Stellingen

1. Glutathione S-transferase heeft niet alleen een detoxificerende werking, maar kan in sommige gevallen ook een potentiërende werking hebben op precursors van toxische stoffen.
   Henderson *et al*. (2000) *Proc Natl Acad Sci USA* **97**:12741-12745.
   Dit proefschrift

2. Een overgevoeligheidsreactie is geen universeel resistentiemechanisme.
   Govrin and Levine (2000) *Curr Biol* **10**:751-757.
   Dit proefschrift

3. Het polyubiquitine gen *Ubi.U4* uit *Nicotiana tabacum* heeft waarschijnlijk meer dan 4 volledige ubiquitine units, hetgeen als gevolg van een kloneringsartefact over het hoofd gezien is.
   Genschik *et al*. (1994) *Gene* **148**:195-202.

4. De door Brommonschenkel *et al*. (2000) gepubliceerde sequentie van het resistentiegen Sw5 is incorrect omdat het een chimaera is van Sw5-a en Sw5-b.
   Brommonschenkel *et al*. (2000) *Molec Plant Microbe Int* **13**:1130-1138.
   Spassova *et al*. (2001) *Mol Breeding* **7**:151-161.

5. De meerwaarde van het behalen van de doktorstitel komt tot uiting in de aanspreektitel. De toevoeging ‘zeer’ aan ‘Weledelgeleerde heer’ geeft hierbij aan hoeveel zweet en tranen het behalen ervan heeft gekost.

6. De traditie om vogels te benoemen naar het geluid dat ze produceren (zoals de grutto en de kievit), zou ervoor pleiten om de Turkse tortel te herbenoemen als Koerdische tortel.
   Peterson *et al*. (1994) Petersons vogelgids van alle Europese vogels.

7. Gezien de spelfouten die gemaakt worden in de namen bij het bestellen van oligo’s, is het verwonderlijk dat er niet meer fouten in de nucleotidesequentie te vinden zijn.

8. De gedachte ‘Big brother is watching you’ is net zo beangstigend als de idee dat veel Nederlanders naar *big brother* kijken.
   vrij naar George Orwell (1948).

9. De uitspraak ‘We are the Borg. Resistance is futile - you will be assimilated’ geeft aan hoe weinig een hoogontwikkeld ras als *the Borg* weet van resistentieveredeling.
   The Borg Collective, Delta quadrant. Star Trek: The next generation.

Stellingen behorende bij het proefschrift van Theo Prins getiteld:
Identification and functional analysis of *Botrytis cinerea* genes
induced during infection of tomato.

Wageningen, 17 oktober 2001

Identification and functional analysis of *Botrytis cinerea* genes induced during infection of tomato

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Theo Prins

Identification and functional analysis of *Botrytis cinerea* genes induced during infection of tomato

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 17 oktober 2001
des namiddags te 16.00 uur in de Aula

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Nothing takes the past away
Like the future
Nothing makes the darkness go
Like the light

(Nothing really matters, Madonna)

Aan mijn ouders

Abbreviations

ABA	ABscisic Acid
AFLP®	Amplified restriction Fragment Length Polymorphism (® KeyGene nv)
AOS	Active Oxygen Species
AP	Aspartic Protease
bp	basepairs
CCLS	Chemical Cross linking Subtraction
CEP	Carboxyl Extension Protein
CWDEs	Cell Wall Degrading Enzymes
ddB	differential display *Botrytis*
ddT	differential display Tomato
DDRT-PCR	Differential Display Reverse Transcriptase PCR
DOP	Degenerate Oligonucleotide primed PCR
DPI	specific NADPH oxidase Inhibitor
DROP	Directional Random Oligonucleotide Primed
EDS	Enzymatic Degrading Subtraction
endoPG	endoPolyGalacturonase
ESTs	Expressed Sequence Tags
GST	Glutathione S-Transferase
h.p.i.	hours post inoculation
HR	Hypersensitive Response
kb	kilobases
kDa	kiloDalton
nt.	nucleotides
OA	Oxalic Acid
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pI	Isoelectric point
pfu	plaque forming unit
PGIPs	PolyGalacturonase Inhibiting Proteins
RACE	Rapid Amplification of cDNA Ends
RDA	Representational Difference Analysis
REMI	Restriction Enzyme Mediated Integration
RH	Relative Humidity
RT	Reverse transcription or Room Temperature
ROIs	Reactive Oxygen Intermediates
SAR	Systemically Acquired Resistance
SOD	SuperOxide Dismutase
TNV	Tobacco Necrosis virus
Ubi	Ubiquitin
UEP	Ubiquitin Extension Protein

Contents

Aim and outline of this thesis

The research described in this thesis focuses on the isolation and functional analysis of unidentified pathogenicity factors of the grey mould fungus, *Botrytis cinerea*. We studied the interaction between *B. cinerea* and one of its hosts, tomato (*Lycopersicon esculentum*), by means of non-biased screening methods for fungal genes that are specifically expressed during pathogenesis.

At the start of this project, not much was known about pathogenicity factors at the molecular level. The publication of a transformation system for *B. cinerea*, in the first year of the project, made it possible to study the role of cloned genes by targeted mutagenesis.

Chapter 1 presents an overview of the infection strategy of *B. cinerea* and related necrotrophs. This chapter highlights the state-of-the-art of our knowledge on *B. cinerea* in relation to physiology and pathogenesis.

The following four experimental chapters describe the screening methods used and the genes that have been identified and functionally analysed.

Chapter 2 describes the adaptation and application of the Differential Display Reverse Transcriptase PCR (DDRT-PCR) method to the analysis of fungal gene expression in the *B. cinerea*- tomato interaction. The sensitivity of the method and the isolated fragments isolated with this method will be discussed.

Chapter 3 describes the differential hybridisation screening of a genomic library of *B. cinerea* with radiolabelled cDNA probes. A small gene family of ubiquitin genes was identified. Their possible role in the interaction is discussed.

Chapter 4 describes the cloning of a glutathione S-transferase (GST) gene by means of hybridisation screening with a subtractive RT-PCR product. A role of GST in detoxification and oxidative stress tolerance is discussed.

Chapter 5 describes a direct PCR-based approach to clone an aspartic protease gene. This enzyme activity was previously suggested to be an important pathogenicity factor. The gene that we cloned encodes an aspartic protease, but it does most likely not correspond to the aspartic protease that is presumably required for pathogenicity.

The general discussion in chapter 6 describes the rationale, as well as the advantages and disadvantages of the non-biased screening methods applied in this thesis. Additional methods are also discussed and compared. The genes that we identified, as described in the experimental chapters, are discussed with emphasis on their role in pathogenesis.

CHAPTER 1

Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens

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Introduction

Botrytis cinerea Persoon: Fries (known as "grey mould fungus") causes serious pre- and post-harvest diseases in at least 235 plant species (Jarvis, 1977), including a range of agronomically important crops, such as grapevine, tomato, strawberry, cucumber, bulb flowers and ornamental plants. Graminaceous monocots are generally considered as poor hosts for grey mould. Disease control frequently relies on chemicals, although efforts to develop biological control strategies are increasingly successful (*e.g*. Köhl *et al*., 1995; Elad, 1996) and biocontrol agents are marketed. The name of the asexual stage or anamorph, *Botrytis cinerea,* is preferred to the name of the teleomorph, *Botryotinia fuckeliana* (de Bary) Whetzel (XIth International Botrytis Symposium, 1996, Wageningen, The Netherlands). The teleomorph has rarely been detected in the field during the last century, but molecular population studies recently provided clear evidence that sexual reproduction occurs more frequently than previously anticipated (Giraud *et al*., 1997). The pathogen is a typical necrotroph, inducing host cell death resulting in serious damage to plant tissues, culminating in rot of the plant or the harvested product. There are extensive descriptions of microscopic and biochemical studies on infection mechanisms (reviewed by Staples and Mayer, 1995). Comprehensive insight in the infection process, however, is hampered by the fact that various groups used different fungal strains and different host species for their studies.

It is relatively recently that molecular-genetic tools such as transformation (Hamada *et al*., 1994), differential gene expression analysis (Benito *et al*., 1996) and gene cloning (van der Vlugt-Bergmans *et al*., 1997a,b) have come available to unravel the factors involved in pathogenicity of *B. cinerea.* Application of these tools has provided substantial new data in a short time and it is expected to yield even more information in the next decade, which will be used to design novel strategies for grey mould control. Here we present an overview of the current insights and hypotheses on the mechanisms employed by *B. cinerea* to infect its host plants. The role of different fungal compounds in consecutive stages of the infection process is described. The balance between induced plant defence responses and the evasion of these responses by the fungus plays an important role in determining the outcome of the attempted invasion. Finally, we briefly discuss the analogy between infection mechanisms utilised by *B. cinerea* and related necrotrophs, which are as yet less accessible for molecular-genetic research.

Disease cycle

For the purpose of this review, different stages are distinguished in the disease cycle of *B. cinerea* (Figure 1). It should be emphasised that, given the wide host range of the fungus, not all processes occur in every infection.

B. cinerea can produce conidia on every host plant. They are ubiquitous in the air and can be transported by wind over long distances before infecting the next host (Jarvis, 1977). Following attachment, the conidium germinates under favourable conditions and produces a germ tube that penetrates the host surface. Whether true infection structures are produced during this process is a matter of debate. After surface penetration the underlying cells are killed and the fungus establishes a primary lesion, in which necrosis and defence responses may occur. In some cases this is the onset of a period of quiescence of an undefined length, in which fungal outgrowth is negligible (reviewed by Prusky, 1996). At a certain stage the defence barriers are breached and the fungus starts a vigorous outgrowth, resulting in rapid maceration of plant tissue, on which the fungus finally sporulates to produce inoculum for the next infection. Under optimal conditions, one infection cycle may be completed in as little as

3-4 days, depending on the type of host tissue attacked. In the following paragraphs we will discuss the various stages in the disease cycle and the role that enzymes and metabolites (mainly of fungal origin) play in these stages.

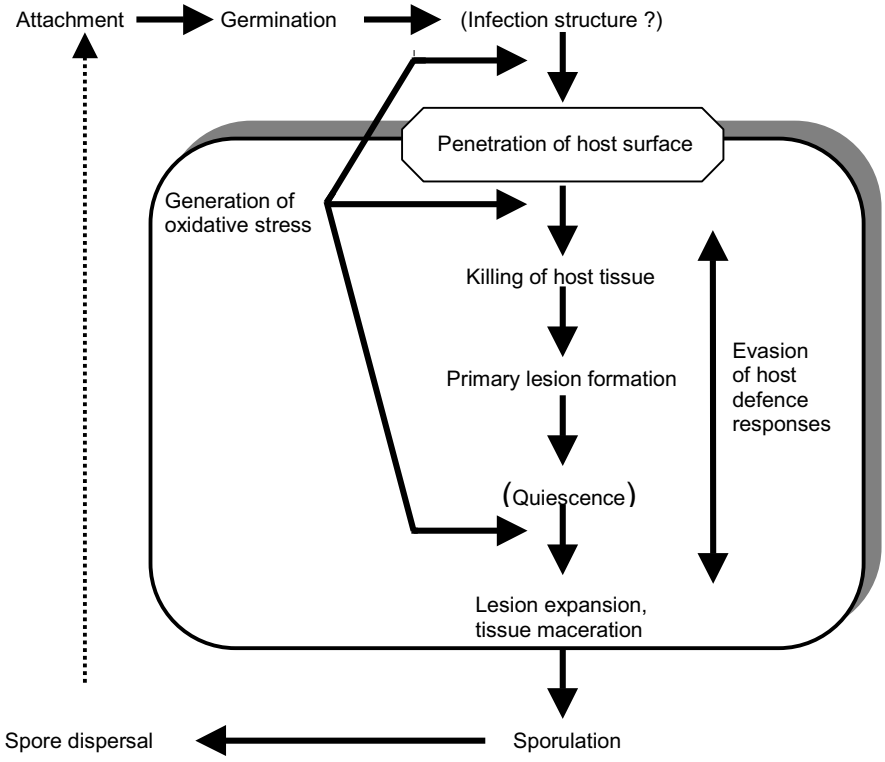

Figure 1. Different stages in the infection process of *B. cinerea*, to be discussed below. The shaded box represents the host tissue.

Attachment of conidia

Dispersal of conidia by insects

Before being able to penetrate host tissue the conidia of *B. cinerea* must land on and attach to the plant surface. The conidia are ubiquitous in the air and are capable of reaching any host by random chance. Specific trafficking by insects towards attachment sites of host tissue has also been described. The fruit fly *Drosophila melanogaster,* the New Zealand flower thrips *Thrips obscuratus*, as well as larvae of the grape berry moth *Lobesia botrana*, were shown to carry viable conidia of *B. cinerea* (Fermaud and Le Menn, 1992; Fermaud *et al.,* 1994; Fermaud and Gaunt, 1995; Louis *et al.,* 1996). Wounds inflicted on host tissue by the various insects increased disease development. The fruit fly not only carried conidia externally on its cuticle but also in the digestive tract, and microsclerotia were detected in the gut (Louis *et al.,* 1996).

Surface characteristics

A number of physical surface interactions is believed to play a role in the attachment of conidia to the plant epidermis. Conidia of *B. cinerea* have a hydrophobic surface which can usually only be wetted by solutions containing mild detergents. Williamson *et al*. (1995) described a granular structure on the surface of *B. cinerea* conidia, with a smooth area along the main axis. Doss *et al.* (1997) visualised numerous short (200-250 nm) protuberances on the rough surface of dry conidia of seven different *Botrytis* species. These protuberances disappeared upon hydration and redrying. The typical basket-weave pattern of hydrophobin rodlets present in *Magnaporthe grisea* (*MPG*1; Talbot *et al*., 1996) was not observed on conidia of any of the *Botrytis* species tested (Doss *et al*., 1997). Hydrophobins, which are involved in the interaction of *M. grisea* with the rice plant surface (Talbot *et al*, 1993; Beckerman and Ebbole, 1996; Talbot *et al*., 1996), thus seem to be absent in the *Botrytis* species studied, unless *Botrytis* hydrophobins aggregate in a structure different from *M. grisea* and other fungi (Kershaw and Talbot, 1998). Molecular research is needed to clarify whether or not *B. cinerea* possesses hydrophobin genes.

