, 1999), the major model in plant biology including plant-microbe interactions. *A. thaliana* is a self-fertilizing annual species with a broad geographical distribution throughout the Northern hemisphere (Hoffmann, 2002). Accessions representing substantial natural variation have been collected from natural populations. Most of the variation is of quantitative nature, exhibiting a continuous range of phenotypic variation. Natural variation in susceptibility to *B. cinerea* among sixteen *A. thaliana* accessions was reported and multiple QTL that govern susceptibility were identified, most of which were specific for an individual *B. cinerea* isolate (Denby et al. (2004). Also, the effects of defined mutations on susceptibility to *B. cinerea* are usually quantitative (reviewed by Glazebrook, 2001, 2005; Thomma et al., 1998, 1999; van Baarlen et al., 2007), illustrating the complex nature of the interaction between *B. cinerea* and its hosts.

A variety of *B. cinerea* pathogenicity factors has been identified (van Kan, 2006), including endopolygalacturonases (BcPGs; reviewed by Kars and Van Kan, 2004). BcPGs are important in the maceration of plant cell walls and decomposition of host tissue (Kars et al., 2005a) and it is plausible to predict that genetic determinants of a plant that contribute to delimiting maceration and tissue decomposition may confer (partial) disease resistance to *B. cinerea.* Plants possess a family of defense proteins known as polygalacturonase-inhibiting proteins (PGIPs) that can interact with and inhibit PGs of several sources (reviewed by Juge, 2006). The constitutive expression of PGIPs in transgenic plants have indeed been shown to reduce *B. cinerea* disease symptoms (Agüero et al., 2005; Ferrari et al., 2003; Joubert et al., 2006; Powell et al., 2000). To identify, in an unbiased manner, genetic factors of plants that contribute to reduction of the damage inflicted by BcPGs, we studied the natural variation in *A. thaliana* accessions in their response to infiltration of pure BcPGs, produced in the yeast *Pichia pastoris* (Kars et al., 2005a). QTL analyses were performed in order to investigate the genetic nature of the observed variation in responses to BcPGs. For

this investigation, we used a recently developed recombinant inbred line (RIL) population (El-Lithy et al., 2006) and created a F_2 population from parents that showed contrasting responses to different BcPGs, i.e. accessions Col-0 and Br-0. A single QTL was detected that controls the response to all four BcPGs tested.

Results

Variation in the response to endopolygalacturonases of Botrytis cinerea in a collection of Arabidopsis thaliana accessions

In order to study the genetic variation in *Arabidopsis thaliana* response to *Botrytis cinerea* endopolygalacturonases (BcPGs), rosette leaves of 47 accessions (Table 1) were infiltrated with BcPG2, BcPG3, BcPG4 and BcPG6. In this pre-screening, variation among the accessions was observed (not shown). Eleven of the 47 accessions, representing the spectrum of variation observed in responses to the BcPGs tested, were selected to further study the genetic variation using BcPG2, BcPG3 and BcPG6 (Table 1). The responses were scored in five classes ranging from 0 (no discernible symptoms) to 4 (full necrosis of the infiltrated area; Figure 1). The two Kashmir accessions (Kas-1 and Kas-2) showed the highest response to the three BcPGs infiltrated, whereas the accessions Estland (Est-0) and Brno (Br-0) showed almost no response (Figure 2). The accession Kondara (Kond) showed almost no response to BcPG2, but showed a high response to BcPG6. Accessions Tsu (Tsu-1), Gabelstein (Ga-0), Landsberg *erecta* (L*er*) and Shahdara (Shah) showed a high response to BcPG2 and BcPG6, but a rather low response to BcPG3 (Figure 2).

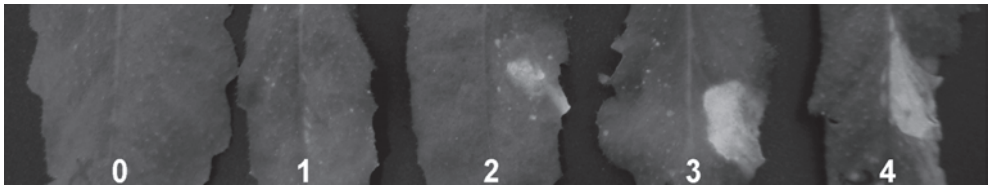


Figure 1. Response to *Botrytis cinerea* endopolygalacturonases in *Arabidopsis thaliana*. The response was visually scored in five classes ranging from 0 to 4, with 0 = no symptoms, 1 = chlorotic spots within the infiltrated zone, 2 = chlorosis covering the infiltrated zone, 3 = abundant chlorosis with necrotic spots, and 4 = complete necrosis.

Table 1. *Arabidopsis thaliana* accessions used in the screening for variation in response to different *Botrytis cinerea* endopolygalacturonases.

Accession	Origin	Stock center number	Accessions used in further studies [a]
Br-0	Brno (Czech Republic)	N6626	x
Bs-1	Basel (Switzerland)	N6627	
Cnt-1	Canterbury (UK)	N1635	
Co-1	Coimbra (Portugal)	N6669	
Col-0	Columbia (USA)	N907	x
Col-4	Columbia (USA)	N933	
Ct-1	Catania (Italy)	N1094	
Cvi	Cape Verdi Islands	N8580	
Cvi-1 (bas)	Cape Verdi Islands	N8580	
Di (Dijon-G)	Dijon (France)	N10159	
Ei-2	Eifel (Germany)	N6689	
Ema-1	East Malling (UK)	N1637	
En-2	Enkheim (Germany)	N1138	
Eri	Eriengsboda (Sweden)	N10042	
Est-0	Estland	N6700	x
Ga-0	Gabelstein (Germany)	N6714	x
Ga-2	Gabelstein (Germany)	N10209	
Gy-0	La Miniere (France)	N6732	
Ka-0	Karnten (Austria)	N6752	
Kas-1	Kashmir (India)	N903	x
Kas-2	Kashmir (India)	N1264	x
Kond	Kondara (Tadjikistan)	N6175	x
Kyoto	Kyoto (Japan)	N10231	
L*er*	Landsberg (Germany)	NW20	x
L*er* Koornneef	Landsberg (Germany)	N8581	
Li-0	Limburg (Germany)	N6775	
Lip-0	Lipowiec/Chrzanow (Poland)	N6780	
Lm-2	Le Mans (France)	N6784	
Lz-0	Lezoux/Puy-de-Dome (France)	N6788	
Ma-0	Marburg/Lahn (Germany)	N6789	
Nd-0	Niederzenz (Germany)	N6803	
Nd-1	Niederzenz (Germany)	N1636	
No-0	Halle (Germany)	N1394	
Nok-1	Noordwijk (Netherlands)	N6807	
Oy-0	Oystese (Norway)	N6824	x
Pi-0	Pitztal Tirol (Austria)	N6832	
Rld-1	Netherlands	N913	
Rsch-0	Rschew/Starize (Russia)	N6848	
Shah	Pamiro-Alay (Tadjikistan)	N929	x
Stw-0	Stobowa (Poland)	N6865	
Ts-1	Tossa del Mar (Spain)	N1552	
Tsu-1	Tsu (Japan)	N1640	x
Wei-0	Weiningen (Switserland)	N6182	
Wei-1	Weiningen (Switserland)	N1639	
Wi-0	Wildbad (Germany)	N6920	
Ws-1	Wassilewskija (Russia)	N2223	
Wt-1	Wietze (Germany)	N1604	

[a] Eleven accessions representing the spectrum of variation in responses were selected for further studies (marked with x).

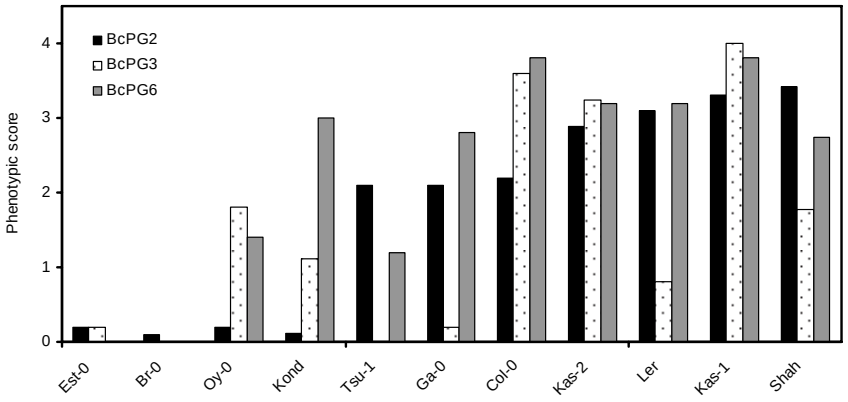


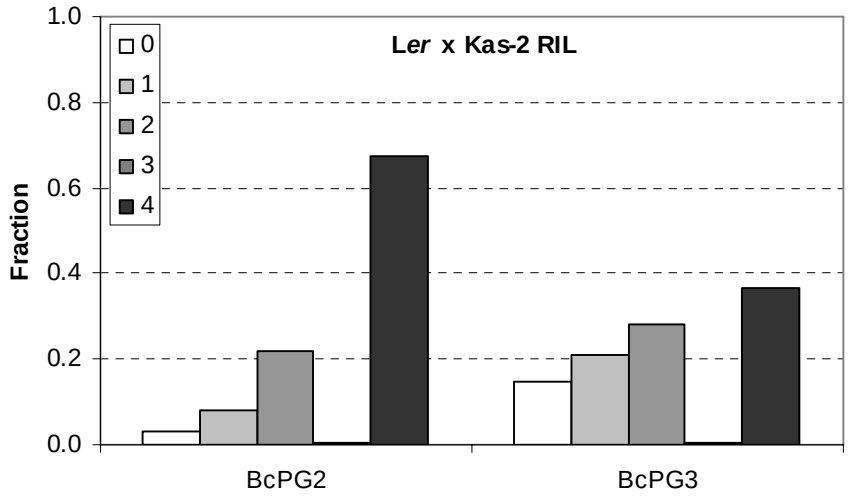
Figure 2. Response to *Botrytis cinerea* endopolygalacturonases BcPG2, BcPG3 and BcPG6 (3U/ml) of eleven selected *Arabidopsis thaliana* accessions. Phenotypic score ranged from 0 (no symptoms) to 4 (complete necrosis). Full names of *A. thaliana* accessions are given in Table 1.

Phenotypes of the mapping populations

Two mapping populations were analyzed to identify loci involved in the variation of response to BcPGs. The first population used was a previously described recombinant inbred line (RIL) population derived from the cross between the accessions Landsberg *erecta* (L*er*) and Kashmir (Kas-2) (El-Lithy et al., 2006). The parental lines of this population showed a marked difference in response to BcPG3, whereas their responses to BcPG2 and BcPG6 were similar (Figure 2). A second population was generated by crossing two accessions that showed more distinct responses, i.e Columbia (Col-0), which showed a high response to the BcPGs tested, and Brno (Br-0), which showed no symptoms upon infiltration with BcPGs (Figure 2).

Plants of the L*er* x Kas-2 RIL population were infiltrated with BcPG2 and BcPG3. The response to BcPG3 was generally lower than to BcPG2, yet the difference between the average responses to both BcPGs was small (Figure 3A). The plants of the Col-0 x Br-0 F_2 population were infiltrated with BcPG2, BcPG3, BcPG4 and BcPG6. The lowest average response in this population was observed with BcPG2, while BcPG4 caused the highest average response with 72% of the individuals scoring in the most severe phenotypic class (Figure 3B).

A



B

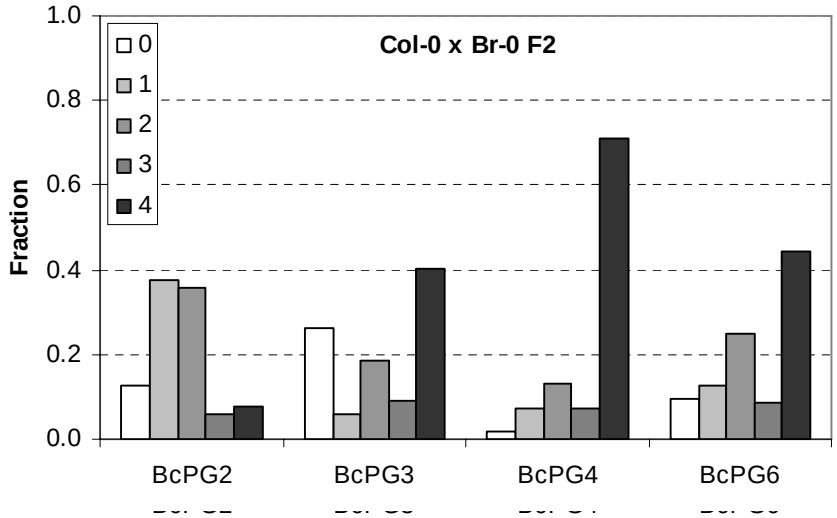


Figure 3. Phenotypic scores of the response to the different *Botrytis cinerea* endopolygalacturonases using **(A)** the L*er* x Kas-2 recombinant inbred line population and **(B)** the Col-0 x Br-0 F2 population. Each bar represents a different phenotypic score class as indicated in the graph legend. Classes range from 0 (no response) to 4 (necrosis). The fraction of plants that were categorized in the different phenotypic score classes is given on the Y-axis.

Correlations were calculated between the biological replicates as well as between the average responses to infiltration with different BcPGs, for both segregating populations (Tables 2 and 3). For the RIL population, correlations between biological replicates were 0.50 or higher. For the F2 population, correlations between biological replicates were between 0.76 and 0.83, except for the three replicates of the BcPG2 infiltration, which had a correlation between 0.54 and 0.65. Generally, the correlations between the average response to infiltration with BcPG2 and the average response to infiltration with any of the other BcPGs were low (<0.40), while the correlations between the average responses to infiltration with BcPG3, BcPG4 and BcPG6 were above 0.70.

Table 2. The correlations between the biological replicates, traits and averages of replicates as calculated for the L*er* x Kas-2 recombinant inbred line (RIL) population.

Trait [a]		BcPG2		BcPG3			AvgBcPG2	AvgBcPG3
	Biological replicate	1	2	1	2	3		
BcPG2	1	1	0.57	0.30	0.27	0.25	0.88	0.32
	2		1	0.27	0.24	0.24	0.90	0.29
BcPG3	1			1	0.64	0.50	0.33	0.84
	2				1	0.62	0.30	0.88
	3					1	0.28	0.82
AvgBcPG2							1	0.35
AvgBcPG3								1

[a] Plants of the Ler x Kas-2 RIL population were infiltrated with *Botrytis cinerea* endopolygalacturonases BcPG2 and BcPG3. AvgBcPGx represents the average response score value of the two BcPG2 or three BcPG3 biological replicates.

QTL mapping using the Ler x Kas-2 recombinant inbred line population

The L*er* x Kas-2 RIL population was analyzed to identify QTLs associated with the variation in response to BcPG2 and BcPG3. The genetic map contained 77 single nucleotide polymorphism (SNP) and single-sequence length polymorphism markers, spanning a total genetic length of 411 cM, with an average marker interval of 5.7 cM (El Lithy et al., 2006). The analysis revealed no significant QTL for the response to BcPG2 and BcPG3.

QTL mapping using the Col-0 x Br-0 F_2 population

In an effort to use the same marker set as in the L*er* x Kas-2 RIL population, the results obtained with the Arabidopsis SNPwave assay (van Eijk et al., 2004) were examined for polymorphic markers between the Col-0 and Br-0 accessions. Of the SNP markers that were polymorphic between Col-0 and L*er*, only 27% appeared to be polymorphic between Col-0 and Br-0. This was considered as insufficient to allow cost-efficient genotyping using this technology. Therefore, an amplified fragment length polymorphism (AFLP) linkage map was

Table 3. The correlations between the biological replicates, traits and averages of replicates as calculated for the Col-0 x Br-0 F_2 population.

Trait [a]		BcPG2			BcPG3		BcPG4		BcPG6		AvgBcPG2	AvgBcPG3	AvgBcPG4	AvgBcPG6
	Biological replicate	1	2	3	1	2	1	2	1	2				
BcPG2	1	1	0.54	0.57	0.23	0.23	0.26	0.24	0.37	0.32	0.82	0.24	0.27	0.36
	2		1	0.65	0.39	0.30	0.29	0.27	0.32	0.36	0.87	0.36	0.29	0.36
	3			1	0.37	0.34	0.27	0.28	0.38	0.38	0.87	0.37	0.29	0.40
BcPG3	1				1	0.83	0.62	0.59	0.71	0.70	0.39	0.96	0.64	0.74
	2					1	0.67	0.65	0.73	0.77	0.34	0.96	0.70	0.78
BcPG4	1						1	0.76	0.63	0.64	0.33	0.67	0.94	0.66
	2							1	0.65	0.67	0.31	0.65	0.94	0.69
BcPG6	1								1	0.83	0.42	0.75	0.68	0.96
	2									1	0.42	0.77	0.69	0.96
AvgBcPG2											1	0.38	0.34	0.44
AvgBcPG3												1	0.70	0.79
AvgBcPG4													1	0.72
AvgBcPG6														1

[a] Plants of the Col-0 x Br-0 F2 population were infiltrated with *Botrytis cinerea* endopolygalacturonases BcPG2, BcPG3, BcPG4 and BcPG6. AvgBcPGx represents the average response score value of the three BcPG2 or two BcPG3, BcPG4 or BcPG6 biological replicates.

generated using five primer combinations resulting in 120 markers scored on this population. The number of markers per primer combination varied from 20 to 27, with an average of 24 (Table 4). A total of 2.1 % of the data points could not be scored co-dominantly and 1.1 % data was missing. All markers, except E11/M62-F-141, could unambiguously be assigned to one of the five linkage groups. The linkage groups could be assigned to the *A. thaliana* chromosomes based on common markers shared with other maps (not shown). The resulting linkage map had a total length of 370 cM and showed good genome coverage, with an average marker interval of 3.2 cM (Figure 4). The segregation ratio of the markers was checked, and none of the markers showed noticeable segregation distortion.

QTL analysis identified a single locus on chromosome 3 of the *A. thaliana* genome governing the responses to all BcPGs tested. The QTL for the response to BcPG2 had a log-likelihood (LOD) value of 4.0, which just exceeded the significance threshold of 3.3, while the LOD values for the QTLs for responses to BcPG3, BcPG4 and BcPG6 were highly significant with values of 52, 27 and 31, respectively (Figures 5A and 5B). The variance in response explained by the QTL ranged from 60 to 81% for BcPG3, BcPG4 and BcPG6, while it was 9.5% for BcPG2.

The TAIR database (www.arabidopsis.org) was used to find the position of the AFLP markers flanking the QTL in the genome sequence. The highest LOD peak lies between the markers E11/M62-F-131 and E11/M50-F-184. This area spans 12 cM and physically covers approximately 1.6 million base pairs located on or between BAC clone F16J14 and P1 clone MPE11. This region contains approximately 366 genes, starting with At3G22450.1 and ending with At3G26100.1. The full list of genes can be retrieved from the TAIR database.

The allele conferring resistance to BcPGs originates from the parental line Br-0. One fourth of the F_2 population plants was homozygous for the Br-0 allele at marker E11/M62-F-111. None of these plants showed symptoms upon infiltration of BcPG3 (score 0), while the heterozygous plants and the plants homozygous for the Col-0 allele all showed a phenotypic score of 3 or higher, indicating that the allele for resistance has a recessive mode of inheritance.

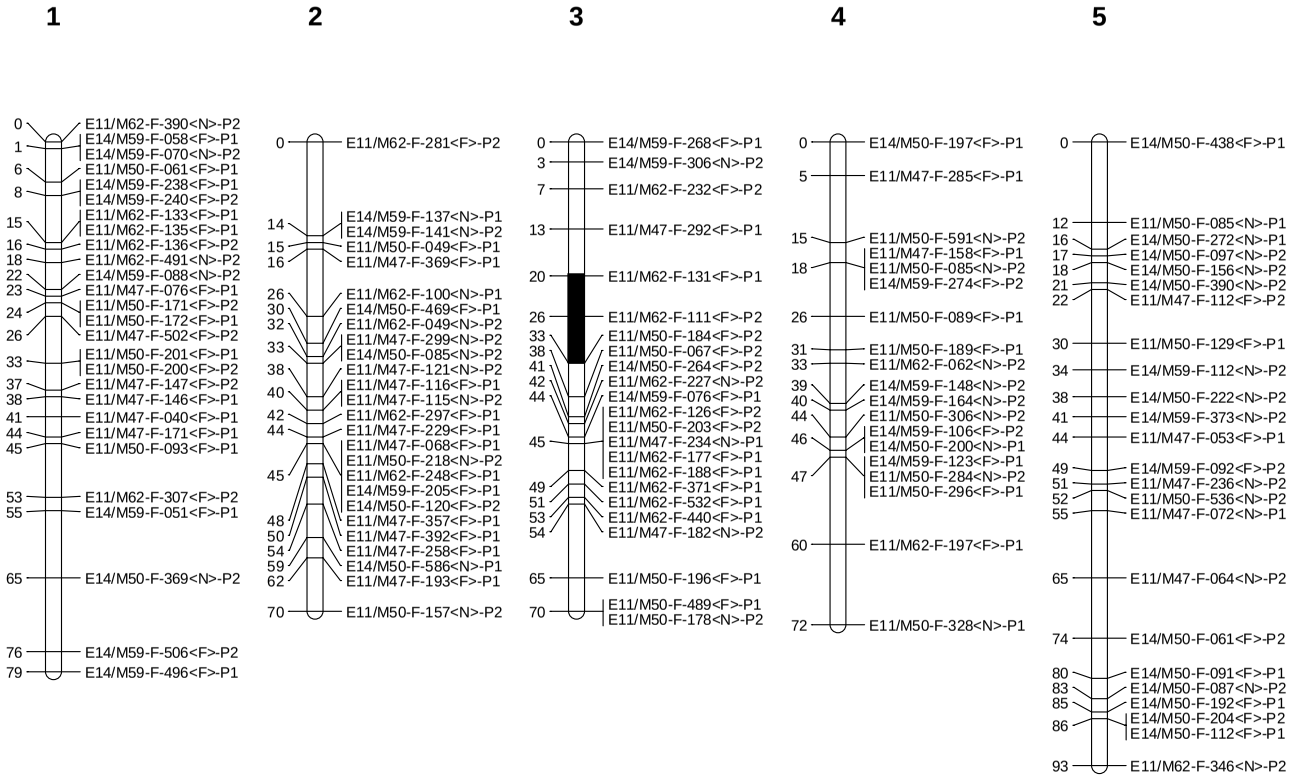


Figure 4. The Col-0 x Br-0 AFLP linkage map showing the position of the QTL governing response to *Botrytis cinerea* endopolygalacturonases BcPG2, BcPG3, BcPG4 and BcPG6 (black bar). Chromosome numbers are given above the schematic representation of each chromosome.