Doss *et al.* (1993, 1995) distinguished two steps in the attachment to host tissue. The first stage, preceding the hydration of conidia, is characterised by relatively weak adhesive forces, presumably resulting mainly from hydrophobic interactions between the host and conidial surfaces (Doss *et al.,* 1993). A stronger binding occurs in the second stage of adhesion, several hours after inoculation, when it becomes increasingly difficult to wash off the conidia. It should be noted, however, that at this stage conidia have developed into germlings. At this stage a fibrillar-like matrix material is present on the tip of germ tubes (Cole *et al*., 1996; see Figure 2). The attachment of germlings to either hydrophobic or hydrophilic substrata coincides with the production of a base-soluble compound, forming a fibrillate sheath around the germlings (Doss *et al.,* 1995), supposedly consisting of an extracellular β-(1,3)(1,6)-n-glucan (cinerean). Cinerean forms a slimy adhering capsule in liquid culture. Once glucose is exhausted from the medium, *B. cinerea* degrades the polymer by cinereanase into glucose and gentobiose, which is subsequently hydrolysed to glucose (Stahmann *et al.,* 1992; Stahmann *et al.,* 1993; Monschau *et al.,* 1997). Besides serving as glue and nutrient storage, cinerean may have a role in protecting hyphae against dehydration and defence mechanisms of the host.

When inoculation was carried out using conidia in aqueous glucose, a sheath of fibrillar-like material (presumably cinerean) was detected on the germ tube surface, whereas in inoculations with dry conidia this sheath is absent (Cole *et al.*, 1996). Since dry-inoculated conidia were also firmly attached to the plant surface, it was concluded that the sheath is not essential for attachment of conidia to the host.

Germination

After landing on the host tissue, a number of factors influence the germination of a conidium. Free surface water or high relative humidity (>93 % RH) is required to germinate and penetrate the host epidermis (Williamson *et al.,* 1995). In addition, moisture assists the pathogen in the uptake of nutrients residing on the host epidermis or pollen grains (Blakeman, 1980). When dry conidia are inoculated on plant surfaces and subsequently incubated in the absence of free surface water, the emerging germ tube usually remains shorter than the length of a conidium before it penetrates the surface (Salinas and Verhoeff, 1995; Williamson *et al*., 1995; Cole *et al*., 1996). Inoculation with conidia in an aqueous suspension, however, usually requires the addition of nutrients, which might mimic the

situation in a wound on the plant epidermis, from which nutrients leach (Harper *et al*., 1981; van den Heuvel, 1981). A highly efficient germination and synchronous infection of tomato leaves is obtained, when conidia are preincubated for 2-4 hours in liquid medium supplemented with phosphate and sugar (Benito *et al.,* 1998). The sugar is probably not only involved in stimulating the germination, but also in oxidative processes leading to host cell death (Edlich *et al*., 1989; see paragraph 'Killing the host').

Gaseous compounds might also be involved in the stimulation of germination. Elad and Volpin (1988) found a correlation between the level of ethylene production by flowers, petals and leaves of different rose cultivars, and the severity of grey mould symptoms. A stimulation of grey mould development by ethylene was also demonstrated in strawberry, tomato, cucumber and pepper (*e.g.* Elad and Volpin, 1988; McNicol *et al*., 1989). This observation is usually ascribed to the weakening of the host tissue and senescence that coincides with ethylene production. The influence of ethylene on *B. cinerea* itself is only rarely investigated. Analogous to *Colletotrichum gloeosporioides* (Flaishman and Kolattukudy, 1994), germination of *B. cinerea* might be influenced by ethylene. Kepczynski and Kepczynska (1977) found that germination of *B. cinerea* conidia on a hydrophobic surface was stimulated by exogenous ethylene, but the germ tube length was unaffected. Application of 2,5-norbornadiene, a competitive inhibitor of ethylene perception in plants, inhibited germination in a reversible manner (Kepczynska, 1993). On the other hand, it was reported that in a hydrophilic environment, ethylene stimulated germ tube elongation without affecting the percentage of germination (Barkai-Golan *et al.,* 1989). It is tempting to speculate that ethylene produced by the plant during leaf senescence or fruit ripening might function as a signal for the conidia on the (hydrophobic) plant surface to germinate and initiate the infection. Subsequently the germ tube elongation might be stimulated by ethylene in the more hydrophilic environment of the invaded plant tissue. Thus, ethylene might favour grey mould development by weakening the host, as well as by stimulating germination of *B. cinerea* conidia and outgrowth of hyphae. Molecular and biochemical approaches are required to elucidate whether *B. cinerea* possesses ethylene receptors.

Differentiation of infection structures on the host surface

There is some debate whether *B. cinerea* forms appressoria during penetration. There is consensus that germ tubes do not differentiate into the highly organised appressoria that are typical for many plant pathogenic fungi (reviewed by Mendgen *et al.,* 1996). Several authors, however, reported an appressorium-like structure in *B. cinerea* but their nomenclature diverges. Van den Heuvel and Waterreus (1983) distinguished germ tube apices, *i.e.* tips of germ tubes appearing as an appressorium-like swelling, appressoria of different forms and infection cushions. Akutsu *et al*. (1981) grouped pre-penetration structures into primary, secondary or hyphal elongations. It is probable that these structures, which were all observed upon inoculation with conidia in an aqueous glucose suspension, correspond to the structure shown in Figure 2, a swollen hyphal tip with an "adhesive pad" (Cole *et al*., 1996). Secretion of a phosphatase by *B. cinerea* grown *in vitro* occurs specifically from vesicles in the hyphal tip (Weber and Pitt, 1997), which is typical for protein secretion in filamentous fungi (Sietsma *et al*., 1995). It remains to be determined whether fungal hydrolytic enzymes, involved in host surface penetration, are also secreted at the hyphal tip and whether these enzymes accumulate prior to the onset of penetration, in the indented area delimited by arrows in Figure 2.

The swelling of the hyphal tip may be the consequence of a rise in the osmotic value in the hyphal tip, resulting in water absorption. In the absence of a rigid layer in the outer wall,

swelling can not result in an equally high turgor as is generated in appressoria of *M. grisea* (Howard *et al*., 1991; de Jong *et al*., 1997). The external sheath, presumably consisting of cinerean (see paragraph 'Attachment of conidia'), may contribute to the swelling by retaining water, as cinerean is extremely hygroscopic. Whether the hydrophobic host surface or the release of volatile plant hormones provide signals to trigger the formation of hyphal swellings is purely speculative.

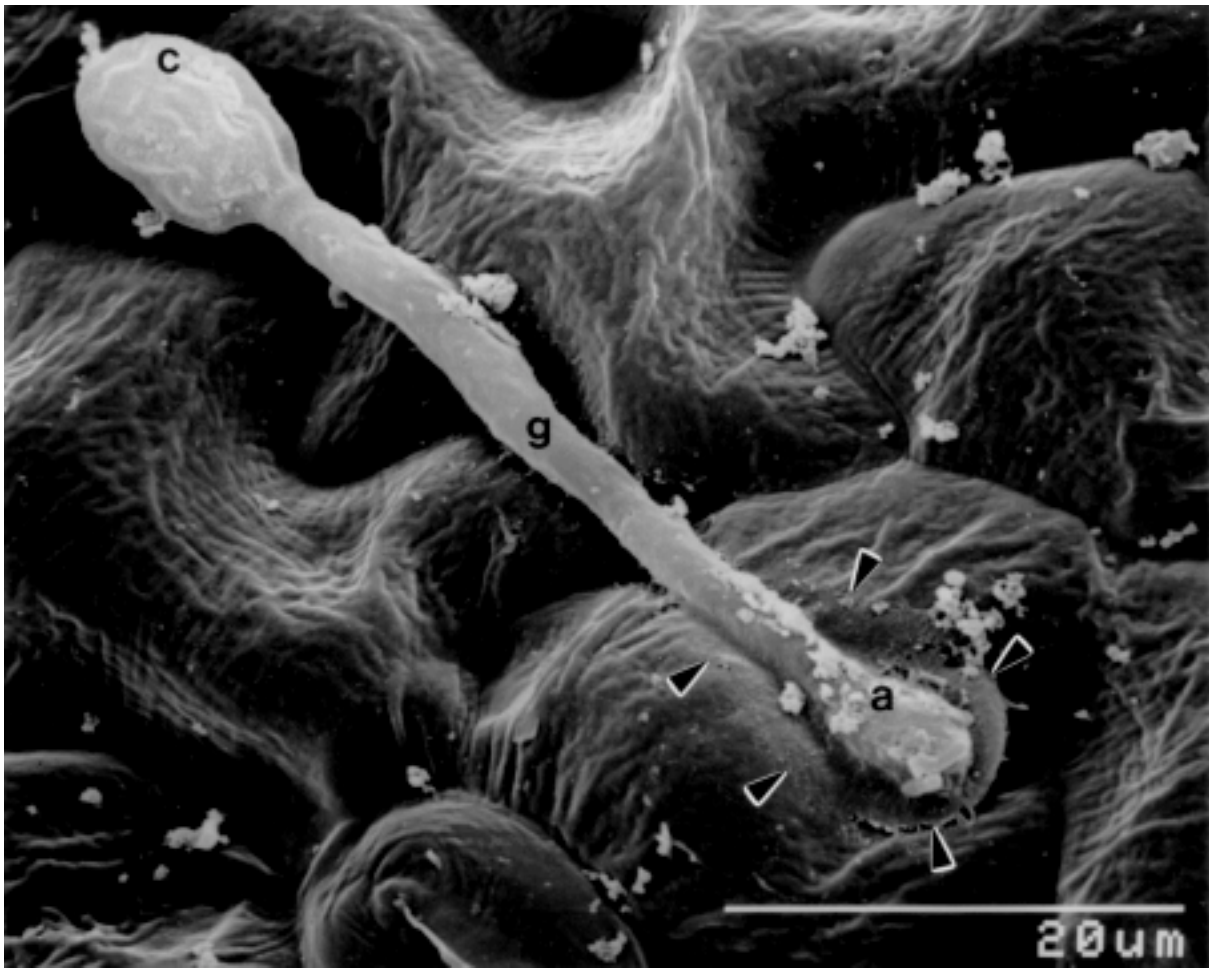

Figure 2. Scanning Electron Micrograph of an early stage of infection (5 h.p.i.) of tomato leaves. A conidium (c) has germinated and formed a germ tube (g), which terminates in a swollen tip (a) surrounded by matrix material, the adhesive pad. The host epidermal surface is partly indented around the site of contact with the adhesive pad (indicated by arrows).

Swelling of hyphal tips is usually not observed when hosts are inoculated with dry conidia (Salinas and Verhoeff, 1995; Williamson *et al*. 1995; Cole *et al*., 1996). In the latter cases, the conidia produced short germ tubes that directly penetrated the epidermal cells, subsequently leading to cell death.

Penetration of the host surface

Invasion of host tissue can be achieved by active penetration or passive ingress. *B. cinerea* is a renowned opportunist that can initiate infection at wound sites, or at sites that have previously been infected by other pathogens. *B. cinerea* can also enter the substomatal cavity

via an open stoma. Only direct penetration of the epidermal surface is discussed in this paragraph. For reasons of simplicity the penetration of dead or wounded tissue, as well as via stomata, is regarded as an expansion rather then a penetration process, and is dealt with in paragraph 'Disease expansion and tissue maceration'.

Cutinases

When conidia land on aerial parts of a plant, the first barrier to overcome is the cuticle, which covers the epidermal cells. Its major structural component, cutin, is a polyester composed of hydroxylated and epoxidised C_{16}- and C_{18}-fatty acids (Martin and Juniper, 1970). Physical damage or mechanical penetration of the cuticle by *B. cinerea* is not usually observed (Williamson *et al*., 1995; Cole *et al*., 1996). Hence, cutinolytic activity is presumably required to penetrate this layer (Salinas and Verhoeff, 1995; van der Vlugt-Bergmans, 1997a). Salinas (1992) investigated whether a particular 18 kDa cutinase is important in this process and raised monoclonal antibodies against the enzyme. Application of the antibody to gerbera flowers prior to inoculation reduced lesion formation by 80 %. A cutinase–deficient gene replacement mutant, however, did not have any discernible reduction in virulence on gerbera flowers nor on tomato fruits, as compared to the wild type (van Kan *et al.,* 1997). Although the observations of Salinas (1992) remain to be explained, it can be ruled out that this particular 18 kDa cutinase is essential in penetration.

It should be taken into account that the 18 kDa cutinase, like all other cutinases studied thus far in plant pathogenic fungi, is most likely an exo-hydrolase. Enzyme activity of cutinases is usually defined by their ability to release soluble fatty acid monomers from the water-insoluble substrate (Purdy and Kolattukudy, 1973). It would be much more efficient for a pathogen to produce an endo-hydrolase, in order to create openings for penetrating a polymer by fungal hyphae. Such endo-cutinase, however, will not release water-soluble products from the insoluble cutin, since cleavage products most likely remain attached in the network. Hence, endo-cutinase activity is difficult to detect in biochemical assays.

One candidate for an enzyme with such activity is a 60 kDa lipase that is induced upon growth in liquid medium with apple cutin as the sole carbon source (Comménil *et al.,* 1998). This lipase possesses low but significant cutinolytic activity and it has clearly distinct kinetic properties from the 'typical' cutinases mentioned above. When polyclonal antibodies raised against this lipase were applied prior to inoculation with *B. cinerea* conidia, germ tubes were no longer able to penetrate the cuticle. The antibodies did not affect germination (Comménil *et al.,* 1998). Whether the lipase plays an essential role in host tissue penetration should be assessed by cloning the corresponding gene, constructing a targeted lipase-deficient mutant and determining its virulence.

Pectinases

Microscopic studies have shown that after penetration of the cuticle, hyphae of *B. cinerea* frequently invade the anticlinal wall between two epidermal cells. The concomitant swelling of the epidermal cell wall (Mansfield and Richardson, 1981) is indicative for the degradation of the pectin in the matrix of the epidermal wall, presumably as a result of water absorption, as will be discussed below in paragraph 'Killing the host'. Biochemical evidence suggested that pectinases might be involved in primary infection. At least one (basic) endopolygalacturonase (endoPG) is expressed constitutively and it was therefore proposed to be involved in early stages of the infection process (Van der Cruyssen *et al*., 1994). Gene cloning revealed that *B. cinerea* contains an endoPG gene family, consisting of six members encoding basic as well as acidic isozymes (ten Have *et al.,* 1998; Wubben *et al*., 1999).

Targeted deletion mutants were made in both genes encoding the basic endoPGs (*Bcpg*1 and *Bcpg*2) by gene replacement. Both types of mutants were still able to cause primary necrotic lesions on non-wounded tomato and bean leaves (ten Have *et al.,* 1998; ten Have *et al*., unpublished), excluding an essential role for BcPG1 and BcPG2 in host surface penetration.

Proteases

Movahedi and Heale (1990a) detected extracellular aspartic protease (AP) activity in ungerminated conidia as well as during germination, prior to the appearance of pectinase activity. Application of the specific AP inhibitor pepstatin during inoculation markedly reduced infection of carrot slices, suggesting an important role for AP during primary infection (Movahedi and Heale, 1990b). Recently, a gene was cloned encoding an aspartic protease, *BcAP*1, and targeted mutants were made to study its involvement in the infection of detached tomato leaf tissue (Chapter 5). No discernible loss of virulence was observed for the BcAP1-deficient mutant, indicating that this protease is not essential for virulence. Since *B. cinerea* probably contains at least one additional AP gene, the importance of aspartic proteases in pathogenesis can not yet be excluded.

Killing the host

B. cinerea kills host cells before they are invaded by hyphae (Clark and Lorbeer, 1976). Recent studies have demonstrated that invasion of plant tissue by *B. cinerea* triggers nuclear condensation and plant membrane damage, two indicators for programmed cell death, in a ring of cells around the hyphae (Govrin and Levine, 2000). These results imply that diffusible factors have a direct or indirect phytotoxic activity. Several phytotoxic compounds that have been proposed to play a role in killing host cells are discussed below.

Toxins

Culture filtrates of *B. cinerea* may induce toxic effects when applied to plant tissue (Rebordinos *et al*., 1996), but there is still little evidence for the existence of a causal fungal toxin *sensu strictu*. A *B. cinerea* isolate excreted in liquid culture a highly substituted lactone, botcinolide, which inhibited wheat coleoptile elongation and induced necrosis or chlorosis on bean, corn and tobacco plants (Cutler *et al*., 1993). Homobotcinolide, a natural botcinolide homologue, did not exhibit phytotoxic effects on bean but it was more potent in inhibiting wheat coleoptile elongation (Cutler *et al*., 1996). Four additional derivatives of botcinolides have been isolated and structurally characterised without elucidation of their biological activity (Collado *et al*., 1996). The tricyclic sesquiterpenes, botrydial and dehydrobotrydial, were purified from liquid culture and were phytotoxic on tobacco leaf discs. However, both metabolites were secreted by *B. cinerea* only when the medium contained a high glucose level (>3 %). Moreover the damage inflicted by the purified metabolites only occurred at concentrations significantly above the natural concentrations found in liquid media (Rebordinos *et al*., 1996). Before one can envisage a significant role of phytotoxins of *B. cinerea* in pathogenesis, more information is needed on the timing of secretion, the occurrence of toxic metabolites *in situ* and their correlation with the aggressiveness of individual fungal strains.

Oxalic acid

Secretion of oxalic acid (OA) is a widespread property of fungi from various taxonomic classes. Its occurrence and ecological function was recently reviewed by Dutton and Evans (1996). A key role for OA in pathogenesis has been postulated for several plant pathogens like *Sclerotinia sclerotiorum* (Godoy *et al*., 1990), *Sclerotium rolfsii* (Kritzman *et al*., 1977), *Mycena citricolor* (Rao and Tewari, 1987), and *Sclerotium cepivorum* (Stone and Armentrout, 1985). The wide range of host species, tissue types and plant growth stages that are parasitised by some of these pathogens, has been ascribed to the phytotoxicity of OA. The sensitivity of bean cultivars to OA was closely correlated with the severity of infection by *Sclerotinia* white mould (Tu, 1985; Tu, 1989). Mutants of *S. sclerotiorum,* which are deficient in OA production, were unable to infect *Arabidopsis* plants (Dickman and Mitra, 1992) and the deficiency could be restored by supplementing the inoculum with OA (Godoy *et al*., 1993). These studies do, however, not prove that OA acts solely and directly as a phytotoxin. It may simply be a component in a cascade of events leading to host cell death. This view is supported by other studies in which the damage induced by *Sclerotinia* could not be explained solely by the toxicity of OA (Callahan and Rowe, 1991; Rowe, 1993).

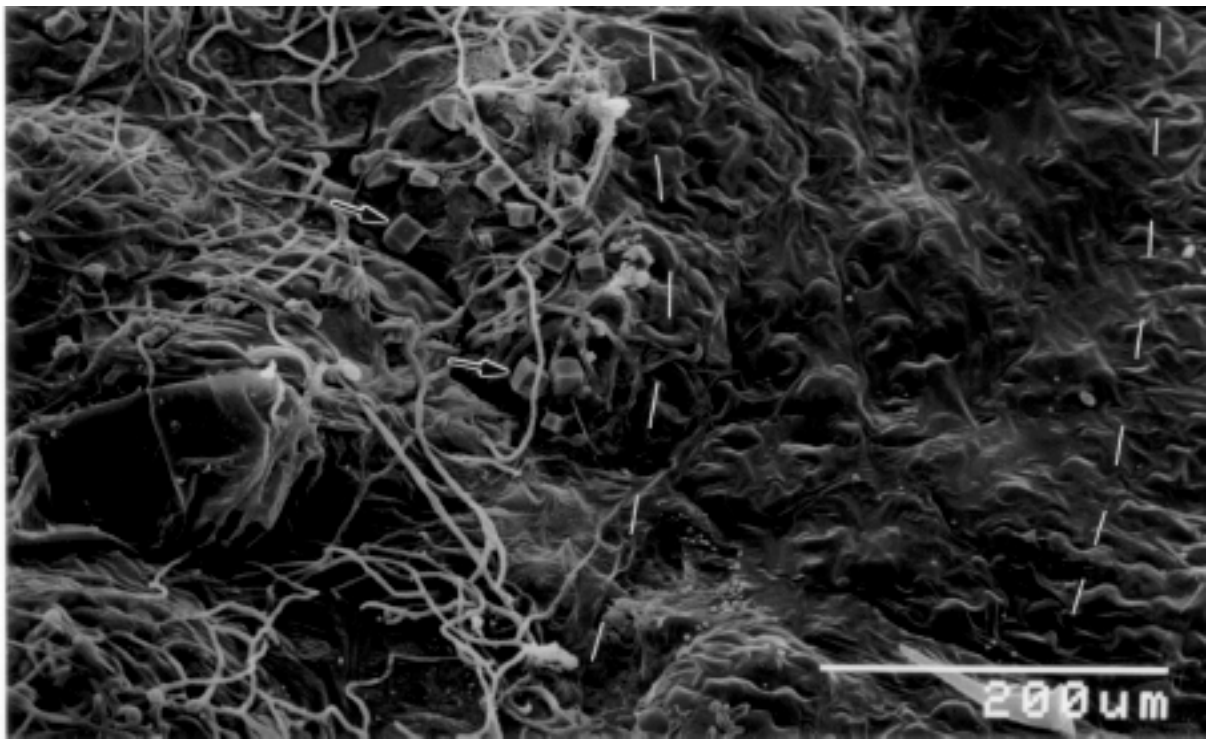

Figure 3. Formation of crystals (indicated by arrows) in tomato leaves infected by *B. cinerea*, visualised by Scanning Electron Microscopy. The white dashed line in the centre of the picture indicates the border of the lesion between the external (ectotrophic) mycelium surrounded by crystals (on the left-hand side), and a concentric zone of collapsed epidermal cells (on the right-hand side). Both leaf zones are colonised by mycelium, growing below the epidermis. The white dashed line at the right-hand side of the picture represents the border between this endotrophically colonised area and the non-invaded leaf tissue.

B. cinerea produces OA both *in vitro* (Gentile, 1954; Schroeder, 1972; Donèche *et al*., 1985) and *in planta* (Verhoeff *et al*., 1988). The sizes of lesions induced by several strains of *B. cinerea* on grapevine and bean leaves strongly correlated with the amount of OA secreted *in vitro* by these strains (Germeier *et al*., 1994). However, in a time course study with

inoculated bean leaf discs the amounts of OA, secreted at 14 hours post inoculation, when the first host cells collapsed, were insufficient to justify the tissue damage observed (Tiedemann *et al*., unpublished). It is thus likely that OA is an important co-determinant of pathogenicity but not the primary phytotoxic agent. In fact, the role of OA might be in a synergistic action with endoPGs during macerating plant tissue, as has been described in various plant pathogens (Bateman and Beer, 1965; Amadioha, 1993; Punja *et al*., 1985; Stone and Armentrout, 1985). Many fungal endoPGs have an activity optimum at low pH (Rombouts and Pilnik, 1980) and are therefore stimulated by the simultaneous secretion of OA. In addition, OA is believed to enhance the pectin degradation resulting from endoPG activity, by sequestering the Ca^{2+} ions from (intact or partially hydrolysed) Ca-pectates in the cell walls. The resulting calcium oxalate complex is insoluble and crystallises in the infected leaf tissue (Figure 3). 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 was described by Mansfield and Richardson (1981).

Induction of active oxygen species

Evidence that Active Oxygen Species (AOS) are involved in the attack by a pathogen, rather then in triggering plant defence, has been presented for several fungi, such as *Drechslera siccans, D. avenae* (Gönner and Schlösser, 1993), *Phytophthora infestans* (Jordan and DeVay, 1990) and *Cercospora beticola* (Daub and Hangarter, 1983). Studies on AOS production in relation to *B. cinerea* pathogenicity are more recent. Various antioxidants were found to reduce grey mould disease development (Elad, 1992; Tiedemann, 1997). In a screening of a number of bean (*Phaseolus vulgaris*) genotypes, a correlation was found between the sensitivity of a particular genotype to oxidative stress and its susceptibility to *B. cinerea* (Tiedemann, 1997). Toxic AOS were detected *in situ* in bean leaf tissue infected with an aggressive strain of *B. cinerea,* and their appearance coincided with the occurrence of host tissue damage (Tiedemann, 1997). Transgenic tobacco plants, expressing an alfalfa ferritin, were more tolerant to oxidative reagents such as paraquat, and they were more resistant to infection by *B. cinerea* than the non-transformed control (Deák *et al*., 1999).

One of the AOS produced at the host-fungal interface is hydrogen peroxide (H_2O_2, see Figure 4). The level of H_2O_2, released from bean leaf discs inoculated with different *B. cinerea* isolates, correlated with the aggressiveness of the isolate on such leaf tissue (Tiedemann, 1997). In *B. cinerea*-infected *Arabidopsis thaliana* leaves, H_2O_2 was detected in the apoplastic space as much as 5-10 cell layers away from the fungal hyphae (Govrin and Levine, 2000).

The mechanism by which *B. cinerea* induces (presumably toxic levels of) AOS in its host is still unclear. Fungal sugar oxidases might provide the source (Edlich *et al*., 1989). It was proposed that the oxidative burst is a consequence of lipid oxidation by the invading fungus (Weigend and Lyr, 1996). Exogenous application of glucose oxidase or H_2O_2, released during glucose oxidation, mimicked this phenomenon. The conclusion of Weigend and Lyr (1996) that a fungal glucose oxidase is responsible for the H_2O_2 accumulation at the host-fungus interface should be validated by molecular-genetic studies. Liu *et al.* (1998) purified and characterised a (presumably intracellular) glucose oxidase from mycelium of *B. cinerea.* More recently, a gene was cloned that encodes a different glucose oxidase, which contains a signal peptide and is therefore probably extracellular (Liu and Tudzynski, unpublished results). This gene will be used for gene replacement studies in order to study the role of extracellular glucose oxidase in pathogenicity.