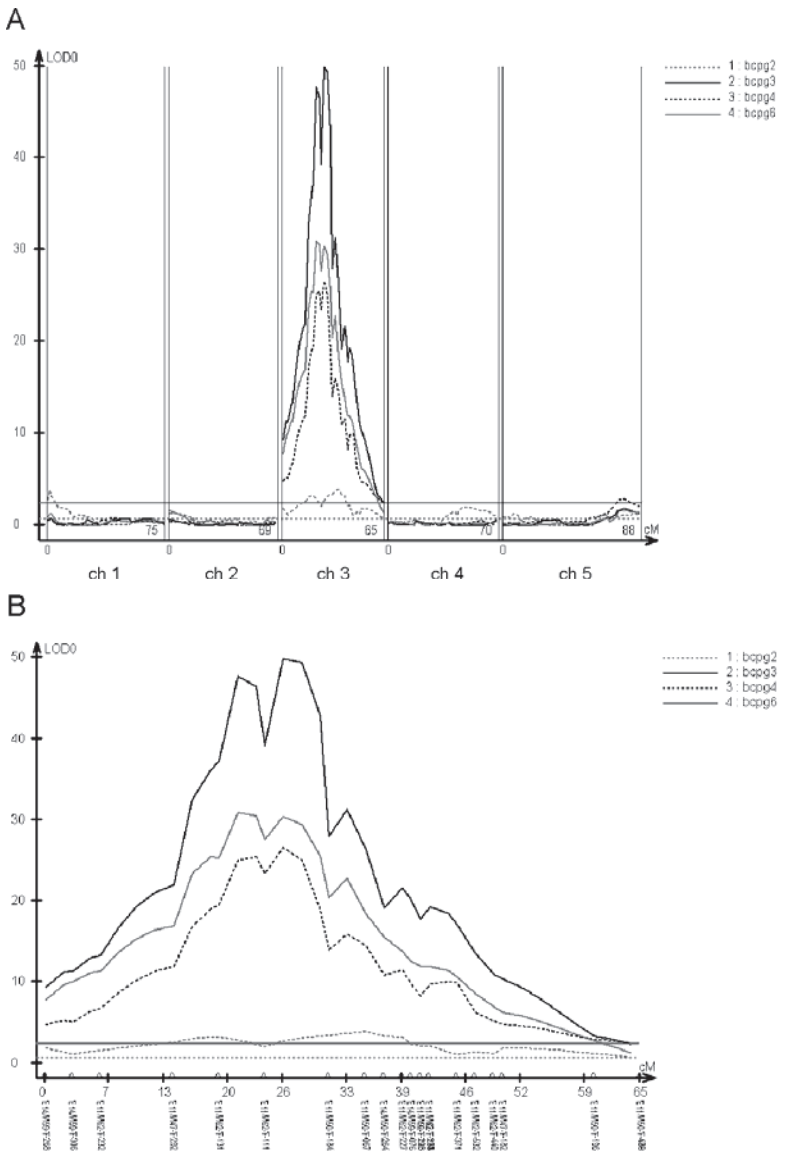


Figure 5. (A) QTL graph for response to *Botrytis cinerea* endopolygalacturonases (BcPGs) in *Arabidopsis thaliana*, using the Col-0 x Br-0 F_2 population. The five chromosomes of *A. thaliana* are plotted on the x-axis. The log-likelihood (LOD) scores are plotted on the y-axis. The grey horizontal line corresponds to the significance threshold of 3.3. **(B)** An enlargement of the graph of chromosome 3, including the molecular marker positions, is shown separately. The LOD scores per trait are given between brackets: BcPG2 (LOD=4), BcPG3 (LOD=52), BcPG4 (LOD=27), BcPG6 (LOD=31).

Table 4. The number of polymorphic AFLP markers found between the *Arabidopsis thaliana* accessions Col-0 and Br-0 using five primer combinations. The polymorphic markers were used to genetically analyze the Col-0 x Br-0 F2 population. Selective nucleotides are given between brackets.

	*Mse*I			
*Eco*RI	M47 (CAA)	M50 (CAT)	M59 (CTA)	M62 (CTT)
E11 (AA)	27	26	-	25
E14 (AT)	-	20	22	-

Discussion

Quantitative trait locus mapping

In this study, we demonstrated the variance among multiple *Arabidopsis thaliana* accessions in responses to *Botrytis cinerea* endopolygalacturonases (BcPGs). *A. thaliana* plants that showed a chlorotic or necrotic response upon infiltration of BcPGs are called sensitive, whereas plants that did not show any symptoms to BcPGs are called resistant. We hypothesize that resistance to BcPGs may confer (partial) disease resistance to *B. cinerea*, however, this remains to be experimentally validated.

QTL analysis of the Col x Br-0 F_2 population revealed a single QTL, which we designate RBPG1 (resistance to *Botrytis* polygalacturonases), with a large effect that mapped to a 12 cM interval on chromosome 3. RBPG1 is correlated with resistance to BcPG2, BcPG3, BcPG4 and BcPG6. The resistant allele originated from Br-0 and showed a recessive mode of inheritance. RBPG1 was not identified in the L*er* x Kas-2 RIL population, suggesting that they do not carry the Br-0 (resistant) allele. This is in agreement with the observation that both the L*er* and Kas-2 accessions showed a high response to the BcPGs tested (Figure 1).

Arabidopsis thaliana loci involved in resistance or susceptibility to Botrytis cinerea

The *A. thaliana* genome contains two paralogous genes, *Atpgip*1 and *Atpgip*2, that occur in tandem in the genome and encode polygalacturonase-inhibitor proteins (PGIPs) which have similar inhibitory activity against BcPG1 (Ferrari et al., 2003). Expression of *Atpgip*1 is induced by oligogalacturonides (degradation products of cell wall pectins) and gene silencing of *Atpgip*1 resulted in reduced inhibitor activity and enhanced susceptibility to *B. cinerea* (Ferrari et al., 2006). The *Atpgip* genes are located on chromosome 5, whereas the RBPG1 locus is located on chromosome 3, suggesting that the AtPGIPs are not (directly) involved in resistance against the BcPGs.

Using a T-DNA insertion-mutagenized *A. thaliana* population, Mengiste et al. (2003) identified several mutants with enhanced susceptibility to *B. cinerea* and showed that the bos1 mutant is not only affected in disease resistance but also in responses to abiotic stress. The *BOS1* gene encodes a R2R3MYB transcription factor (AtMYB108), located on

chromosome 3 (At3g06490; Mengiste et al., 2003), however it maps upstream (telomeric) of the marker interval that delimits the RBPG1 locus. Three other *B. cinerea*-susceptible mutants, bos2, bos3 and bos4, were identified and the corresponding *BOS* loci mediate defense responses through mechanisms independent of the *BOS1* gene (Veronese et al., 2004). The *BOS2* and *BOS3* loci mapped on chromosome 4 and 1, respectively, whereas the location of *BOS4* remains to be determined.

Denby et al. (2004) identified multiple QTL governing susceptibility to *B. cinerea* isolates in progeny from a cross between the accessions Col-0 and L*er*. The two *B. cinerea* isolates used in their study revealed a total of 12 different QTL on different chromosomes, none of which mapped at a similar position as RBPG1. The QTL analysis reported by Denby et al. (2004) was performed with the accessions Col-0 and L*er*, that in our experiments showed only little variation in their response to the different BcPGs. The QTL study performed by Denby et al. (2004) differed substantially from our study in design and methodology. Denby et al. (2004) performed inoculation studies with *B. cinerea* conidia and quantified lesion sizes at a single time point. In the present study, we tested plants with pure proteins instead of inoculating with the pathogen, thereby eliminating the contribution of other *B. cinerea* virulence factors such as phytotoxins or reactive oxygen species (van Kan, 2006), as well as eliminating the contribution of defense responses of *A. thaliana* that are unrelated to the action of BcPGs and the damage that they inflict.

Candidate genes in the RBPG1 locus

The RBPG1 locus comprises a 12 cM region that contains 366 genes. As the mechanism underlying the response remains elusive, it is challenging to generate a short list of candidate genes that may confer the resistant phenotype. Genes in this region can be assigned to functional categories that may be relevant to the phenotype such as recognition [e.g. LRR disease resistance family proteins], signaling and transcriptional regulation [e.g. protein kinases and phosphatases, zinc finger proteins, heterotrimeric G-protein subunit, F-box proteins, bHLH family proteins, myb-family transcription factors, lesion-inducing proteins, pathogenesis-related transcriptional activators, ethylene-responsive factors] and cell wall composition [pectin esterase, pectate lyase, glycosyl transferase and xyloglucan transferase]. The latter category is interesting to inspect in further detail for candidate genes as differences between plant genotypes in the damage inflicted by endopolygalacturonases may result from differences in cell wall architecture which make the substrate less accessible for hydrolysis. However, it cannot be excluded that genes from the other categories mentioned contribute to the phenotype by modulating expression of genes elsewhere in the *A. thaliana* genome. The QTL serves as a starting point to identify the gene(s) involved in response to BcPGs. However, additional markers are needed to reduce the size of the confidence interval thereby reducing the number of candidate genes. The

Col-0 x Br-0 RIL population (single seed descent F_8, n>300) that was recently generated will allow us to study the correlation between resistance to BcPGs, conferred by the RBPG1 locus, and (partial) disease resistance to infection by *B. cinerea*.

Experimental procedures

Plant material and growth conditions

Seeds of 47 *Arabidopsis thaliana* accessions (Table 1) and the Landsberg *erecta* (L*er*, NW20) x Kashmir (Kas-2, N1264) recombinant inbred line (RIL) population were kindly provided by Maarten Koornneef (Laboratory of Genetics of Wageningen University, The Netherlands). The RIL population described by El-Lithy et al. (2006) consists of 164 RILs. Seeds of 81 randomly chosen RILs were used in this study.

A F_2 population was created by reciprocally crossing the accessions Columbia-0 (Col-0, N907) and Brno (Br-0, N6626). The F1 seeds were grown as described below and allowed for self-fertilization to obtain 340 F_2 plants. A total of 183 randomly chosen F_2 plants were infiltrated with *Botrytis cinerea* endopolygalacturonases (BcPGs) as described below. Since infiltrations are not destructive, all the F_2 plants were self-fertilized and the generation of a RIL_8 was pursued using single-seed descent. Seeds were sown in Petri dishes on water-saturated filter paper and kept at 4 ºC for 4 days to break seed dormancy. After vernalization seeds were transferred to a climate chamber at 20 ºC with a photoperiod of 8 h and 70% relative humidity. After 2 days, germinated seeds were planted in 7-cm pots with standard soil and grown under the same conditions as described above.