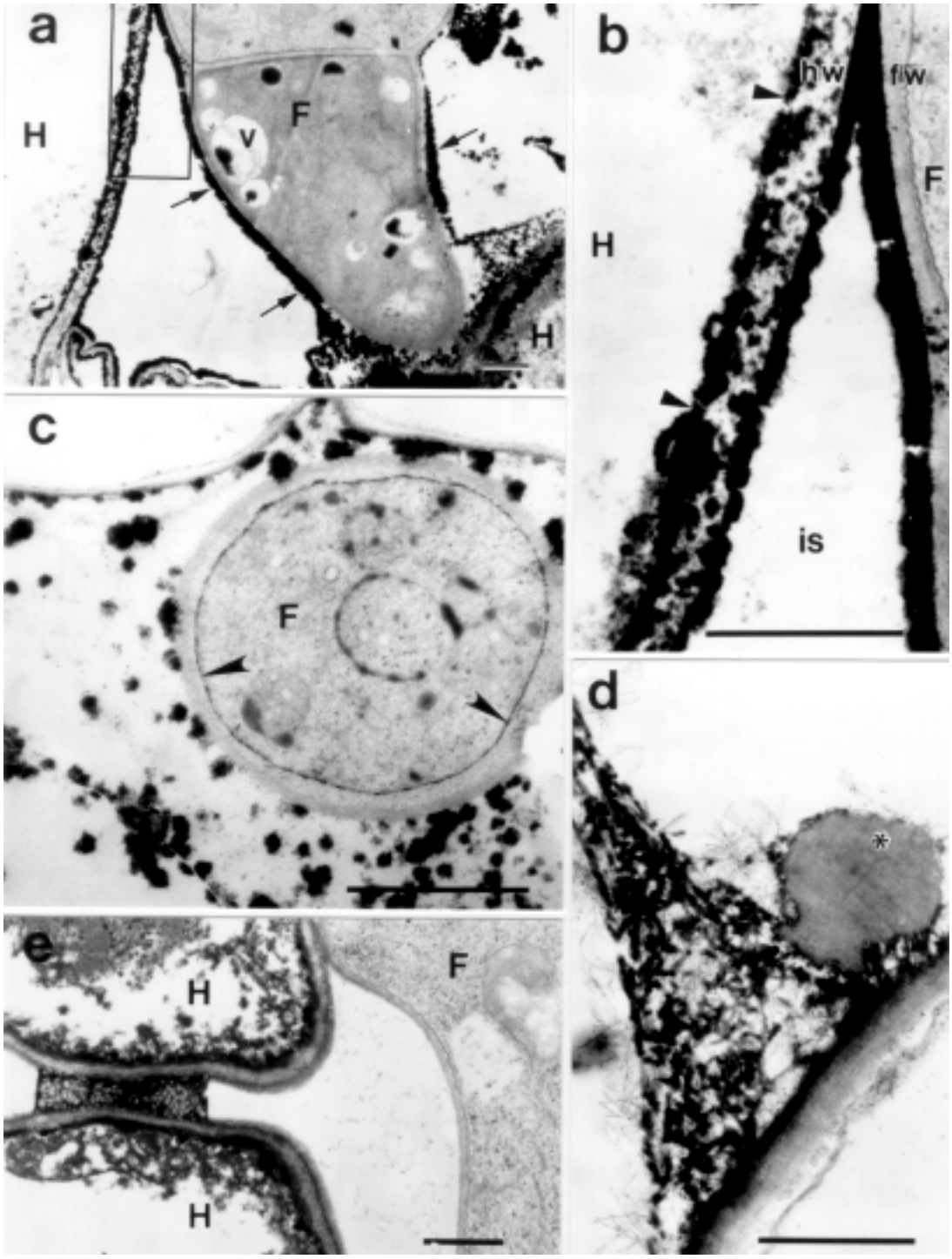

Figure 4. Localisation of H_2O_2 in infected tomato leaf with cerium chloride (a-d) and visualisation by Transmission EM. An electron-dense precipitate of cerium perhydroxide (indicated by arrows) is formed in the presence of H_2O_2. (a), overview picture of the interface between host cells (H) and *B. cinerea* hyphae (F). A fungal vacuole is indicated by (v). (b), close-up of a section of (a), H_2O_2 is produced in the host cell (H) at the plasma membrane and diffuses through the host cell wall (hw) into the intercellular space (is). Electron-dense precipitate is observed in the periplasmic space (indicated by arrow-heads) and at the outside of the host cell wall (hw), as well as at the outside of the fungal wall (fw). The host cell is electron translucent and appears to be dead. (c), H_2O_2 production in hyphae. Electron-dense precipitate is observed inside a fungal cell that appears vital. (d), control, illustrating H_2O_2 production in lignifying xylem tissue. The asterisk indicates a cell wall thickening. (e), control of infected tissue that was not treated with cerium chloride. Panels a, b, c and e were stained 48 h.p.i., panel d was stained 24 h.p.i.

Besides the production of AOS by fungal enzymes, it is probable that also the host contributes to a large extent to the generation of AOS, because of their extremely short half-life time. A prime source for generation of AOS is the plant 'oxidative burst' system, which consists of a plasma membrane-bound NADPH oxidase, inducible by fungal elicitors and requiring extracellular Ca^{2+} in the range of 1.5 mM (Schwacke and Hager, 1992). Infiltration of the specific NADPH oxidase inhibitor DPI into leaves of *Arabidopsis thaliana* prior to inoculation with *B. cinerea* resulted in a reduction of AOS production and a slower colonisation of host tissue by the fungus (Govrin and Levine, 2000). Oligogalacturonides released from the plant cell wall by pectinases of *B. cinerea* are potential elicitors of an oxidative burst (Legendre *et al*., 1993). This was demonstrated earlier in cotton leaf tissue, generating H_2O_2 in response to treatment with endoPG, in the presence of divalent cations (Mussell, 1973). Furthermore, OA may serve as a precursor of AOS in different ways, either through its enzymatic degradation by oxalate oxidase or by the chelation of ferric iron (Dutton and Evans, 1996). Although it is well established that oxidative damage is evoked by *B. cinerea* in the initial stages of attack, it remains to be clarified which factors determine the induction, generation and regulation of its phytotoxic components and how crucial this process is for penetration and colonisation.

Formation of primary lesions, defence responses in the host

One potent defence mechanism of plants against invasion by pathogens is the deposition of lignin, occurring during hypersensitive response. A more limited response involves the deposition of callose at the site of penetration. Brett and Waldron (1996) proposed that disturbance of the plant plasma membrane, which maintains a calcium gradient across the membrane, results in a net calcium influx, which in turn triggers callose deposition by the calcium-dependent β(1-3) glucan synthase complex. Both lignin and callose formation may put up a barricade that prevents further penetration of the pathogen into the protoplast.

The host surface penetration and the subsequent rupture of the plant cell walls by cell wall degrading enzymes (CWDEs) of *B. cinerea* triggers a cascade of processes in the fungus as well as the host. This paragraph deals with molecules, generated at the host-fungus interface, which trigger plant defence responses and with the impact of such responses on the progress of the infection.

Elicitors

Among the plant cell wall breakdown products, cutin monomers may act as endogenous elicitors. Dihydroxypalmitic acid, the major component of potato cutin, is the active monomer that stimulates ethylene production, alkalisation, membrane depolarisation, and transcriptional activation of defence-related genes (Schweizer *et al*., 1996). Such elicitors may be released by the action of the 18 kDa cutinase discussed in paragraph ‘Penetration of the host surface’. Fragments of host-derived pectic polysaccharides released by fungal pectinases may act as elicitors of different defence responses (Legendre *et al*., 1993; Brett and Waldron, 1996).

In addition it was shown that several components released from fungal hyphae may serve as exogenous elicitors of defence responses in *in vitro* assays with cell suspensions like ergosterol (Granado *et al*., 1995), chitin fragments (Felix *et al*., 1993) and glycopeptides (Basse and Boller, 1992; Fath and Boller, 1996). The host may respond by transient alkalisation and membrane depolarisation, coinciding with an oxidative burst.

Induction of necrosis

The initial establishment of primary necrotic lesions coincides with (and is in fact the result of) host defence activation in the neighbouring tissue in response to the death of an invaded cell. It is as yet unclear whether cell death caused by a necrotroph, such as *B. cinerea*, is equivalent to cell death during a hypersensitive response (HR) to a biotrophic pathogen (reviewed by Lamb and Dixon, 1997). Recent studies by Govrin and Levine (unpublished results) have shown that an oxidative burst occurs in plant tissue several cell layers away from the fungal hyphae. Cytological staining provided evidence for rapid nuclear condensation and irreversible membrane damage, indicative of a programmed cell death process (Govrin and Levine, 2000).

Largely the same defence responses are activated during an infection by *B. cinerea* as during HR to avirulent races of a biotrophic pathogen: lignification (Maule and Ride, 1976; Heale and Sharman, 1977), biosynthesis of phytoalexins (*e.g.* Bennett *et al*., 1994), and PR proteins (*e.g.* Benito *et al*., 1998). The total spectrum of defence 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, the lesions enter a lag phase in which they become darker but do not expand. Only a proportion of the primary lesions is eventually able to develop into aggressive, expanding lesions that rapidly colonise the entire tissue (van den Heuvel, 1981; De Meyer and Höfte, 1997; Benito *et al.*, 1998). Even when only a small proportion of the primary lesions expands, the entire tissue can be destroyed within 24-48 hours. In the non-expanding lesions the fungus is not killed, since viable fungal mycelium could be recovered from all lesions (Benito, unpublished results).

Thus, an active defence contributes to restricting the fungus within the primary lesions. This was further emphasised by the work of Benito *et al*. (1998) who incubated inoculated tomato leaves at low temperature (4° C) and in darkness. Tomato is a rather cold-sensitive plant and its induced defence responses are limited and ineffective at 4° C. *B. cinerea*, however, is renowned for its ability to infect plant tissues, even when they are stored cold. Under these conditions the plant serves as a living albeit rather inert substrate, whereas *B. cinerea* can readily grow and has an advantage over the host. The primary penetration sites become water-soaked instead of necrotic, and all primary lesions develop into expanding lesions, often at fairly even growth rate (Benito *et al*., 1998; ten Have *et al*., 1998).

Systemic acquired resistance

It has recently been reported that an effective defence to *B. cinerea* may be pre-activated by a mechanism resembling Systemic Acquired Resistance (SAR, reviewed by Ryals *et al*., 1996). De Meyer and Höfte (1997) demonstrated that bacterization of bean roots with a *Pseudomonas aeruginosa* strain resulted in a reduced number of expanding *B. cinerea* lesions on the leaves. The production of salicylic acid by the bacterium was required for triggering the SAR to *B. cinerea* (De Meyer and Höfte, 1997). Transgenic plants expressing a bacterial salicylate hydroxylase gene (NahG, Gaffney *et al*., 1993) may be used to validate whether lesion restriction is mediated by salicylate signalling, providing further evidence for the importance of SAR in restricting *B. cinerea* lesion expansion. The potent grey mould biocontrol agent *Trichoderma harzianum* T39 (Elad *et al*., 1994; O'Neill *et al*., 1996) appears to act in part by conferring SAR (De Meyer *et al*., 1998).

More recent studies have indicated that effective lesion restriction occurs in non-induced wild type *Arabidopsis thaliana* ecotypes. The ability to restrict *B. cinerea* lesion outgrowth was abolished in mutant *A. thaliana* genotypes carrying the *coi*1 mutation (deficient in the

jasmonate signalling pathway; Thomma *et al*., 1998) and the *lsd*1 mutation (involved in HR in gene-for-gene interactions; Govrin and Levine, unpublished results).

Even when lesion restriction occurs, the defence responses are eventually unable to stop *B. cinerea* colonisation completely. As a necrotroph, *B. cinerea* is well adapted to surviving in a hostile environment in which toxic plant metabolites and antifungal proteins accumulate. Various screenings were performed *in vitro* to identify plant proteins that are capable of reducing growth of *B. cinerea.* Many plant defence proteins appeared to be potent growth inhibitors of *B. cinerea* like chitinase, β-1,3-glucanase, and a class of low molecular weight proteins known as defensins (*e.g*. Broekaert *et al*., 1989; Terras *et al*., 1993; Chung *et al*., 1997; Tabei *et al*., 1997; Salzmann *et al*., 1998). Several of the corresponding genes have been or will be used in molecular resistance breeding, but no major success has yet been reported.

Quiescence

In non-green tissues, *B. cinerea* often causes quiescent infections (reviewed by Prusky, 1996), in which no symptoms are discernible at first. Most prominent examples are soft fruits such as strawberry, raspberry and grape. In these hosts, *B. cinerea* frequently infects during the flowering stage and resides in the developing fruit tissue. No fungal growth occurs and the mycelium degenerates into fragments of irregular shaped cells. Fungal growth only resumes at the onset of fruit ripening. It has often been considered that high levels of fungitoxic or fungistatic compounds in immature fruits contribute to grey mould quiescence, since the level of these compounds decreases during the ripening process. Therefore, attempts have been undertaken to increase the levels of compounds of interest, or to prevent their degradation during ripening. The level of the stilbene phytoalexin resveratrol in grapes was shown to be correlated with grey mould resistance (Langcake, 1981; Bavaresco *et al*., 1997). The effect of over-expressing stilbene synthase genes from *Vitis* in transgenic plants on the level of resistance towards *B. cinerea* was evaluated. A significant, partial resistance was obtained in tobacco (Hain *et al*., 1993) but not in tomato (Thomzik *et al*., 1997).

Besides phytoalexins, fruits usually contain high levels of proteinaceous inhibitors of fungal cell wall degrading enzymes, the PolyGalacturonase Inhibiting Proteins (PGIPs) and their level decreases during ripening. In view of this correlation and the significant role that polygalacturonases play in the infection (see paragraph ‘Disease expansion and tissue maceration’), efforts to produce transgenic plants overexpressing PGIPs have been undertaken to obtain resistance towards *B. cinerea* (Graham *et al*., 1996). This strategy has thus far met with limited success if any. One of the problems in this strategy is that PGIPs have a differential activity towards individual fungal endoPGs (Desiderio *et al*., 1997). This makes it relevant to utilise PGIPs that are most potent against the *B. cinerea* endoPG isozymes that are important in virulence (see paragraph ‘Disease expansion and tissue maceration’).

Evasion of chemical defence

Plant pathogenic fungi have developed various mechanisms to overcome the deleterious effect of preformed (phytoanticipins) or induced (phytoalexins) chemical defence agents. The major strategy is an enzymatic detoxification of these compounds (reviewed by VanEtten *et al*., 1995; Osbourn, 1996; Osbourn *et al*., 1998). Although the ability to detoxify these chemicals is widespread among phytopathogens, an essential role for the successful

colonisation of a host has so far only been proven in a few cases (reviewed by Tudzynski and Tudzynski, 1998).

Because of its broad host range, *B. cinerea* encounters a wide spectrum of antimicrobial compounds synthesised by the various host plants. The ability of *B. cinerea* to degrade or detoxify phytoalexins was intensively studied already over two decades ago (see review of the "older" literature by Mansfield, 1980). In some cases this ability obviously contributed to the virulence, *e.g.* in *Capsicum frutescens* (capsidiol, Stoessl *et al*., 1972) and *Phaseolus vulgaris* (phaseollin, van den Heuvel, 1976). *B. cinerea* is also able to degrade the phytoalexins rishitin, medicarpin and maackiain, but this ability did not directly influence its ability to infect the respective host plants (Mansfield, 1980).

The best studied example for phytoalexin detoxification by *B. cinerea* that correlates with virulence, is the detoxification of the *Vitis* phytoalexins pterostilbene and resveratrol. The ability of fungal isolates to detoxify these phytoalexins was correlated to their virulence (Sbaghi *et al*., 1996). Conversely, the resistance level of *Vitis* genotypes against grey mould is correlated to their phytoalexin content (Langcake, 1981; Jeandet *et al*., 1992). *B. cinerea* produces a substrate-specific laccase (stilbene oxidase) that is able to oxidise both compounds to non-toxic derivatives (Pezet *et al*., 1991). The enzyme has been purified and characterised (Pezet, 1998) and the mechanism of stilbene degradation has been elucidated in detail (Breuil *et al*., 1998). The elicitation of stilbene biosynthesis in *Vitis* plants by a soil bacterium can be used as a biological control mechanism against *B. cinerea* infection of grape (Bernard *et al*., 1998). Thus, in spite of the capability of *B. cinerea* isolates to degrade the *Vitis* phytoalexins, these compounds remain effective chemical defence compounds. The exact role of stilbene oxidase in pathogenicity of *B. cinerea* remains to be validated by a molecular-genetic approach, by cloning and deletion of the stilbene oxidase gene.

B. cinerea is also able to detoxify preformed antimicrobial compounds. Verhoeff and Liem (1975) showed that *B. cinerea* can degrade the tomato saponin α-tomatine, and suggested that this ability is correlated with the resistance against this phytoanticipin and with latency on tomato. Recently, Quidde *et al*. (1998) analysed the degradation of α-tomatine by a field isolate of *B. cinerea* in detail. They showed that the saponin is not completely deglycosylated (as suggested by Verhoeff and Liem, 1975), but that only the terminal xylose is removed, yielding β_1-tomatine. β_1-tomatine appeared to be far less toxic than α-tomatine, confirming that this deglycosylation step represents a detoxification. The corresponding enzyme, a "tomatinase", was purified and characterised biochemically. A field survey showed that most *B. cinerea* isolates tested (from various host plants and geographic origin) possessed tomatinase activity, with only one exception (Quidde *et al*., 1998). Interestingly, the strain lacking tomatinase activity was highly sensitive to α-tomatine, completely non-pathogenic on tomato, but highly aggressive on *Phaseolus*, strongly suggesting that the ability to detoxify α-tomatine is correlated with the virulence of *B. cinerea* on tomato. However, this strain might have further defects that cause the specific loss of virulence on tomato; a disruption of the tomatinase gene will be necessary for a final proof.

B. cinerea is also able to detoxify saponins other than α-tomatine. Quidde *et al*. (1999) demonstrated that the fungus can deglycosylate digitonin, avenacin, and avenacosides. They purified an "avenacinase" and showed that it is highly specific to avenacin and has no side activity, *e.g.* against avenacosides. Deletion of a putative "saponinase" gene (*sap1*) led to loss of avenacinase activity only. The role of an "avenacinase" for virulence of *B. cinerea* is open, since avenacin has so far only been detected in *Avena* roots, which are not infected by *B. cinerea.* Probably comparable saponins occur in other plants that are potential hosts for the fungus. Taken together, the available data demonstrate that *B. cinerea* synthesises at least three different saponinases: two glucosidases (avenacinase/avenacosidase) and a xylosidase (tomatinase, digitoninase). Since such analyses are limited by the availability of substrates, it

is very likely that *B. cinerea* possesses an even larger set of enzymes for the effective detoxification of phytoanticipins.

In addition to substrate-specific detoxifying enzymes, a less specific enzyme might be involved in counteracting the effect of plant defence compounds that inhibit the growth of *B. cinerea*, namely glutathione S-transferase (GST). This enzyme has been studied especially in mammals and in plants in the past few years (Marrs, 1996). GST is able to conjugate glutathione to toxic compounds that accumulate in the cytoplasm. The resulting conjugate is subsequently transported to the vacuole or secreted. Many organisms contain a set of GST isozymes, which accept a variety of toxic substrates, including xenobiotics, phenolics, heavy metals and H_2O_2. A GST gene was cloned from *B. cinerea* (Prins *et al*., 2000: Chapter 4), which is present in a single copy in the genome. A targeted gene disruption was performed and the resulting mutant was tested for a possible loss of virulence on tomato leaves. The mutant was still pathogenic, indicating that the GST gene is not essential for infecting this host tissue (Prins *et al*., 2000: Chapter 4). In view of the wide substrate range of GSTs, it is difficult to predict against which toxic compounds the *B. cinerea* GST is supposedly conferring protection, and whether this has consequences for the infection on particular host plants.

Besides detoxifying antifungal chemicals that accumulate in plant tissue, a second mechanism has been proposed for fungi to overcome the growth inhibiting effects of these compounds. Energy-dependent secretion by ABC-transporters was postulated to provide plant pathogenic fungi with a tool to prevent the fungistatic or fungitoxic effects of such compounds (de Waard, 1997). ABC transporters have been cloned from *B. cinerea* and their role in resistance towards plant defence compounds and virulence on various hosts is being studied (Schoonbeek *et al.*, 2001). Some ABC-transporters are located in the vacuolar membrane and are involved in pumping complexes of glutathione and toxic compounds, generated by GST, into the vacuole (Ishikawa *et al*., 1997).

The oxidative burst that occurs at the host-pathogen interface (see Figure 4 and paragraphs ‘Killing the host’ and ‘Formation of primary lesions, defence responses in the host’) imposes an oxidative stress on the host as well as the pathogen. During the infection on tomato leaves, the host catalase mRNA level increased from the moment of necrotic lesion appearance (van der Vlugt-Bergmans *et al*., 1997b). The pathogen needs to be able to cope with external oxidative stress in order to survive in the necrotic tissue. Successful detoxification of extracellular AOS by *B. cinerea* was indicated by the observation that an intracellular catalase of *B. cinerea* is not expressed *in planta* in necrotic tissue, in spite of the fact that it can be induced *in vitro* in liquid culture by exogenously applied hydrogen peroxide (van der Vlugt-Bergmans *et al*., 1997b). The removal of potentially toxic external AOS by *B. cinerea* is likely to be mediated by extracellular enzymes, such as Superoxide Dismutase (SOD) and catalase. Genes encoding SOD (Quidde, Weltring and Tudzynski, unpublished results) and extracellular catalase (Schouten and van Kan, unpublished results) have been cloned, and are currently characterised. If SOD and extracellular catalase are involved in self-protection of the pathogen against the oxidative stress that it imposes on the host, targeted replacement of the corresponding genes in *B. cinerea* might yield a mutant that is killed by the oxidative burst occurring within the necrotic lesion.

Taken together, the biochemical and preliminary molecular-genetic data demonstrate the enormous metabolic versatility of *B. cinerea*, enabling the fungus to overcome a wide spectrum of antifungal plant defence mechanisms. Whether this metabolic capability contributes to the broad host-range of the fungus is under investigation in various laboratories.

Disease expansion and tissue maceration

Besides needing to cope with host defence responses, as described in paragraph 'Evasion of chemical defence', *B. cinerea* must be able to macerate plant tissue and utilise it for its own growth. The initial step in expansion of primary lesions is presumably the killing of neighbouring cells by mechanisms similar to the ones described in paragraph 'Killing the host'.

In order to grow out of the primary lesion, the plant tissue must be actively degraded. First, the lignified barrier surrounding the primary lesion may need to be breached. It has not yet been studied how this process is achieved, but it is likely to involve peroxidases and other enzymes with lignin degrading capacity. It was proposed that laccase activity secreted by *B. cinerea* can interfere with plant defence responses by oxidising phenolic compounds that serve as lignin precursors or as phytoalexins (Viterbo *et al*., 1992). Moreover, some fungal laccases are capable of degrading lignin (Evans and Betts, 1991). Cucurbitacins, a group of compounds from Cucurbitaceae, inhibited the production of extracellular laccase activity by *B. cinerea* and conferred resistance to the pathogen (Bar Nun and Mayer, 1990).

Once through the lignin barrier, access to the surrounding tissue is obtained and this tissue is rapidly colonised. Cell wall degradation facilitates the entry of the pathogen and it provides nutrients for growth. For this purpose, *B. cinerea* possesses a set of cell wall degrading enzymes (CWDEs). The enzymes are produced during all stages of infection. Among the CWDEs of *B. cinerea* described by various groups are one or more pectin lyases (Movahedi and Heale, 1990b), pectin methylesterase (Reignault *et al.*, 1994), endopolygalacturonase (Johnston and Williamson, 1992), exopolygalacturonase (Johnston and Williamson, 1992) and cellulase (Barkai-Golan *et al.*, 1988).

It has been reported by several groups that treatment of plants with calcium fertiliser contributes to inhibition of grey mould development (Elad and Volpin, 1988; Wisniewski *et al.,* 1995; Chardonnet *et al.,* 1997; Klein *et al.,* 1997), although the effect may be tissue dependent. In cucumber, calcium treatment was able to reduce disease, by reduction of enzymatic pectin degradation on fruits but not on leaves (Chardonnet and Donèche, 1995). It is most likely that the protective effect of calcium is caused by the reinforcement of Ca-pectate complexes into egg-box structures, which are less accessible to CWDEs.

Recently, a number of *B. cinerea* genes have been cloned that encode CWDEs: pectin lyase (Mulder, unpublished), rhamnogalacturonan-hydrolase (Chen *et al.*, 1997) and six genes encoding endoPGs (ten Have *et al.*, 1998, Wubben *et al.*, 1999). The endoPG genes, denoted *Bcpg*1-6, constitute a well studied and, most probably, complete gene family. The expression patterns of the individual endoPG genes *in planta* depend on the host tissue that is infected, on the progress of infection, as well as on the external conditions at which infection occurs (ten Have *et al.*, 2001). Expression patterns *in planta* are in accordance with the expression data obtained in liquid cultures (Wubben *et al.*, 2000) and they can largely be explained by combinations of inducing and repressing conditions in the host tissue. *Bcpg*1 is expressed constitutively in all tissues and on all carbon sources tested. Other genes (*Bcpg*4 and 6) are induced by the end product of PG activity, galacturonic acid, while the *Bcpg*3 gene is induced at low pH and the *Bcpg*5 gene is induced by growing on apple pectin. These studies suggest that *B. cinerea* can degrade pectate under different environmental conditions. It may be argued that there is a relation between the host range and the need to possess such a large gene family. The related plant pathogenic fungus *Sclerotinia sclerotiorum* also has a substantial endoPG gene family, as well as a broad host range (Fraissinet-Tachet *et al.*, 1995). However, Southern analysis showed that homologues of all members of the *B. cinerea* endoPG gene family are present in *Botrytis* species that can only infect a single host plant species (Wubben *et al.*, 1999). This observation makes it unlikely that the mere presence of a

family of endoPG genes is sufficient to explain the broad host range of *B. cinerea*, as compared to other *Botrytis* species.

Two genes encoding endoPGs (*Bcpg*1 and *Bcpg*2), were eliminated by gene replacement and both mutants were reduced in virulence on tomato as well as on broad bean leaves (ten Have *et al*., 1998; ten Have *et al*., unpublished). The effect of the deletion was most pronounced for the *Bcpg*1 gene. This gene is expressed constitutively and could therefore be important in facilitating intercellular growth at the outer edge of the diseased tissue. Another explanation for the reduction in virulence may be purely nutritional. The absence of one enzyme will hamper the release of pectin degradation products that serve as nutrients, consequently resulting in slower growth of the fungus through the tissue. These studies clearly show that *B. cinerea* CWDEs can be involved in the invasion of host tissue surrounding the primary infected area. In various other plant pathogenic fungi, targeted deletion of CWDE genes resulted in strains with no discernible loss of virulence (Schaeffer *et al.*, 1994; Bowen *et al.*, 1995; Sposato *et al.*, 1995; Apel-Birkhold and Walton, 1996; Gao *et al.*, 1996; Görlach *et al.*, 1998; Scott-Craig *et al.*, 1998). The only other fungus in which a significant role of an endoPG in pathogenesis has been reported, is the saprophyte *Aspergillus flavus*, where endoPG deletion resulted in a reduced ability to invade cotton bolls (Shieh *et al.*, 1997).

We hypothesise that *B. cinerea* should be regarded as a "pectolytic" fungus. The host range of *B. cinerea* is confined to dicotyledons and non-graminaceous monocotyledons. All these hosts have a rather high content of pectin in the cell walls, when compared to the graminaceous non-host plants (Carpita and Gibeaut, 1993). In view of the role of endoPGs in virulence of *B. cinerea*, a suitable control strategy might be based on the use of PGIPs, which can inhibit endoPGs from *B. cinerea* (Johnston *et al.*, 1994). Therefore, PGIP genes may be used in molecular resistance breeding (Graham *et al*., 1996), taking into account the differential activity of PGIPs towards individual fungal endoPGs (Desiderio *et al*., 1997).

Phytohormone production by *B. cinerea*

Changes in the level of one individual plant hormone, or in the balance between different hormones, cause developmental changes in the plant which may result in symptoms such as stunting, overgrowth, leaf curls, and can even lead to plant death. Many of these symptoms are similar to those caused by plant pathogenic fungi. Interestingly, some plant pathogenic fungi are able to synthesise phytohormones in culture. Infected plant tissue often contains higher levels of certain phytohormones than uninfected plants (Isaac, 1992). However, it is unclear whether the hormone increase originates from the plant or from the fungus, whether pathogens influence the plant-directed hormone synthesis, or whether external application of hormones influences the disease. These knowledge gaps hamper a clear evaluation of the role of plant hormones in disease aetiology.

B. cinerea is often regarded as a pathogen that mainly attacks weakened or senescing tissues. Hence, it was logical to determine the effects of applying exogenous hormones that stimulate plant senescence or ripening. Increased susceptibility to grey mould was shown to be associated with factors that enhance ageing of the host tissues, such as abscisic acid (ABA) and ethylene (Elad, 1988; McNicol *et al*., 1989; Elad and Evensen, 1995). ABA was also found to be a strong inhibitor of the accumulation of the phytoalexins rishitin and lubimin in potato tuber. The disease development on rose flowers was promoted by application of ABA, although germination and germ-tube elongation of *B. cinerea* were unaffected by ABA (Shaul *et al*., 1996).