Phenotypic scoring

Plants of six to eight weeks old were infiltrated with BcPGs purified from culture filtrates of *Pichia pastoris* expressing BcPGs, as previously described (Kars et al., 2005a). Prior to infiltration, BcPG enzymes were diluted in 10 mM sodium acetate, pH 4.2, to an enzyme activity of 3U/ml. In the initial screening, 47 *A. thaliana* accessions (Table 1) were infiltrated with BcPG2, BcPG3, BcPG4 and BcPG6. Multiple rosette leaves per plant were infiltrated with one BcPG on either side of the mid-vein. Eleven selected accessions (Table 1, underlined) were subsequently infiltrated with BcPG2, BcPG3 and BcPG6 (BcPG4 was not available in sufficient quantities at the time of infiltration).

Plants of the L*er* x Kas-2 RIL population were infiltrated with BcPG2 and BcPG3 (BcPG4 and BcPG6 were not available in sufficient quantities at the time of infiltration). Six plants per RIL were infiltrated with BcPG2 in duplicate and BcPG3 in triplicate using individual leaves.

Plants of the Col-0 x Br-0 F_2 population were infiltrated with BcPG2 in triplicate and with BcPG3, BcPG4 and BcPG6 in duplicate.

The response to each infiltration was visually scored ranging from 0, for symptomless, non-responding (resistant) plants similar to Br-0, to 4 for responding plants similar to Kas-2 (in steps of 1). The score was as follows: 0 = no symptoms, 1 = chlorotic spots within the infiltrated zone, 2 = chlorosis covering the infiltrated zone, 3 = abundant chlorosis with necrotic spots, and 4 = complete necrosis (Figure 1).

DNA isolation and genotyping

DNA was isolated from freeze-dried leaves of individual F_2 plants using a modified CTAB procedure (Stewart and Via, 1993).

The molecular marker data and the linkage map of the RIL population were described by El-Lithy et al. (2006). The population was genotyped using two types of molecular markers, i.e. 45 single nucleotide polymorphisms (SNPs) and 30 single-sequence length polymorphisms. In addition, two morphological markers were scored, i.e. the erecta and ga5-gibberellin-deficient mutations. The genetic map of the RIL population spanned 411 cM, with an average marker interval of 5.7 cM.

Genotypic data on the F_2 population (Col-0 x Br-0) were generated using amplified fragment length polymorphism (AFLP) markers by Keygene N.V., Wageningen, The Netherlands. The AFLP analysis was performed as described by Vos et al. (1995) using the restriction enzymes *Eco*RI and *Mse*I. AFLP markers were amplified using adapter specific primers containing two (E+2) or three (M+3) selective nucleotides. Five different E+2/M+3 primer combinations were used (Table 1). AFLP amplification reactions were performed in a Perkin Elmer 9600 thermocycler (Perkin Elmer Corp., Norwalk, CT, USA). The amplified DNA products were separated on a MegaBACE 1000 capillary electrophoresis system (Amersham BioSciences). Proprietary AFLP marker analysis software (Keygene NV) was used to score the markers co-dominantly on the basis of peak intensities. Data that could not be scored co-dominantly unambiguously were scored dominantly. The genetic linkage map of the F_2 population was constructed using the JoinMap 3.0 program (Stam, 1993; Van Ooijen and Voorrips, 2001), applying the Kosambi mapping function.

Quantitative trait loci analysis

Quantitative trait loci (QTL) mapping was performed using the software packages MapQTL version 4.0 (Van Ooijen et al., 2002) and WinQTLcart version 2.5 (Wang et al., 2006). The map distances between markers generated by Joinmap, genotypic data and phenotypic scores (trait data) were entered in the QTL program. QTL mapping was performed using scores of individual infiltrations, infiltrations per plant and the average scores of all plants per RIL. For each BcPG, the QTL analysis was performed on the individual replicates and the averages of the replicates. The data were analyzed using the interval mapping method (IM). This method calculates the log-likelihood (LOD) values every 1 cM along the chromosome.

QTL were significant when the LOD score exceeded the significance threshold (P = 0.05), which represents 95% confidence intervals for normally distributed data. Empirical thresholds for interval mapping were obtained by permutations (10.000), as implemented in the package (Churchill and Doerge, 1994). The resulting genome wide LOD thresholds for all traits in the RIL population and the F_2 population were 2.6 and 3.3, respectively.

Acknowledgements

We thank Rik van Wijk for the many useful discussions on QTL mapping, for his help with statistical analysis and critical reading of the manuscript. We acknowledge Pierre de Wit and Laure Perchepied for critical reading of the manuscript and Maarten Koornneef for his advice, for providing the *A. thaliana* accessions and the molecular marker data on the RIL population. We are grateful to Bert Essenstam and Pieter Kuiken (Unifarm) for excellent plant care. The research was funded by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (project WGC.5034) and supported by De Ruiter Seeds B.V. (Bergschenhoek, The Netherlands) and Keygene N.V. (Wageningen, The Netherlands).

Chapter 7

General Discussion

General Discussion

The experimental results presented in this thesis have further increased our understanding of the infection of plants by *Botrytis cinerea*. The first section discusses the use of efficient mutagenesis strategies in the functional analysis of fungal genes. The second section discusses the contribution of pectin-degrading enzymes (i.e. pectin methylesterases, endopolygalacturonases, pectin lyase and pectate lyase) to virulence. The third section discusses the macerating and necrotizing activity of *B. cinerea* endopolygalacturonases (BcPGs) and the elicitation of responses in host plants by either BcPGs or by pectin-degradation products derived by the action of BcPGs. In the last section, the possible mechanisms of *Arabidopsis thaliana* resistance to BcPGs are discussed.

Efficient mutagenesis strategies in the functional analysis of fungal genes

Commonly used strategies for gene analysis in fungi are random insertional mutagenesis and targeted mutagenesis by gene disruption, gene replacement or gene silencing (for *Botrytis* reviewed by van Kan, 2006). Different random mutagenesis strategies (reviewed by Brown and Holden, 1998) have been used for plant pathogenic fungi, including restriction enzyme mediated integration (Brown and Holden, 1998; Kahmann and Basse, 1999; Maier and Schäfer, 1999), *Agrobacterium tumefaciens*-mediated insertion (de Groot et al., 1998; Michielse et al., 2005) and transposon tagged insertion mutagenesis (Hua-Van et al., 1998, 2001; Villalba et al., 2001). Subsequent screening of the transformants for virulence results in the unbiased detection of genes involved in virulence. The gene(s) of interest can subsequently be isolated using the tag sequence.

Targeted mutagenesis is generally preferred for the analysis of genes that are predicted to be involved in virulence based on known enzyme activity or previous information from other pathosystems. With the availability of three selectable marker genes that confer resistance to antibiotics (hygromycin, nourseothricin and glufosinate), the generation of single, double or triple gene-specific mutants in *B. cinerea* is feasible and provides a possibility to study synergism of different genes in plant-pathogen interactions (chapter 2). In this thesis we have used a gene replacement strategy to mutate genes encoding pectinases that were considered to be involved in the virulence of *B. cinerea* (chapters 2, 3 and 4). We favoured gene replacement by double recombination over gene disruption by single recombination (Prins et al., 2000) as gene replacement avoids partial gene duplication and the production of truncated but possibly still functional proteins. The PCR-based strategy, described in chapter 2, is a time-efficient procedure as it circumvents the need for cloning steps or ligating rare restriction sites to fuse the target gene fragments to the selection marker cassette (Kämper et al., 2004). Following the fusion of the target gene fragments and the selection marker cassettes by overlap extension PCR, the fusion product is transferred by protoplast transformation to the recipient fungus and becomes integrated

by homologous recombination with high efficiency. For the *B. cinerea* pectin methylesterase (*Bcpme*) genes, homologous recombination occurred at a frequency of >60% (chapter 2).

A PCR screening strategy was designed to distinguish, at an early stage, proper homologous recombinants from transformants with an ectopic integration using primer combinations as described in chapter 2. This strategy reduces the number of transformants that need to be taken through the laborious procedures of single spore isolation and DNA isolation for Southern analysis and is currently routinely used for the functional analysis of *B. cinerea* genes.

The contribution of pectin-degrading enzymes to virulence of Botrytis cinerea

When a *B. cinerea* conidium lands on the plant surface, a germ tube is rapidly formed which develops into an appressorium-like structure that penetrates the cuticle. Mechanical force alone does not seem to be sufficient to penetrate the host surface (Salinas and Verhoeff, 1995) and secreted enzymes are anticipated to soften the tissue prior to actual penetration (Collmer and Keen, 1986). The fungus grows through the anticlinal cell wall that separates two epidermal cells. This wall contains a high proportion of pectin suggesting that pectinases are important to the pathogen to pave the way for ingress. This thesis describes the functional analysis of several cell wall-degrading enzymes that are secreted by *B. cinerea*, in particular the endopolygalacturonases (BcPGs) and pectin methylesterases BcPMEs (chapters 2, 3 and 4). The differences in biochemical properties, substrate preference and substrate degradation profiles of BcPGs, as well as their distinct temporal expression patterns suggest that each of the BcPGs has a different role in the cell wall degradation process and that BcPMEs are possibly redundant. In the following section, I summarize the characteristics of the different pectinases studied and discuss their proposed roles during pathogenesis. based on our findings.

Enzymes likely to be involved in pectin degradation during surface penetration and invasion of the anticlinal cell wall are BcPG1 and BcPG2. The *Bcpg*1 and *Bcpg*2 genes are expressed in the first 24 hours after inoculation and the enzymes are able to cause major damage *in situ* in plant tissue (chapters 3 and 4). We have produced these enzymes *in vitro* and showed that damage becomes apparent immediately after infiltration of these enzymes. Infiltration of liquids into leaves causes the infiltrated sectors to turn translucent due to the presence of water in the intercellular space, but after evaporation the leaves show a normal appearance within 5 minutes. Leaves infiltrated with BcPG1 or BcPG2 remained translucent suggesting that the injected solution did not evaporate, as was the case after infiltration of buffer. In the minutes following BcPG1 or BcPG2 infiltration, the leaf structure changed and the tissue between the veins collapsed. The veins remained seemingly intact for several hours, possibly because they contain cell wall components that cannot be hydrolyzed by BcPGs, such as lignin and cellulose. After 48 hours the collapsed tissue became necrotic

and a chlorotic halo was observed around the margins of the infiltrated sectors. The destructive properties of the enzymes and the expression pattern of the genes are in agreement with the observed severe reduction in virulence of *Bcpg*1 and *Bcpg*2 knockout mutants (ten Have et al., 1998; chapter 3).

During the infection of tomato leaves, *Bcpg*3 transcripts are not detectable and mutants in *Bcpg3* showed no reduced virulence (chapter 4). *In vitro* produced BcPG3 was not able to cause damage when infiltrated in tomato leaves, suggesting that BcPG3 does not play a role in the cell wall degradation of tomato leaves. However, BcPG3 is capable of causing damage in *A. thaliana* leaves (chapter 6), indicating that BcPG3 may have preference for pectin of particular host plants. Pectin degradation profiles show that BcPG3 has a role in the conversion of small pectin polymers (oligogalacturonides, OGAs) into monomers (galacturonic acid, GA) that serve as nutrients for fungal growth.