B. cinerea is able to produce several plant hormones (see Tudzynski, 1997). ABA is one of the naturally occurring phytohormones produced by *B. cinerea* (Marumo *et al*., 1982; Kettner and Dörffling, 1995; Wu and Shi, 1998) and other phytopathogenic fungi such as *Cercospora rosicola* (Assante *et al*., 1977), *Ceratocystis*, *Fusarium* (Dörffling and Petersen, 1984; Tuomi *et al*., 1995) and *Alternaria brassicae* (Dahiya *et al*., 1988).

Among 95 strains of *B. cinerea* tested, 39 produced ABA in axenic culture at a yield up to several mg/l (Hirai *et al*., 1986). Kettner and Dörffling (1995) determined the levels of ABA in tomato leaves infected with ABA producing or non-producing *B. cinerea* strains and concluded that at least four processes control the level of ABA: 1. Release of ABA or its precursor by the pathogen; 2. Stimulation of ABA biosynthesis in the host plant; 3. Inhibition of plant ABA metabolism; 4. Promotion of biosynthesis of fungal ABA by the host tissue.

The ABA level in tomato leaves increased after infection, both with an ABA-producing and a non-producing *B. cinerea* isolate, albeit much more in response to the ABA-producing strain (Kettner and Dörffling, 1995). This indicates that ABA favours infection, but the ability of the fungus to produce ABA is not a prerequisite for infection of tomato leaves. In studies on the interaction of ABA-producing *B. cinerea* isolates with *Salix* leaves, a positive correlation was found between the frequency of infection and the amount of ABA produced in the infected tissue (Tuomi *et al*., 1993). However, the ABA quantities detected in the tissue were too high to be secreted by the fungus, and presumably resulted mainly from increased ABA production by the plant.

Several *B. cinerea* strains isolated from infected fruits are able to produce ethylene in liquid culture. The ethylene levels are physiologically significant, suggesting that ethylene produced by *B. cinerea* itself may play a role in the interaction between host and pathogen (Qadir *et al*., 1997).

Cytokinin-like substances with cytokinin activity were detected in culture fluids of pathogenic *B. cinerea* isolates and other pathogens (Talieva and Filimonova, 1992). Since exogenous application of cytokinin causes abnormalities in plants such as tumor formation, overgrowth, galls and rusts, a possible involvement of fungal cytokinin in development of plant infection was suggested.

A systematic study of phytohormone production by *B. cinerea* and its role in pathogenicity is only in its early stages. It is difficult to determine whether the observed effects are due to growth regulators produced by the invading fungus itself, or by the plant in response to the stress evoked by the pathogen. Cloning of the biosynthetic genes of *B. cinerea* phytohormone pathways, followed by gene expression and gene replacement experiments are needed to resolve the role of hormone production by the fungus in pathogenesis.

Other *Botrytis* species and other necrotrophs

Botrytis *species with a narrow host range*

In contrast to *Botrytis cinerea* that can infect at least 235 plant species, virtually all other (± 20) pathogenic *Botrytis/Botryotinia* species have a narrow host range, which is restricted to one or a few species of the corroliferous monocotyledons, the *Ranunculaceae* or the *Leguminosae* (Jarvis, 1977). The symptoms and development of diseases caused by the other *Botrytis* species on their host are often similar to those caused by *B. cinerea*, *i.e.* primary necrotic lesions of which a proportion subsequently expands into healthy tissue, thereby destroying large parts of the plant. Most of the infection mechanisms of narrow host range *Botrytis* species are probably similar to the mechanisms described above for *B. cinerea*.

Epidemics of *B. elliptica* in lilies and *B. tulipae* in tulips can occur simultaneously in regions in The Netherlands where tulips and lilies are co-cultivated. When *B. elliptica* is inoculated onto the non-host tulip, it is able to cause primary necrotic lesions that are barely distinguishable from those caused by *B. tulipae*. The lesions of *B. elliptica*, however, do not expand on tulip whereas those of *B. tulipae* do. The reciprocal is true for *B. tulipae* on the non-host lily. *B. cinerea* frequently occurs as a secondary opportunistic pathogen on tulip and lily leaves previously invaded by either of the other *Botrytis* species.

The determinants of the limited host range of *Botrytis* species, other than *B. cinerea,* are yet unclear. Specificity is apparently not determined by recognition of the host or signal exchange needed to establish an infection. In many cases, host-specific *Botrytis* species are able to cause primary lesions on a non-host, but these primary lesions fail to expand. The decisive step whether a plant is regarded as a host or a non-host thus lies in the lesion expansion. The effective restriction of the pathogen within the primary lesion needs to be breached, by neutralising the fungitoxic and fungistatic (phenolic) metabolites that have accumulated within the lesion and by breaking through the fortified lignin barriers surrounding the lesion.

The broad host range of *B. cinerea* might thus be a consequence of its ability to neutralise defence mechanisms of a large number of host species (as discussed in the paragraph about 'Evasion of chemical defence'), whereas other *Botrytis* species are possibly only able to neutralise the defence mechanisms of one particular plant species, *i.e.* their specific host. Only very few studies have been reported on the ability of host-specific *Botrytis* species to neutralise fungitoxic compounds from their respective hosts.

As discussed in the paragraph about evasion of chemical defence, Quidde *et al.* (1998) characterised a tomatinase-deficient *B. cinerea* field isolate that was non-pathogenic on tomato, yet retained the ability to infect bean as any other isolate. This suggests that host specialisation or preference may occur even within the *B. cinerea* population. There is recent evidence that genetically isolated, sexually reproducing, sympatric populations (denominated *transposa* and *vacuma*) co-exist within the species *Botryotinia fuckeliana* in the Champagne region (Giraud *et al*., 1997). One of the populations appeared to be more stable over time than the other and it was proposed that this represented a resident population, which was better adapted to the host plant(s) in the area (Giraud *et al*., 1997). It will be highly interesting to investigate whether separate fungal populations have a preference for certain (single or groups of) host plant species. If such subgroups are indeed specifically adapted to subsets of host plants, the statement that *B. cinerea* is able to infect at least 235 host species (Jarvis, 1977) may represent an oversimplification.

Sclerotinia sclerotiorum

Sclerotinia sclerotiorum is a wide host range pathogen with many similarities to *B. cinerea*. Both fungi belong to the family Sclerotiniaceae. The major difference is that *S. sclerotiorum* ("white mould") does not normally produce asexual spores. In the field it usually infects the base of the stem by ascospores released from apothecia that develop in the top layer of the soil in the spring. There are probably many things in common among the infection mechanism utilised by the two pathogens. Alike *B. cinerea* (see paragraph 'Disease expansion and tissue maceration'), *S. sclerotiorum* secretes a spectrum of CWDEs with differential regulation patterns (Fraissinet-Tachet *et al*., 1995; Fraissinet-Tachet and Fèvre, 1996; Martel *et al*., 1996). Whether these enzymes play a role in the infection process could thus far not be validated, since gene replacement has not yet been achieved in *S. sclerotiorum*.

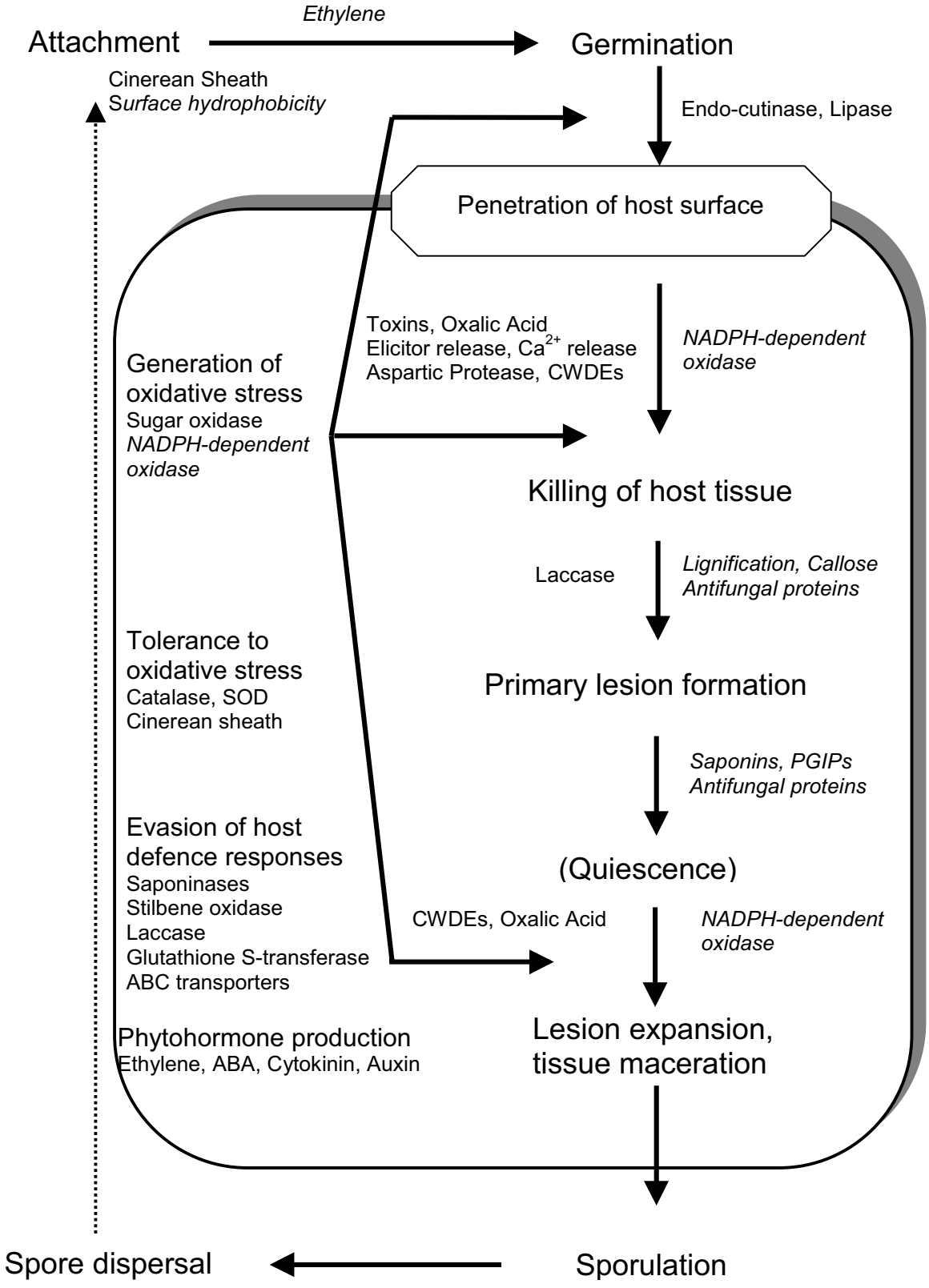

Figure 5. Schematic model of the involvement of various enzymes and metabolites in different stages of the infection process of *B. cinerea*. The shaded box represents the host tissue. Compounds produced by the pathogen are indicated in normal font, compounds originating from the host plant are indicated in italics.

Mutants of *S. sclerotiorum* that are deficient in producing oxalic acid (OA) are significantly less virulent than the wild type and the loss of virulence can be restored by adding exogenous OA in the inoculum (Godoy *et al*., 1993). The genes responsible for the OA deficiency in *S. sclerotiorum* have not been identified. It is likely that OA has an analogous role in pathogenesis of *B. cinerea* (see paragraph 'Killing the host'), but final validation of this hypothesis should come from the construction of an OA-deficient *B. cinerea* mutant.

Summary and concluding remarks

Tools have come available to start validating the numerous microscopic and biochemical observations that have been made on the infection strategies of *B. cinerea*. Figure 5 recapitulates our current view on the separate stages of the infection process and summarises the involvement of various fungal enzymes and metabolites in these stages, as discussed in this chapter. Some of the corresponding genes have been analysed in recent years, others will soon follow.

The major challenge is in integrating the various factors in a comprehensive scheme of events. Such knowledge will be useful for developing new, rational disease control strategies.

Botrytis cinerea is a complex pathogen that can probably not be controlled by simple measures. This review demonstrates that the pathogen is versatile and uses a combination of factors during pathogenesis. In spite of the fact that necrotrophs appear to avoid the (usually subtle) interactions with their host that are typical for biotrophs, there seems to be a delicate balance between the attack mechanisms of the fungus and the defence of the host. Rather than combating the fungus vigorously by one single approach, it should be attempted to alter this balance in favour of the plant. Such a strategy will likely consist of a sophisticated combination of biological control agents, appropriate (partially) resistant plant genotypes and chemicals that either enhance the plant defence response or interfere with crucial steps in the infection process.

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CHAPTER 2

Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction

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Abstract

Establishment of a plant-pathogen interaction involves differential gene expression in both organisms. In order to isolate *Botrytis cinerea* genes whose expression is induced during its interaction with tomato, a comparative analysis of the expression pattern of the fungus *in planta* with its expression pattern during *in vitro* culture was performed by differential display of mRNA (DDRT-PCR). Discrimination of fungal genes induced *in planta* from plant defence genes induced in response to the pathogen was attempted by including in this comparative analysis the expression patterns of healthy tomato leaves and of tomato leaves infected with two different pathogens, either *Phytophthora infestans* or tobacco necrosis virus (TNV). Using a limited set of primer combinations, three *B. cinerea c*DNA fragments, ddB-2, ddB-5 and ddB-47, were isolated representing fungal genes whose expression is enhanced *in planta.* Northern blot analysis showed that the transcripts detected with the cDNA clones ddB-2 and ddB-5 accumulated at detectable levels only at late time points during the interaction. The cDNA clone ddB-47 detected two different sizes of transcripts displaying distinct, transient expression patterns during the interaction. Sequence analysis and database searches revealed no significant homology to any known sequence. These results show that the differential display procedure possesses enough sensitivity to be applied to the detection of fungal genes induced during a plant-pathogen interaction. Additionally, four cDNA fragments were isolated representing tomato genes induced in response to the infection caused by *B. cinerea,* but not by *P. infestans.*

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The nucleotide sequence data reported are in the EMBL and GENBANK Nucleotide Sequence Databases under the accession numbers Z70214 (ddB-2), Z70215 (ddB-47), Z70217 (ddB-5) and Z70216 (ddT-19).

Introduction

Botrytis cinerea Pers.: Fr. is an important fungal pathogen with a wide host range. It causes important economic losses because of pre- and post-harvest diseases in different agricultural crops (Jarvis, 1977). Extensive biochemical and physiological research on the infection processes has been performed. Most of the studies have dealt with different fungal isolates and different host plants, making it difficult to determine the general relevance of specific aspects studied in each system. As a necrotrophic pathogen it colonises preferentially dead or senescent plant tissues from which it spreads into healthy tissues (Mansfield, 1980). Wounds can serve as entry sites (Fermaud and Menn, 1989; Wilson, 1963) but direct penetration of healthy plant tissues by infection pegs differentiated from appressoria or from the tips of germtubes also occurs frequently (McClellan and Hewitt, 1973; Salinas *et al*., 1989). Research on the fungal processes involved in pathogenesis has focused on extracellular enzymes with a presumed role in the infection process, such as a cutinase (Salinas, 1992), an aspartic protease (Movahedi and Heale, 1990) and a large number of cell wall-degrading enzymes (Johnston and Williamson, 1992; Pieterse *et al*., 1993). Moreover, *B. cinerea* has been reported to produce substances toxic to plant cells (Jarvis, 1977) and it is able to detoxify antifungal plant compounds (Mansfield, 1980). Thus far, no unequivocal evidence that any of these processes is essential for pathogenesis has been obtained. Establishment of the infection results from complex interactions between host and pathogen, involving processes in the fungus (activation of mechanisms for penetration and colonisation of the host tissue) and in the plant (activation of the host defence responses). In both organisms altered gene expression occurs from the onset of the attempted fungal invasion onwards. Among the fungal genes whose expression is specifically induced or increased during the interaction with its host, one would expect to identify factors essential for pathogenesis.

The analysis of differential gene expression has thus far largely relied on hybridisation procedures (Sargent, 1987). In the analysis of plant-fungal pathogen interactions these procedures have been utilised successfully to isolate infection structure-specific genes (Bhairi *et al*., 1989; Hwang and Kolattukudy, 1995) and genes specifically induced during pathogenesis (Pieterse *et al*., 1993; Talbot *et al*., 1993). These procedures are laborious and time-consuming and their sensitivity is limited, only allowing the detection of relatively abundant mRNAs. Recently, a new procedure was described for differential gene expression analysis, differential display of mRNA (Liang and Pardee, 1992). This method has several advantages as compared to subtractive and differential hybridisation procedures: it is quicker and, since it is PCR-based, its sensitivity is higher (Liang and Pardee, 1992). Since the first report, several modifications and optimisations have been introduced and the technique has been applied to the analysis of very different biological systems (reviewed by Liang and Pardee, 1995). Because of its higher sensitivity this method could be particularly useful for the analysis of systems in which the amount of biological material is limiting (Zimmermann and Schultz, 1994). This becomes relevant when one intends to study the differential gene expression of a fungal pathogen *in planta* during early stages of the infection process.

Here we report the application of DDRT-PCR to the analysis of fungal gene expression *in planta* in the interaction *Lycopersicon esculentum-B. cinerea.* The comparative gene expression analysis performed has yielded several fungal cDNAs representing genes whose expression is enhanced *in planta.*

Results

Sensitivity of DDRT-PCR

First a standard inoculation procedure of *B. cinerea* on detached tomato leaves was established to obtain highly efficient and synchronised infections. Under the experimental conditions used, the first symptoms were visible 19-20 h after inoculation as small necrotic brownish spots all over the leaf surface. The size of the lesions remained unchanged during the following two days, although the lesions became darker. 72 h after inoculation a few lesions (1 % - 5 % of the total number) started to spread. From these few lesions the fungus colonised the leaf and 120 h after inoculation total leaf necrosis was observed (Benito *et al*., 1998). The attention was focussed on the fungal gene expression during the early stages of infection (penetration) and during the onset of formation of spreading lesions. Material collected 16 h after inoculation (when penetration was occurring but there was no detectable symptom yet) and 72 h after inoculation was selected for analysis. Densitometric scanning of northern blots hybridised with a *B. cinerea*-specific constitutive probe indicated that the proportion of fungal RNA at both time points was about 3-5 % of the total interaction RNA (data not shown). In order to determine if it was possible to detect by DDRT-PCR fungal mRNAs in a fungal-plant mRNA mixture, a reconstruction experiment was carried out. mRNAs from *B. cinerea in vitro* cultures and from healthy tomato leaves were reverse transcribed using anchor primer RT3. The plant cDNA and serial dilutions of the fungal cDNA were mixed and PCR was performed using these mixtures as templates and the RT3 primer in combination with RAPD primer P35 as the anchored and upstream primers, respectively.

As shown in Figure 1, most of the bands detected using cDNAs from healthy leaves (lane 1:0) and cDNAs from *B. cinerea grown in vitro (lane* 0:1) as template in PCR, were also detected in a single lane when equal amounts of both cDNAs are mixed and used as template (lane 1:1). When cDNA from *B. cinerea* grown in vitro was diluted in relation to cDNA from healthy leaves, the intensity of the bands representing *Botrytis* cDNAs decreased with increasing dilution, while the intensity of the bands representing plant cDNAs remained constant (lanes 1: 1/10 to 1: 1/100). Even at the highest dilution tested, several *Botrytis* cDNA-derived bands were still detectable. This observation indicated that the DDRT-PCR procedure shows a sensitivity high enough to analyse our experimental system: the reconstruction sample containing the 1/30 dilution would be comparable to a sample from an infected leaf in which 3 % of the interaction mRNA is *B. cinerea* mRNA.

Using the DDRT-PCR procedure in the analysis of an interaction

Since in the interaction two organisms are present, cDNAs from both fungus and plant are detected by 'differential display'. Most of these cDNAs represent fungal or plant genes that are constitutively expressed. Their origin can be discriminated by comparison of the expression pattern displayed in the interaction with the expression pattern of the fungus grown *in vitro* or the expression pattern of a non-infected tomato plant. Interaction-specific cDNAs are also detected which represent either fungal genes induced *in planta* or plant defence genes induced in response to the pathogen. These last two categories of cDNAs can be discriminated if samples from control infections with a second pathogen are included. It was assumed that all plant defence responses evoked in tomato by *B. cinerea* would also be triggered by another necrotising pathogen, such as *Phytophthora infestans.*

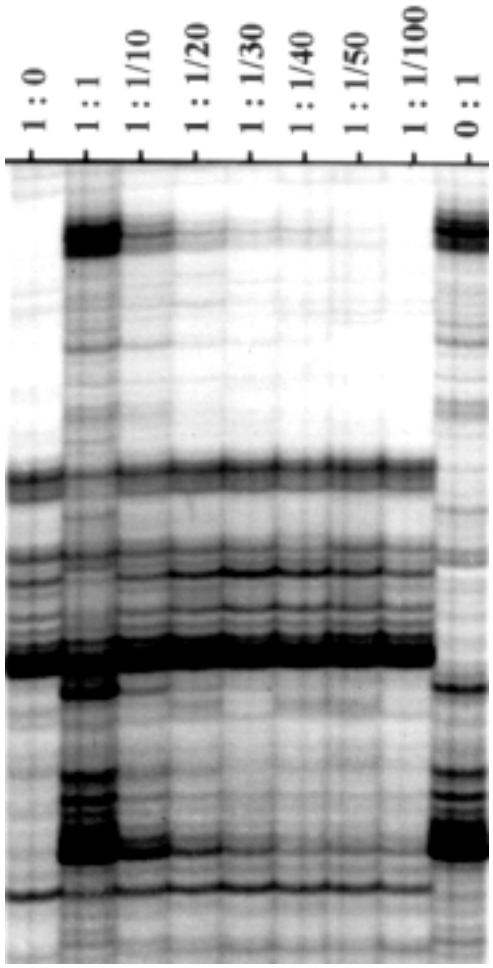

Figure 1. Reconstruction experiment performed to determine the sensitivity of the DDRT-PCR procedure. cDNAs synthesised with primer RT3 on mRNAs from healthy tomato leaves and *B. cinerea* grown *in vitro* were mixed and subjected to PCR amplification using the same anchor primer in combination with the 10-mer primer P35. Each lane shows the amplified products obtained using different proportions (ratios indicated above each lane) of tomato-*B. cinerea* cDNAs mixtures as templates.

Taking all these considerations into account a first set of experiments was carried out to determine the best conditions for the analysis of gene expression patterns during the interaction *B. cinerea*-tomato. *B. cinerea* was inoculated on tomato leaves and mRNA was extracted from infected leaves harvested 16 h after inoculation. mRNA was also purified from *in vitro* cultured *B. cinerea* mycelium, from uninfected tomato leaves and from tomato leaves with *P. infestans,* harvested 40 hours after inoculation. At this time point there was no visible symptom of *P. infestans* infection but 8-12 h later necrotic spots started appearing in the centre of water-soaked areas on the leaf under the experimental conditions used.

PCR was performed on cDNAs synthesised on these mRNA samples analysing several parameters. The best results in terms of reproducibility, optimal number of bands and resolution were achieved when reverse transcription was performed on poly(A)$^+$ RNA at 35° C, in combination with PCR at an annealing temperature of 42° C and the products were resolved in a 6 % non-denaturing polyacrylamide gel. In all experiments, duplicate reactions were included of the *B. cinerea*-tomato interaction samples (using cDNAs derived from two independent cDNA synthesis reactions) in order to reduce the number of false-positives.

Figure 2 shows a representative example of the band patterns obtained, and illustrates the theoretical basis of our approach. In addition to bands representing constitutively expressed mRNAs from *Botrytis* and from tomato, novel bands were detected in samples derived from *B. cinerea*-infected tomato leaves (indicated by arrows). Some of these bands were also detected in samples from *P. infestans*-infected tomato leaves (arrows 2 and 3) and probably represent plant defence genes induced in response to both pathogens. Other bands were apparently specific to the *B. cinerea*-tomato interaction and constituted candidates to reflect *B. cinerea* mRNAs induced *in planta* (arrow l).

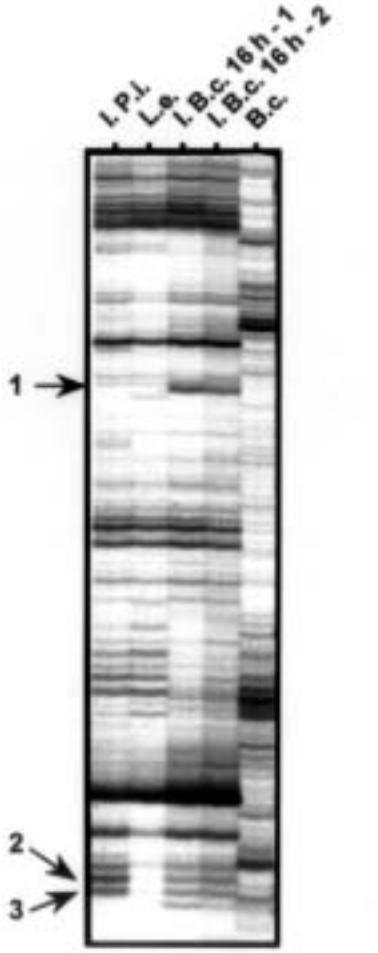

Figure 2. Area of a differential display gel showing the amplified products obtained with primers RT2 and P1 using as templates cDNAs derived from mRNAs obtained from *P. infestans*-tomato interaction 40 h after inoculation (I.P.i.), *B. cinerea*-tomato interaction (I.B.c.) 16 h after inoculation (duplicate reactions, 16 h-1 and 16 h-2), healthy tomato leaves (L.e.) and *B. cinerea* grown *in vitro* (B.c.). The arrows indicate interaction-specific bands.

A total of 104 primer combinations were tested (four anchor primes and 26 RAPD primers). Although differences in the number of bands were observed between different primer combinations, an average of 100-120 bands were detected in lanes derived from plant cDNA samples, while 70-80 bands per lane were detected in samples derived from *B. cinerea* grown *in vitro*. About 15-20 % of the latter bands were also detected in the *B. cinerea*-tomato interaction lane for most of the primer combinations.

Forty bands were apparently specific to the *B. cinerea*-tomato interaction, of which 35 were effectively recovered from the gel and reamplified. Twenty of these fragments were labelled and tested individually by hybridisation on northern blots and on total genomic DNA dotblots. No fragment hybridised specifically to *B. cinerea* genomic DNA, 15 hybridised to plant genomic DNA and 5 hybridised neither to *Botrytis* nor to plant genomic DNA. Of the fragments hybridising to plant genomic DNA, four (ddT-19, ddT-20, ddT-21 and ddT-49) could be detected on northern blots containing plant mRNAs specifically induced during the interaction with *B. cinerea* (Figure 3). Three gave complex hybridisation patterns in all the lanes containing plant RNA, three detected plant ribosomal RNAs and five detected plant mRNAs constitutively expressed.