In vitro produced BcPG4 caused damage when infiltrated in leaves, however, the *Bcpg*4 gene is expressed at a late stage of the infection (chapter 3). *Bcpg*4 gene replacement mutants showed no altered virulence when compared to the wild type strain (chapter 4). BcPG4 is not required for host tissue colonization but rather contributes to the complete decomposition of the primary pectin hydrolysis products, generated by BcPG1 and BcPG2, into smaller fragments.

The role of BcPG5 remains elusive. The enzyme could not be produced in *Pichia pastoris*, hence we were unable to study the enzyme characteristics and its pectin degradation profiles. *Bcpg*5 gene replacement mutants showed no reduction in virulence. Moreover, no expression of the *Bcpg*5 gene was observed during infection of tomato leaves. This supports the conclusion of ten Have et al. (2001) that BcPG5 functions specifically on certain hosts or tissues.

BcPG6 seems not to be required for host tissue colonization. During infection of tomato leaves *Bcpg*6 transcripts accumulate during late stages of the infection. Pectin degradation profiles suggest that alike BcPG4 and BcPG3, BcPG6 may act in the central part of the lesion and contributes to the complete decomposition of pectin hydrolysis products.

We can only speculate on the possible phenotypes of mutants lacking multiple *Bcpg* genes. Attempts to generate a double mutant lacking *Bcpg1* and *Bcpg2* were unsuccessful. It remains to be determined whether such a double mutant will be unable to infect plants. More likely, the double mutant will show increased reduction of virulence, as compared to the single mutants, but it will probably still be able to cause some disease.

Ten Have (PhD thesis, 2000) hypothesized that some BcPG family members could act in concert and some BcPGs possibly could have functional overlap. The expression patterns, pH optima, substrate preferences and pectin degradation profiles of BcPG1 and BcPG2 suggest functional overlap. The same may be true for BcPG3 and BcPG6 (chapter 3). The

data also suggest possible concerted action of BcPG1 with one of the processive enzymes, BcPG3 or BcPG6. Also BcPG2 may act in concert with BcPG3 or BcPG6.

Valette-Collet et al. (2003) suggested that also concerted action of BcPMEs and BcPGs may facilitate degradation of plant cell walls more efficiently than a BcPG alone, but it appears that BcPGs are well capable of degrading highly methylated pectin (chapter 3). *Bcpme1* and *Bcpme2* transcript levels are detected relatively early during the infection process and increase from 24 hours onwards. However, single and double gene *Bcpme* replacement mutants did not show any discernable reduced virulence and they remained able to grow on highly methylated pectin equally efficient as the wild type. The *B. cinerea* genome sequence revealed the presence of a third *Bcpme* gene. The role of this gene in pectin demethylation and virulence remains to be studied.

PGs and pectate lyases (PEL) act on the same substrate, but they have different pH activity profiles. PGs require an acidic environment for optimal activity (chapter 3), whereas lyases are considered to be inactive in acidic environment. The expression pattern of the *B. cinerea* pectate lyase gene *Bcpel1* is similar to that of *Bcpg6*, suggesting that BcPEL1 contributes to further breakdown of pectin hydrolysis products into smaller fragments at later stages of infection, possibly in the central part of lesions, where the acidic environment has been neutralized.

Pectin lyase (PNL) hydrolyses pectin (methylated pectate) and therefore may act in concert with PGs. The *Bcpnl* expression pattern is similar to that of *Bcpg2* (chapter 4), suggesting a role in an early phase of the infection process. However, like the pectate lyases, pectin lyases are unlikely to contribute significantly to pectin degradation in the early stages of infection (chapter 1) due to the acidification of the environment by *B. cinerea*. The role of the *Bcpnl* gene in virulence remains to be studied.

Botrytis cinerea endopolygalacturonases: necrotizing activity or elicitation?

As described above, BcPGs can hydrolyse the pectin polymer causing degradation of the pectic network in the primary cell wall, and eventually causing maceration and necrosis. Prior to the development of necrosis, three possible physiological responses may occur in the plant. First, hydrolysis of the pectin backbone releases oligogalacturonides (OGAs) that may elicit defence responses. Mainly the mid-size OGAs (degree of polymerisation 11-20) are reported to induce plant defence responses (Cervone et al., 1989; De Lorenzo et al., 1994; Ferrari et al., 2007; Ridley et al., 2001). Second, the protein itself may be recognized by plant cells and activate defence responses, as was shown for BcPG1 in grapevine suspension cells by Poinssot et al. (2003). Third, the action of the BcPGs may cause disintegration of the cell wall structure and subsequent tissue collapse.

It was relevant to discriminate between tissue collapse due to enzyme activity (i.e. pectin degradation which causes disintegration of the cell wall) or cell death in response to elicitor

activity of either the protein itself or OGAs derived from pectin degradation. Poinssot et al. (2003) used heat-treatment to inactivate BcPG1, but this did not completely abolish enzyme activity. A more reliable method to inactivate enzyme activity is by altering the catalytic site using targeted mutagenesis as was done for the *Colletotrichum lindemuthianum* endopolygalacturonase ClPG1 (Boudart et al., 2003). Transient expression of a catalytic site-mutant form of ClPG1 by agroinfiltration in tobacco did not cause any symptoms, whereas expression of the wild type ClPG1 caused total cell dissociation and eventually necrosis (Boudart et al., 2003), proving that ClPG1 enzyme activity is essential for symptom development. Experiments with the inactive mutant protein BcPG2-D192A produced in *P. pastoris* showed that infiltration of the purified mutant protein did not cause any visible symptoms and therefore enzyme activity seems to be required to inflict necrosis *in planta*. Furthermore, our observation that BcPG1 and BcPG2 cause mesophyll tissue of broad bean to collapse within as little as 10 minutes (chapter 3) suggests that the elicitor activity of BcPG1 reported by Poinssot et al. (2003) is probably not of biological relevance during infection by *B. cinerea*. Neither seems there any biological relevance for elicitor activity of the other BcPGs as these are expressed at a late stage of infection when the plant tissue is already macerated and dead.

Linear non-methylated OGAs also do not play any detectable role in BcPG-mediated necrosis as these molecules caused no discernible symptoms upon infiltration at any concentration tested (chapter 3). However, the pectin composition in the plant cell wall differs between plant species, tissues, cell types and even in different regions of the wall surrounding an individual cell (e.g. Freshour et al., 1996). Differences in the degrees of methylation and/or acetylation, in the extent of cross-linking or the embedding in other polymers may influence the type of OGAs released upon hydrolysis by BcPGs. Even in the event that OGAs of a specific type may act as elicitors of defence, this mechanism is likely to be much slower than the tissue collapse observed upon exposure to BcPG2. Thus any elicitation of defence is likely to be overruled by the destructive action of pectinases such as BcPG1 and BcPG2.

The results described in this thesis all point to the conclusion that the symptoms caused upon BcPG infiltration are the consequence of enzyme action causing loss of cell wall integrity, culminating in tissue collapse and subsequent cell death.

Resistance to Botrytis cinerea endopolygalacturonases in Arabidopsis thaliana

Among eleven *A. thaliana* accessions that were infiltrated with purified BcPGs two accessions showed distinct responses (chapter 6). Col-0 showed a strong response to BcPGs, visible as tissue collapse and eventual necrosis of the entire infiltrated sector, whereas Br-0 hardly showed any symptoms. One of the active plant defence responses upon *B. cinerea* infection is the accumulation of polygalacturonase-inhibiting proteins

(PGIPs), cell-wall proteins that are able to counteract the action of fungal polygalacturonases. PGIPs are considered to contribute to resistance against polygalacturonase-producing pathogenic fungi (reviewed by De Lorenzo et al., 2001).

In vitro, PGIPs originating from different plant species caused inhibition of most BcPGs except of BcPG2 (Table 1; chapter 5), whereas *in vivo* interaction studies clearly showed inhibition of BcPG2 by *Vitis vinifera* PGIP1 (chapter 5), suggesting that environmental conditions in the plant influence inhibitory activity. There are two possible ways in which PGIPs can inhibit the action of PGs; they can either bind to the fungal PGs (Federici et al., 2001, King et al., 2002), or they bind to pectin (Spadoni et al., 2006) and the pectin-PGIP complex subsequently interacts with the PG, in both ways preventing binding of the polygalacturonases to pectin.

Table 1. Polygalacturonase-inhibiting proteins (PGIPs) extracted from various plant species and their inhibitory effect on *Botrytis cinerea* endopolygalacturonases (BcPGs).

Plant species	**BcPG1**	**BcPG2**	**BcPG3**	**BcPG4**	**BcPG6**
Apple	+	-	+	+	+
Bean	+	-	+	-	-
Leek	+	-	-	+	-
Lemon	+	-	+	-	+
Onion	+	-	-	-	-
Pear	+	-	+	+	+
Pepper	+	-	+	-	+

+ = BcPG inhibition by PGIP; - = no PG inhibition by PGIP

In view of the differences in response to BcPGs in different *A. thaliana* accessions, we considered the possibility that differences in inhibitory activity between the PGIPs present in the different accessions might be involved in determining these differences in response. The *A. thaliana* genome contains two AtPGIP encoding genes. Over-expression of both genes contributed to partial resistance to *B. cinerea* (Ferrari et al., 2003). The AtPGIP sequences in the Col-0 and Br-0 accessions were examined and polymorphisms in AtPGIP1 were identified (Table 2). The polymorphism at amino acid position 261 (Proline in Col-0, Threonine in Br-0) is likely to affect the protein folding, but its effect on the activity or specificity of both PGIP allelic forms remains to be determined. Binary vector constructs were generated for both AtPGIP1 alleles, as well as constructs in which parts of the alleles were exchanged in order to be able to study the effect of individual polymorphic sites. Transformation of *A. thaliana* was conducted, but the transgenic plants remain to be analysed for expression levels, inhibitory activity towards different BcPGs and resistance to *B. cinerea*.

Table 2. Polymorphisms detected in AtPGIP1 of *Arabidopsis thaliana* accessions Col-0 and Br-0.

Accession	Tissue damage upon BcPG infiltration	AtPGIP1 amino acid position		
		8	27	261
Col-0	Yes	C	Q	P
Br-0	No	LST*	L	T

* Insertion of two amino acids and substitution of one amino acid

QTL analysis on the *A. thaliana* Col-0 x Br-0 F2 population identified the quantative trait locus RBPG1 that controls resistance to BcPG2, BcPG3, BcPG4 and BcPG6. RPBG1, however, does not map at the position where the *Atpgip* genes are located, suggesting that these genes are not (directly) involved in resistance to BcPGs.

The QTL comprises a 12 cM region that contains 366 genes. Candidate genes that are possibly related to the resistant phenotype are genes involved in the synthesis of pectin and other cell wall polysaccharides, or genes encoding plant cell wall proteins (reviewed in Showalter, 1993; Hückelhoven, 2007). *B. cinerea* is hardly able to infect woody plants, presumably because they contain a high percentage of cellulose and hemicellulose instead of pectin. In the pectin structure that was proposed by Vincken et al. (2003), homogalacturonan is a side chain of rhamnogalacturonan that connects neighbouring cells. This model predicts that cleavage of homogalacturonan would disrupt the connection between plant cells and would lead to cell separation. The structures of *A. thaliana* (Col-0) leaf cell-wall polysaccharides are typical of those of many other plants, except for the higher proportion of soluble pectic polysaccharides (Zablackis et al., 1995). Studies are ongoing to investigate the cell wall composition of the accessions Br-0 and Col-0. *A. thaliana* mutants in which the cell wall composition is altered (Mouille et al., 2007; Persson et al., 2007; Reiter et al., 1997) can be studied for response to BcPGs.

The research described in this thesis contributes to a better understanding of the *B. cinerea*-host interaction with emphasis on the pectin degrading enzymes. The availability of purified BcPGs with clearly distinct biochemical properties opens new opportunities to study the architecture of pectin in a wide variety of plants. A better understanding of the plant cell wall architecture will create new opportunities for the development of crops with enhanced disease resistance. In view of the importance of BcPGs in pathogenesis of *B. cinerea*, it may be envisaged that plants with cell walls that are more resistant to BcPG-inflicted damage will show a higher level of resistance to *B. cinerea.* Resistance to BcPGs may not reduce the primary infection of *B. cinerea*, but will probably slow down the lesion outgrowth. This may be sufficient to enable the host plant to activate other types of defence mechanisms that effectively control grey mould infection. The RBPG1 locus offers the perspective of isolating a gene that may be exploited to generate *B. cinerea*-resistant plants.

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Summary

Samenvatting

Summary

Botrytis cinerea is a fungal plant pathogen that causes soft rot in many plant species. During the infection process, from the moment a conidium lands on the plant surface until complete host colonization, the fungus secretes numerous enzymes and metabolites that may contribute to virulence (reviewed in chapter 1). Among the extracellular enzymes that are produced during the early stages of infection are pectin-degrading enzymes that facilitate the penetration of the plant surface and growth into the middle lamella, and contribute to decomposition of plant tissue and its conversion into fungal biomass. Pectin is one of the major structural components of the middle lamella and can be degraded by enzymes that can either depolymerize the substrate (polygalacturonases, pectin and pectate lyases) or alter its structure (pectin acetylesterases and pectin methylesterases). In previous research, the *B. cinerea* endopolygalacturonase (*Bcpg*) gene family was partially characterized and targeted mutagenesis showed that mutants deficient in *Bcpg1* were reduced in virulence.

The main objective of the work described in this thesis was to further elucidate the process of cell wall-degradation by *B. cinerea*, by performing expression studies and functional analysis of genes encoding pectinases, with emphasis on pectin methylesterases and endopolygalacturonases (chapters 2, 3 and 4).

A PCR-based targeted mutagenesis method was developed for the functional analysis of *B. cinerea* genes. This method circumvents the need for cloning while generating constructs for gene-replacement and yielded a high proportion of transformants in which homologous recombination had occurred (chapter 2). Using this method, *B. cinerea* single and double mutants lacking the pectin methyl-esterase genes *Bcpme1* and *Bcpme2* were created and tested on various host plants in order to evaluate their role in virulence. No reduction in virulence was observed in the mutants and they remained able to grow on highly methylated pectin equally efficient as the wild type (chapter 2). Mutants lacking the *Bcpg2* gene were delayed in the primary infection by approximately 24 hours and were reduced in lesion expansion rate by 50 to 85% (chapter 3).

The enzymes BcPG1, BcPG2, BcPG3, BcPG4 and BcPG6 were produced in the heterologous host *Pichia pastoris*, purified and biochemically characterized. All BcPGs possess the characteristics of true endopolygalacturonases, however, polygalacturonic acid (PGA) degradation experiments showed that BcPG3 and BcPG6 are processive enzymes, capable of hydrolyzing the smallest oligogalactu-ronides (OGAs) into galacturonic acid (GA). PGA hydrolysis by BcPG1, BcPG2 and BcPG4 resulted in the transient accumulation of mid-size OGA polymers, indicating that they are less capable of hydrolyzing small OGAs into GA (chapter 3). The necrotizing activity of all purified BcPGs upon infiltration in different plant species was tested. BcPG1 and BcPG2 caused major damage *in situ*, resulting in rapid tissue collapse (within minutes) and necrosis (after two days). In order to investigate

whether tissue collapse and subsequent necrosis was caused by the enzymatic activity of BcPG2 or in response to protein recognition, an enzymatically inactive mutant of BcPG2 was generated. Infiltration of the BcPG2-D192A mutant protein did not cause any discernible symptoms and therefore we concluded that enzyme activity is required to inflict necrosis *in planta* (chapter 3).

The *B. cinerea* endopolygalacturonase genes *Bcpg*3, *Bcpg*4, *Bcpg*5 and *Bcpg*6 were also functionally analyzed by gene replacement, but mutants in these genes showed no reduction in virulence (chapter 4). The *Bcpg1* and *Bcpg2* genes were expressed early during *B. cinerea* infection of tomato leaves, whereas *Bcpg4* and *Bcpg6* were expressed at later stages of lesion expansion and tissue colonization. *Bcpg3* and *Bcpg5* transcripts remained undetected during the infection of tomato leaves (chapter 4).

All six BcPGs and enzymatically inactive mutant forms of BcPG1 and BcPG2 were transiently expressed in *Nicotiana benthamiana* using agroinfiltration (chapter 5). Expression of BcPG2 caused leaf tissue to become brittle, fragile and eventually necrotic, whereas its inactive mutant form BcPG2-D192A did not cause any symptoms. Co-infiltration studies showed that BcPG2 was inhibited *in planta* by a polygalacturonase inhibiting protein from *Vitis vinifera*, VvPGIP1. Surprisingly, physical interaction between these two proteins *in vitro* could not be demonstrated, using multiple established methods.

The natural variation among *Arabidopsis thaliana* accessions in their responses to infiltration with purified BcPGs was genetically analyzed in segregating progenies from crosses between parents that differed in sensitivity to BcPGs. A QTL, designated RBPG1, that controls the response to BcPG2, BcPG3, BcPG4 and BcPG6 was identified in the Col-0 x Br-0 F2 population. The Br-0 allele at this QTL is recessive and significantly diminishes the response to the BcPGs. The QTL is positioned on chromosome 3 of the *A. thaliana* genome and the locus comprises a 12cM region that contains 366 genes. The QTL is a starting point to identify the gene involved in response to BcPGs and study its role in resistance to *B. cinerea*.

The general discussion recapitulates how the research in this thesis contributes to a better understanding of the *B. cinerea*-host interaction with emphasis on the pectin degrading enzymes. The availability of purified BcPGs with distinct biochemical properties opens new opportunities to study the architecture of pectin in a wide variety of plants. A better understanding of the plant cell wall architecture will create new opportunities to design crops with enhanced disease resistance. The RBPG1 locus offers the perspective of isolating a gene that may be exploited to generate *B. cinerea*-resistant plants.

Samenvatting

Botrytis cinerea is een pathogene schimmel die rot veroorzaakt in vele verschillende plantensoorten. Tijdens het infectieproces, vanaf het moment dat een spore landt op het plantoppervlak tot aan volledige kolonisatie, scheidt de schimmel verscheidene enzymen en metabolieten uit die mogelijk bijdragen aan de virulentie (beschreven in hoofdstuk 1). Onder de extracellulaire enzymen die worden geproduceerd tijdens de vroege stadia van de infectie zijn pectine- afbrekende enzymen die de penetratie van het plantoppervlak en de groei in de middenlamel mogelijk maken en zodoende bijdragen aan de afbraak van plantenweefsel en hun omzetting in schimmel biomassa. Pectine is een van de belangrijkste structurele componenten van de middenlamel en kan worden afgebroken door enzymen die enerzijds het substraat kunnen depolymeriseren (polygalacturonases, pectine en pectaat lyases) of anderzijds de pectinestructuur kunnen veranderen (pectine acetylesterases en pectine methylesterases). In eerder onderzoek werd de *B. cinerea* endopolygalacturonase (*Bcpg*) genfamilie gekarakteriseerd en door middel van gerichte mutagenese werd aangetoond dat *B. cinerea* mutanten waarin *Bcpg1* uitgeschakeld is minder virulent zijn.

Het voornaamste doel van het onderzoek beschreven in dit proefschrift was om het proces van celwandafbraak door *B. cinerea* verder op te helderen door het uitvoeren van genexpressie studies en functionele analyse van pectinases, met name pectine methylesterases en endopolygalacturonases (hoofdstukken 2, 3, 4).

Een op PCR gebaseerde gerichte mutagenese methode werd ontwikkeld voor de functionele analyse van *B. cinerea* genen. Deze methode maakt het mogelijk om zonder klonering genconstructen te maken die direct gebruikt worden om een (deel van een) gen te vervangen (zgn. ‘gene replacement’). De methode leverde met hoge frequentie transformanten op waarin homologe recombinatie was opgetreden (hoofdstuk 2). *B. cinerea* mutanten werden gemaakt waarin pectine methylesterase genen *Bcpme1* en *Bcpme2* waren uitgeschakeld. Deze mutanten werden getest op verschillende planten om de rol van *Bcpme* genen in virulentie te evalueren. De mutanten vertoonden geen reductie in virulentie en groeiden even goed op hoog-gemethyleerd pectine als het wild type isolaat (hoofdstuk 2). Mutanten waarin het *Bcpg2* gen was uitgeschakeld waren ongeveer 24 uur vertraagd in de vorming van primaire lesies en de snelheid waarmee deze lesies uitgroeiden was met 50 tot 85% afgenomen (hoofdstuk 3).

De enzymen BcPG1, BcPG2, BcPG3, BcPG4 en BcPG6 werden geproduceerd in de gist *Pichia pastoris*, gezuiverd en biochemisch gekarakteriseerd. Alle enzymen hebben de karakteristieken van échte endopolygalacturonases, hoewel polygalacturonzuur (PGA) afbraak experimenten aantoonden dat BcPG3 en BcPG6 processieve enzymen zijn die de kleinste oligogalacturoniden (OGAs) kunnen hydrolyseren tot galacturonzuur (GA). Hydrolyse van PGA door BcPG1, BcPG2 of BcPG4 resulteerde in de tijdelijke accumulatie van middelgrote OGAs, hetgeen er op wijst dat deze enzymen slechter in staat zijn om

kleine OGAs te hydrolyseren (hoofdstuk 3). De necrotiserende activiteit van zuivere BcPGs werd getest door verschillende plantensoorten te infiltreren. Met name BcPG1 en BcPG2 veroorzaakten veel schade *in situ*, resulterend in een snelle instorting van plantenweefsel (binnen enkele minuten) en necrose (na twee dagen). Om te onderzoeken of de zichtbare beschadiging van het plantenweefsel en daaropvolgende necrose werd veroorzaakt door de enzymatische activiteit van BcPG2 of door een (afweer)reactie op de herkenning van het BcPG2 eiwit, werd een enzymatisch inactief eiwit geproduceerd. Infiltratie van het BcPG2-D192A mutant eiwit veroorzaakte geen symptomen. Daaruit concluderen we dat enzym-activiteit essentieel is voor het veroorzaken van schade in de plant (hoofdstuk 3).

De *B. cinerea* endopolygalacturonase genen *Bcpg*3, *Bcpg*4, *Bcpg*5 and *Bcpg*6 werden ook functioneel geanalyseerd. Mutanten in deze genen vertoonden geen reductie in virulentie (hoofdstuk 4). De *Bcpg1* en *Bcpg2* genen kwamen vroeg tijdens de *B. cinerea* infectie van tomatenbladeren tot expressie, terwijl *Bcpg4* en *Bcpg6* transcripten in latere stadia van lesie expansie en weefsel kolonisatie werden gedetecteerd. *Bcpg3* en *Bcpg5* transcripten werden niet aangetoond tijdens de infectie van tomatenbladeren (hoofdstuk 4).

Alle zes de BcPGs en de enzymatisch inactieve mutante vormen van BcPG1 en BcPG2 werden tot expressie gebracht in *Nicotiana benthamiana* door Agroinfiltratie (hoofdstuk 5). Expressie van BcPG2 maakte het bladweefsel broos en necrotisch, terwijl de inactieve mutant BcPG2-D192A geen symptomen veroorzaakte. Co-infiltratie studies toonden aan dat BcPG2 *in planta* werd geremd door een eiwit van druif, VvPGIP1. Verrassend genoeg kon *in vitro* geen fysische interactie tussen beide eiwitten worden aangetoond, gebruikmakend van verschillende methoden.

De natuurlijke variatie van *Arabidopsis thaliana* accessies in hun respons op infiltratie van BcPGs werd genetisch geanalyseerd in nakomelingen van kruisingen tussen ouders die verschilden in gevoeligheid voor BcPGs. Een QTL, genaamd RBPG1, dat de respons op BcPG2, BcPG3, BcPG4 en BcPG6 controleert werd geïdentificeerd in de Col-0 x Br-0 F2 populatie. Het Br-0 allel van dit QTL is recessief en vermindert de respons op BcPGs. Het QTL ligt op chromosoom 3 van *A. thaliana* en het locus bevat een 12cM regio met daarin 366 genen. Het QTL is een startpunt om genen die betrokken zijn bij de respons op BcPGs te identificeren en de rol van het QTL in resistentie tegen *B. cinerea* te onderzoeken.

De algemene discussie vat samen hoe dit onderzoek bijdraagt aan een beter inzicht in de *B. cinerea*-plant interactie. De beschikbaarheid van zuivere BcPGs met verschillende biochemische eigenschappen opent nieuwe mogelijkheden om de architectuur van pectine nader te bestuderen. Een beter inzicht in de plantencelwand zal nieuwe kansen scheppen voor het ontwikkelen van gewassen met verhoogde ziekteresistentie. Het RBPG1 locus biedt perspectief om een gen te isoleren dat gebruikt kan worden om *B. cinerea*-resistente planten te maken.

Dankwoord

About the author

Over de auteur

Publications

Dankwoord

Het laatste stuk schrijfwerk voor dit proefschrift rest mij nog. Gelukkig een stuk dat niet onderhevig is aan discussies over woordkeuzes, alhoewel ik zelf ook al een aardige taalpurist begin te worden. Maar wat dat betreft kan niemand aan Jan tippen. Jan, die disussies zullen me lang heugen, maar ik wil je daar toch voor bedanken, want op dat vlak heb ik veel van je geleerd. Daarbij heb je me erg vrij gelaten in het werk en het onderzoek wat ik zeer gewaardeerd heb. Misschien achteraf meer dan op de momenten zelf, want het feit dat je aan het einde van mijn eerste jaar aankondigde dat je ruim een half jaar op sabbatical zou gaan naar Nieuw-Zeeland sloeg destijds in als een bom. Gelukkig ging het goed en konden we ook op afstand goed met elkaar communiceren. Dat bleek erg handig want vooral het afgelopen jaar (de laatste hoofdstukken) hebben we weer veel op die manier gewerkt. Pierre, jij bent eigenlijk altijd op afstand gebleven maar je hebt me de mogelijkheid gegeven om te promoveren en (net als Jan) me de ruimte gegeven om me ook buiten het onderzoek te ontwikkelen. Van die ruimte heb ik gretig gebruik gemaakt en dat heeft me een veel bredere blik gegeven. Je moet weten dat ik je daar dankbaar voor ben!

Op het lab en kamers (voor en na de verhuizing) was het met de Botrytiofylen Ester, Arjen, Lia, Saskia, Edith, Martijn, Peter, Sander en Yaite rommelig maar gezellig, d.w.z. totdat Arjen met zijn metal-herrie begon! ☺ Vooral met Ester (m'n bench-maatje) en Sas had ik een leuke tijd. Ik vond het erg jammer dat jullie het lab verlieten, want die gezelligheid en kletspraat heb ik lang gemist! Lia, je was m'n kamergenoot en analist op ons project en ik heb veel aan je te danken. De talloze blots die je gedraaid hebt….petje af. En je hulp bij de grote aantallen infiltraties niet te vergeten (ik heb ze nooit willen tellen om de motivatie niet te verliezen, maar zijn er duizenden geweest). Melysia, you were the first stagiair working on our project. Together we have made many mutants. Unfortunately, not one showed a reduced virulence, but at least we were able to publish the *Bcpme*-work. I wish you well and I am sure you will pursue the scientific career that you have in mind. Verder waren er veel studenten die ik kort tijd heb mogen begeleiden: Emilie, Johan, Joske, Ronald, David, Aurélie, Skiona, Nana, Karolina, Alexia, Lisong, Clément en Michael. Jullie allen ook bedankt voor de leerzame periode (want het viel me niet mee met vier studenten tegelijk).

Niets was zo "precious" als mijn plantjes en die wilde ik ook maar aan één persoon toevertrouwen: Bert. Je hebt voor vele plantjes gezorgd met veel zorg en aandacht. Ik weet niet wat ik zonder jou had moeten beginnen! Pieter dank voor het transport, want je bracht ze altijd waar en wanneer ik ze wilde hebben.

Geja, Kim, Rob en Jacques (Fungal Genomics), jullie natuurlijk bedankt voor de goede samenwerking op ons gezamelijke project. Van jullie heb ik nog wat kennis van eiwit-werk en pectine afbraak mee gekregen. Geja, ook buiten het werk konden we het goed vinden, daarom vind ik het heel leuk dat je m'n paranimf wilt zijn. Melané and Albert from Stellenbosch, I wish to thank you for our collaboration and the work you performed on the co-

infiltrations of VvPGIP and BcPG2 and the analysis of their *in vivo* and *in vitro* interaction.

Andere mensen binnen fyto wil ik bedanken voor 't creëren van een aangename sfeer. Labuitjes waren altijd leuk en ontspannen en dat weekend naar de 'witte wyven' ook. Koffie-pauzes waren nooit zo aan mij besteed, want ik was dan meestal in de kassen te vinden. Sinterklaas heeft er menig keer iets van gezegd in zijn gedichten, maar het is eigenlijk nooit écht verandert. Met één iemand van de vakgroep heb ik wel veel gemeen gehad en dat was Rays. Het was fijn om even de kamer naast me binnen te kunnen lopen. Jij zat altijd achter je computer, te midden van je fruitafval en het groeiende onkruid. Ik heb eigenlijk nooit echt begrepen wat je daarmee bezielde, maar we konden het samen goed vinden en lekker kletsen over van alles en nog wat (vooral ook omdat we in de zelfde fase van ons AIO-schap zaten). Ik wil je bedanken voor die tijd. Je stelligheid, je tekeningen en je uitleg over chinese gedichten zal ik zeker niet vergeten. Ali, Ria en Hans wil ik bedanken voor de administratieve ondersteuning. Zo ook het EPS-secretariaat. Vooral toen ik met de AIO-raad bezig was hadden we veel contact. Bedankt voor alles. Tsja, de AIO-raad en alles er omheen…dat vond ik heel leuk om te doen. Na twee jaar voorzitterschap heb ik met zoveel mensen contact gehad dat het er te veel zijn om op te noemen. Ik hoop dat wij met zijn allen toch iets hebben kunnen betekenen voor zowel de onderzoeksschool als de EPS-AIOs. Uit die tijd zijn er een paar overgebleven die intussen tot mijn vrienden behoren. Mireille, Martin, Lizel en Stefan, het was altijd gezellig met jullie en ook al zijn we nu een beetje uitgewaaierd over de wereld…UK, USA, Zuid-Afrika, La Reunion en Frankrijk ik hoop jullie snel weer eens te treffen en we houden in elk geval contact! Miek (paranimf), wij hebben in Davis al veel gedeeld en altijd contact gehouden. Ik vind het fijn dat we telkens de draad weer op kunnen pakken hoe vaak of weinig we elkaar ook zien. Roos, merci pour ton aide morale dans la derniere ligne droite, het is fijn tussendoor even lekker néerlandais te kunnen beppen.

Naast het werk had ik vele bezigheden, waaronder STUVO en GeNeYouS. Dat was een leuke tijd en een heerlijke afleiding van het onderzoek. Studievoorlichting in de avonduren was tof om te doen en het was dolle pret als we er weer met de Picasso op uit trokken. Met de GeNeYouS, Simon, Mirre, Terry en anderen heb ik veel georganiseerd en een reuze tijd gehad. Bedankt daarvoor!

Als laatste wil ik nog even het woord richten aan mijn familie, schoonfamilie en vrienden. Jullie interesse in mijn werk heeft mij telkens weer een beetje extra motivatie gegeven en was fijn duwtje in de rug op de moeilijkere momenten. Al met al heeft het er toe geleid dat er nu een mooi proefschrift voor jullie ligt. Ik ben blij dat jullie trots op me zijn. Oma, bij jou kom ik mijn lekenpraatje nog even testen. Rik, liefie, jij bent de allerbelangrijkste geweest voor me in de afgelopen jaren. Het was druk met twee proefschriften in de maak, maar jij kon het daarnaast gelukkig ook nog opbrengen mij te steunen en me te ontzien als ik dat nodig had. Hopelijk gaan we nu een rustigere periode tegemoet en kunnen we een beetje meer genieten van het leven en elkaar, want daar zijn we wel aan toe. Ik hou van je.

About the author

Ilona Kars was born on 12 March 1972 in Sliedrecht, the Netherlands. After completing highschool in 1990, she started her study Plant Biotechnology at the Laboratory School in Delft. In her final year, she performed her practical training at the Molecular Plant Science Institute of Leiden University. Under the supervision of Dr. Jan Kijne and Dr. P. Reinhoud she studied the molecular changes in tobacco suspension cells during the cryopreservation by vitrification. Since she obtianed her diploma in 1994, Ilona worked nearly four years as assistant molecular biology at the laboratory of the breeding company De Ruiter Seeds in Bleijswijk, Bergschenhoek and El Ejido (Spain). In 1998, she left De Ruiter Seeds for the biotechnology company Keygene N.V., Wageningen, where she generated genetic maps of maize (AFLP) and worked on a genomics project on the ripening of tomato (cDNA-AFLP). In 1999, Ilona decided to continue her studies and started the study Master of Science International Biotechnology at De Montfort University, Leicester (United Kingdom), which she completed the following year. In 2000, she moved to the United States of America to work as young researcher in the laboratory of Prof. Dr. Richard W. Michelmore on resistance gene candidates to downy mildew in lettuce. March 2001, she returned to the Netherlands to start her PhD study at the Laboratory of Phytopathology, Wageningen University, in the group of Dr. Jan A.L. van Kan and Prof. Dr. Pierre de Wit where she performed research on the cell wall degrading enzymes of *Botrytis cinerea*. After she completed her thesis research mid-2005, she found a job at Vitabric Holland B.V in Wageningen, where she worked as R&D Manager with a small team on the development and improvement of liquid flower foods and cut flower transport treatments. However, in January 2007, she decided together with her partner to move to Toulouse, France, where she works since in the group of Prof. Dr. Dominique Roby at the ‘Laboratoire des Interactions Plantes Micro-organismes’ (INRA/LIPM) as a postdoc on *Xanthomonas campestris* pv. *campestris* resistance in *Arabidopsis thaliana*.

Over de auteur

Ilona Kars werd op 12 maart 1972 in Sliedrecht geboren. Na het behalen van het MAVO diploma (1988) werd gekozen voor de HAVO (diploma 1990) om vervolgens te beginnen met de studie Plantenbiotechnologie aan het Hoger Laboratorium Onderwijs in Delft. Tijdens deze opleiding deed zij haar afstudeeropdracht bij Dr. Jan Kijne en Dr. P. Reinhoud op het Instituut voor Moleculaire Plantkunde van de Rijksuniversiteit Leiden, waar ze onderzoek deed naar de moleculaire veranderingen in tabakscellen tijdens cryopreservatie door vitrificatie. Na het afstuderen in 1994 werkte Ilona vier jaar als assistent moleculaire biologie bij het zaadveredelingsbedrijf De Ruiter Seeds in Bleijswijk, Bergschenhoek en El Ejido (Spanje). In 1998 is zij overgestapt naar het biotechnologische bedrijf Keygene N.V. in Wageningen, waar ze als wetenschappelijk medewerker werkte aan o.a. genetische kaarten van maïs (AFLP) en een genomics project over rijping van tomaat (cDNA-AFLP). In 1999 nam Ilona het besluit haar studie te vervolgen. Hetzelfde jaar begon zij met de studie Master of Science International Biotechnology aan de De Montfort University te Leicester (Groot-Brittannië), welke het jaar er op met goed gevolg werd afgesloten. In 2000 verhuisde ze naar de Verenigde Staten om daar een jaar als toegevoegd onderzoeker te werken in het laboratorium van Prof. Dr. Richard W. Michelmore aan het opsporen van nieuwe kandidaat genen voor resistentie tegen valse meeldauw in sla. Maart 2001 keerde ze terug naar Nederland om bij het Laboratorium voor Fytopathologie van Wageningen Universiteit als promovenda te gaan werken in de groep van Dr. Jan A.L. van Kan en Prof. Dr. Pierre de Wit aan celwand afbrekende enzymen van de schimmel *Botrytis cinerea*. Na vier jaar promotieonderzoek vond ze medio 2005 een baan bij Vitabric Holland B.V (producent snijbloemenvoeding en transportmiddelen) in Wageningen. Hier werkte ze als R&D Manager met een klein onderzoeksteam aan product vernieuwing en verbetering alvoor ze in Januari 2007 besloot met haar partner naar Toulouse in Frankrijk te verhuizen waar ze sindsdien bij Prof. Dr. Dominique Roby in het Laboratoire des Interactions Plantes Micro-organismes (INRA/LIPM) werkt als postdoc aan *Xanthomonas campestris* pv. *campestris* resistentie in *Arabidopsis thaliana*.

Publications

Kars, I., Wagemakers, L., Bentsink, L., Witsenboer, H., and van Kan, J.A.L. (to be submitted) The quantitative trait locus RBPG1 controls the response to *Botrytis cinerea* polygalacturonases in *Arabidopsis thaliana*.

Joubert, D.A., Kars, I., Wagemakers, L., Bergmann, C., Kemp, G., Vivier, M.A., and van Kan J.A.L. (2007) A polygalacturonase inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for in vitro interaction. Mol. Plant-Microbe Interact., 20, 392-402.

Kars, I., McCalman, M., Wagemakers, L., and van Kan, J.A.L. (2005b) Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme1* and *Bcpme2* are dispensable for virulence of strain B05.10. Mol. Plant Pathol., 6, 641-652.

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A.E., and van Kan, J.A.L. (2005a) Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J., 43, 213-225.

Kars, I., and van Kan, J.A.L. (2004) Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In *Botrytis:* Biology, Pathology and Control (Y. Elad, B. Williamson, P. Tudzynski and N. Delen, eds.). Dordrecht: Kluwer Academic Publishers, pp. 99-118.

Reinhoud, P.J., Versteege, I., Kars, I., Van Iren, F., and Kijne, J.W. (2000) Physiological and molecular changes in tobacco suspension cells during development of tolerance to cryopreservation by vitrification. In Cryopreservation of tropical germ plasm (F. Engelmann, H. Takagi, eds.). Int. Plant Genet. Res. Inst., Rome, pp. 57-66.

The work in this thesis was performed at the Laboratory of Phytopathology, Binnenhaven 5, 6709 PD Wageningen, the Netherlands, and within the graduate school for Experimental Plant Sciences (EPS).

This research was funded by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs (project WGC.5034).

Printing of this thesis was supported by the J.E. Jurriaanse Stichting.

Printed at Ponsen & Looijen B.V., Wageningen, the Netherlands.

Plaatsvervangend Rector Magnificus:
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Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Ilona Kars**
Date: **21 September 2007**
Group: **Laboratory of Phytopathology, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Cell wall degrating enzymes of *Botrytis cinerea*: a major target for disease control	Jun 2001
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*	Sep 2004
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Wageningen University	Jan 24, 2002
EPS PhD student day, Utrecht University	Mar 27, 2003
EPS PhD student day, Vrije Universiteit Amsterdam	Jun 3, 2004
EPS PhD student day, Radboud University Nijmegen	Jun 2, 2005
Int. PhD students day CNRS, Gif-sur-Yvette, France	May 19, 2005
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Leiden University	Dec 17, 2001
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Jan 10, 2003
EPS theme 2 sympsium 'Interactions between Plants and Biotic Agents', Wageningen University	Dec 12, 2003
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Experimental Plant Sciences meeting 2001, Lunteren	Mar 26-27, 2001
NWO-ALW Experimental Plant Sciences meeting 2003, Lunteren	Apr 7-8, 2003
NOW-ALW Platform Molecualr Genetics meeting 2003, Lunteren	Nov 3-4, 2003
NWO-ALW Experimental Plant Sciences meeting 2004, Lunteren	Apr 6-7, 2004
NWO-ALW Experimental Plant Sciences meeting 2005, Lunteren	Apr 4-5, 2005
Willie Commelin Scholten dag, Utrecht University	Jan 22, 2004
National Botrytis workgroup meeting, Boskoop	Sep 18, 2002
National Botrytis workgroup meeting, Wageningen	Apr 12, 2005
► **Seminars (series), workshops and symposia**	
Flying seminar Jane Glazebrook	Apr 24, 2001
Flying seminar Dan Klessing	Mar 19, 2002
Seminar Teun Munnik	May 17, 2002
Seminar Jonathan Jones	May 29, 2002
Seminar Sophien Kamoun	Jul 8, 2002
Flying seminar Jeff Dangl	May 9, 2003
Dutch-German Workshop on molecular aspects of pathogenicity of *Botrytis cinerea*, Münster	Feb 19, 2002
Dutch-German Workshop on molecular aspects of pathogenicity of *Botrytis cinerea*, Wageningen	Jun 30, 2003
Dutch-German Workshop on molecular aspects of pathogenicity of *Botrytis cinerea*, Kaiserslautern	Jun 6, 2005
NIBI Career Perspectives Day	Jun 11, 2003
CBS/Wageningen Phytopathology symposium	Jun 27, 2003
Spotlite Congres Wetenschap & Carriere	Feb 9, 2004
1st GeNeYouS symposium 'Decisions in genomics', Utrecht	Jan 20, 2004
Genomics Momentum, Rotterdam	Aug 30-Sep 1, 2004
Symposium on Systems Biology in Honor of Prof. Dr. Pierre de Wit	Nov 11, 2004
2nd GeNeYouS symposium at Organon, Oss	Apr 27, 2005
Biocareer event 2005, Jaarbeurs Utrecht	May 12, 2005
► **Seminar plus**	
► **International symposia and congresses**	
1st International Pectins and Pectinases symposium, Rotterdam, The Netherlands	May 6-10, 2001
6th European Conference on Fungal Genetics, Pisa, Italy	Apr 6-9, 2002
Workshop Botrytis, Christchurch, New Zealand	Feb 1-2, 2003
8th International Congress of Plant Pathology, Christchurch, New Zealand	Feb 2-7, 2003
11th International Congress on Molecular Plant-Microbe Interactions, St. Petersburg, Russia	Jul 18-26, 2003
XIII International Botrytis symposium, Antalya, Turkey	Oct 21-25, 2004
XXIII Fungal Genetics Conference, Asilomar, CA, USA	Mar 15-20, 2005
► **Presentations**	
Autumnschool 'Disease Resistance in plants', Wageningen (poster and presentation)	2002
National Botrytis workgroup meetings (two presentations)	2002, 2005
Dutch-German Workshop on molecular aspects of pathogenicity of *Botrytis cinerea* (three presentations)	2002, 2003, 2005
Workshop Botrytis, Christchurch, New Zealand (presentation)	2003
8th ICPP, Christchurch, New Zealand (poster and presentation)	2003
11th Intern. Congress on Molecular Plant-Microbe Interactions, St. Petersburg, Russia (poster presentation)	2003
XIII International Botrytis symposium, Antalya, Turkey (two presentations)	2004
XXIII Fungal Genetics Conference, Asilomar, CA, USA (poster)	2005
► **IAB interview**	Jun 3, 2004
► **Excursions**	
Subtotal Scientific Exposure	*29.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
EPS PhD Summer school 'Environmental signalling: Arabidopsis as a model', Utrecht	Aug 27-29, 2001
EPS PhD Autumn school 'Disease Resistance in plants', Wageningen	Oct 14-16, 2002
EPS PhD Summer school 'The analysis of natural variation within crop and model plants', Wageningen	Apr 22-25, 2003
► **Journal club**	
► **Individual research training**	
Subtotal In-Depth Studies	*3.0 credits**

4) Personal development	*date*
► **Skill training courses**	
Scientific writing	Oct 2001-Feb 2002
EndNote	Feb 12, 2002
Digital Scientific Artwork	Jun 26-27, 2002
Hoorcollege geven	May, 2004
Career Orientation	Oct 2004
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
President EPS PhD council	2002-2004
GeNeYouS International committee	2004-2006
Treasurer GeNeYouS	2004-2005
Country board member Young European Biotech Network (YEBN)	2004-2005
Subtotal Personal Development	*10.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**51.0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*