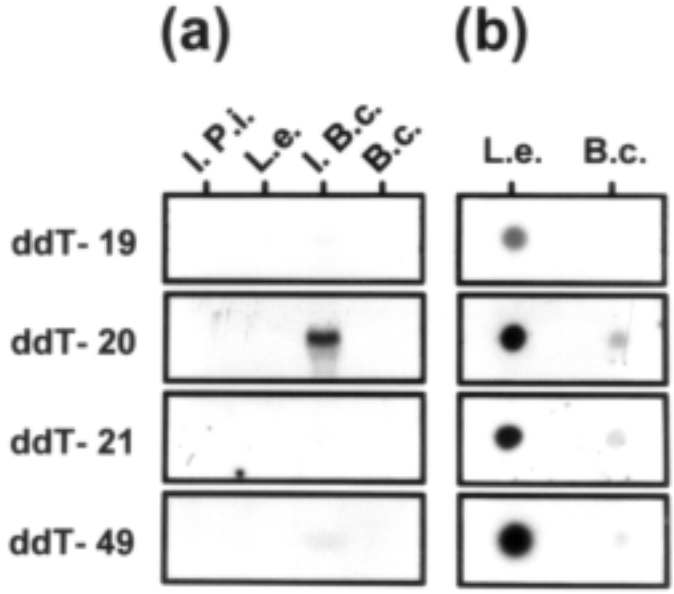

Figure 3. Northern blot (a) and dotblot (b) analysis of four differential display fragments representing plant induced mRNAs. (a) Northern blots contained 20 µg of total RNA extracted from *P. infestans* infected tomato leaves collected 40 h after inoculation (I.P.i.), healthy tomato leaves (L.e.), *B. cinerea-infected* tomato leaves collected 16 h after inoculation (I.B.c.) and *B. cinerea* grown *in vitro* (B.c.). (b) The dotblots contained 2 µg tomato (L.e.) or *B. cinerea* (B.c.) total genomic DNA.

Fragment ddT-19 was cloned and the insert derived from a single plasmid was labelled and used as a probe as before on a genomic DNA dotblot and on a northern blot, giving the same pattern obtained with the crude reamplified product. Figure 4a shows the nucleotide sequence of the cloned fragment. The 202 bp fragment includes the two primers used for the initial differential display analysis. Comparison of its deduced ammoniated sequence with the database demonstrates that fragment ddT-19 encodes part of the enzyme *trans*-cinnamate 4-monooxygenase from tomato, showing in this region a 87 % identity with the equivalent enzymes from *Phaseolus aureus* (Mizutani *et al*. 1993) and *Helianthus tuberosus* (Teutch *et al*., 1993) (Figure 4b). This enzyme catalyses the second step in the common phenylpropanoid pathway (Hahlbrock and Scheel, 1989). Transcription of the mRNA encoding this enzyme appears to be enhanced in tomato in response to the infection caused by *B. cinerea,* but not by *P. infestans,* as shown in Figure 3, at the time points considered in this study.

(a)

```
GATCAAGTCCTACACACACAAGGGGTTGAGTTTGGGTCACGTCCA   45
      V  L  H  T  Q  G  V  E  F  G  S  R  P

CGTAACGTTGTGTTCGATATATTCACGGGGAATGGACAAGACATG   90
R  N  V  V  F  D  I  F  T  G  N  G  Q  D  M

GTGTTCACAATATATGGTGATCATTGGAGAAAAATGAGACGTATC  135
V  F  T  I  Y  G  D  H  W  R  K  M  R  R  I

ATGACAGTACCATTTTTCACTAACAAAGTTGTGCACCAATATAGT  180
M  T  V  P  F  F  T  N  K  V  V  H  Q  Y  S

GATATGTGGGAAAAAAAAAAAA                         202
D  M  W  E
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(b)

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                          10        20        30
ddT-19             VLHTQGVEFGSRPRNVVFDIFTGNGQDMVF
                   ************ ********** ******
Tcmo-P   VVSSPDLAKEVLHTQGVEFGSRTRNVVFDIFTGEGQDMVF
Tcmo-H   VVSSPELAKEVLHTQGVEFGSRTRNVVFDIFTGKGQDMVF
         80        90        100       110

                  40        50        60
ddT-19   TIYGDHWRKMRRIMTVPFFTNKVVHQYSDMWE
         * ** ******************* **   **
Tcmo-P   TVYGEHWRKMRRIMTVPFFTNKVVQQYRHGWEAEAAAVVD
Tcmo-H   TVYGEHWRKMRRIMTVPFFTNKVVQQYRYGWEAEAAAVVD
         120       130       140       150
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Figure 4. (a) Nucleotide sequence of fragment ddT-19 (accession number Z70216) and its deduced amino acid sequence. The primers used for the differential display reaction are underlined. (b) Comparison of the ddT-19 fragment deduced amino acid sequence with the sequence of the enzyme *trans*-cinnamate 4-monooxygenase from *P. aureus* (Tcmo-P) (accession number P37115) and *H. tuberosus* (Tcmo-H) (accession number Q04468). Conserved residues in the three sequences are indicated by asterisks. The deduced sequence of fragment ddT-19 is numbered separately.

A second control interaction system provides higher stringency in the comparative gene expression analysis

From the results presented above it became evident that it is important to use even more appropriate control infections in the analysis. Although slower, TNV induces symptoms in tomato visually similar to those induced by *B. cinerea,* characterised by the development of small necrotic lesions 72 h after inoculation. Material from this interaction was included as a second control in the analysis.

Tomato leaves inoculated with TNV were collected 60 h after inoculation, when no symptom is yet visible. This mRNA sample was included in the differential display analysis. Furthermore, a sample of the *B. cinerea*-tomato interaction at 72 h after inoculation was included. The DDRT-PCR analysis was now performed with 52 primer combinations (two anchored primers, RT1 and RT2, and 26 RAPD primers). A total of 22 *B. cinerea*-tomato interaction-specific cDNAs were detected. Seventeen were effectively recovered from the gel and tested individually. Figure 5a shows the area of the differential display gels where fragments were detected that were further characterised in this work (indicated by arrows). In order to discriminate rapidly the origin of the reamplified fragments, each one was labelled and hybridised to a Southern blot containing *Hin*dIII digests of genomic DNA from tomato and from *B. cinerea.* Four fragments hybridised exclusively to *Botrytis* genomic DNA and

were selected for further analysis. These fragments were cloned and inserts from the individual plasmids were used to confirm the hybridisation pattern on Southern blots and then probed on a time course northern blot for *in planta* expression analysis. The originally reamplified fragments ddB-5 and ddB-47 appeared to be homogeneous while fragments ddB-2 and ddB-12 were mixtures of at least two different DNA fragments: one reproducing the hybridisation pattern obtained initially and a second one which hybridised neither with tomato nor with *Botrytis* genomic DNA. The cloned ddB-12 fragment did not give any detectable signal on the northern blot. Single mRNAs expressed at low levels were detected at late time points with the cloned ddB-2 (1.8 kb) and ddB-5 (1.3 kb) fragments. ddB-47 detected two mRNAs of 1.4 kb and 1.0 kb, both showing a high level of induction *in planta* (Figure 5c). The time course northern blot was also probed with the *B. cinerea* β-tubulin gene in order to make an estimation of the proportion of fungal RNA in the total interaction RNA at each time point. The cloned fragments ddB-2, ddB-5 and ddB-47 hybridised to single *B. cinerea* genomic DNA bands in the Southern blots (Figure 5b).

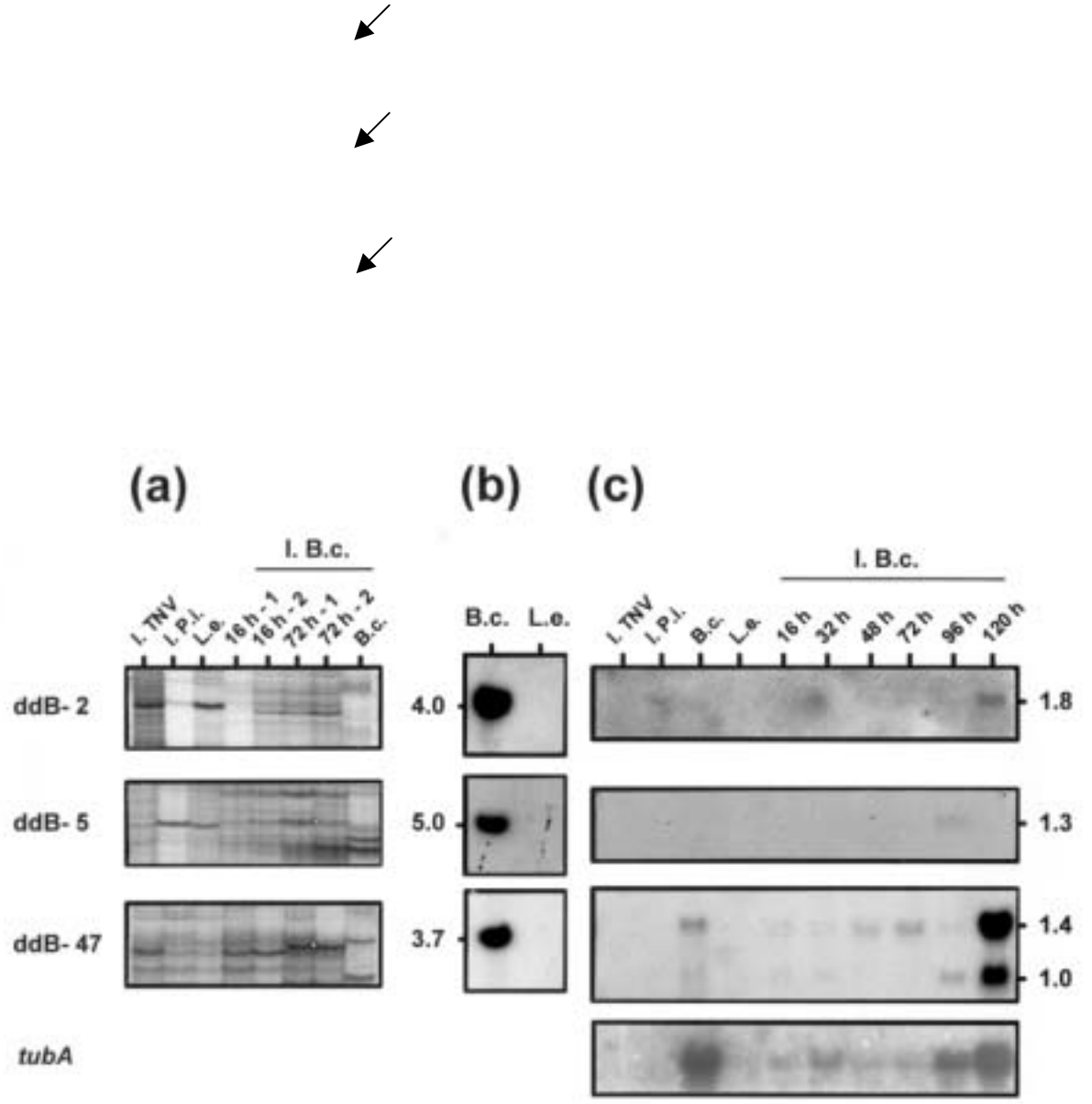

Figure 5. (a) Areas of the differential display gels where fragments ddB-2, ddB-5 and ddB-47 were detected (indicated by arrows). Each lane shows the amplified products obtained by DDRT-PCR on mRNA samples derived from: TNV-tomato interaction 60 h after inoculation (I.TNV), *P. infestans*-tomato interaction 40 h after inoculation (I.P.i.), healthy tomato leaves (L.e.), *B. cinerea*-tomato interaction (I.B.c.) 16 after inoculation (two independent reactions, 16 h-1 and 16 h-2) and 72 h after inoculation (two independent reactions, 72 h-1 and 72 h-2), an *B. cinerea* grown *in vitro* (B.c.). (b) Southern blot analysis of fragments ddB-2, ddB-5 and ddB-47. Each blot contained 5 μg of *B. cinerea* (B.c.) or tomato (L.e.) genomic DNA digested with *Hin*dIII. (c) Expression analysis performed with fragments ddB-2, ddB-5 and ddB-47 by northern blot hybridisation. Each lane contained 20 μg of total RNA. Codes for the samples are as indicated in (a). Analysis of the *B. cinerea*-tomato interaction was extended including samples derived from infected leaves collected at 32, 48, 96 and 120

h after inoculation. The same blot was probed with the *B. cinerea* β-tubulin gene *(tubA)*. Northern blots probed with fragments ddB-2 and ddB-5 were exposed during four days. Blot probed with fragment ddB-47 and with the tubulin-derived probe were exposed overnight. Numbers on the left and right sides of panel (b) and (c) respectively indicate sizes in kb.

Figure 6 shows the sequence of fragments ddB2, ddB-5 and ddB-47. The 263 bp ddB-2 fragment and the 222 bp ddB-47 fragment contained both the anchored primer and the 10-mer primer used for the original differential display analysis while the 143 bp ddB-5 fragment contained only the 10-mer primer at both extremes. Sequence analysis and comparison with the databases did not reveal any significant homology with any known sequence, neither at the nucleotide nor at the amino acid level.

ddB-2

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TGCATTGGTCACAAAGAATACGCAATATCAATACCAAGATGCAGC    45
GGCTGGTGCTTCTAACAGCTTGCAAGATTGAAGATACCCTTAAAA    90
AGTCCAAGGATATTATTTGCGCACGTCCCAACTTAAAACATTCAG   135
CTGCAGAGCATTTAAGCTCCGACTATACTATCTTTGAGGCTCCTT   180
CTAAAGTTGCCATTGGCCTTGAACGTCTTATGCTTGAAGCATCTA   225
GCTTCGGCTTCCGTGTTCGTTACCCGCAAAAAAAAAAA          263
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ddB-5

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CTGCTTGATGATTTGGAATATCTTGTAGACGAAGAGGGACGATTG    45
AGGGGTAGCGGAGAGACTTCGGAAGAGAAGATGGAAGATACGAAT    90
AGGTAAAAACATCGGGTTTGGGAAAATGGCTTCTATTATCTGTAC   135
ATCAAGCAG                                       144
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ddB-47

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GATCGCATTGGAACAGGAGGAGAAGATGAGAAGGGCGAAGTTGGC    45
TGAAGAGGAGAAGGCGGATGAGGAGGAGGAGGCACAGGATGAATC    90
CGGAGATGAGGAGGAGAGTGATAATGAAGAAGAGAAGAAGGATGA   135
GAAGAAGTAAATTAGCATCCGAGTTTGTACATATCTACTTTCTCT   180
CCATCTGCCTTCGTCATCCCGTTTTCCCCAAAAAAAAAAAAA      222
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Figure 6. Nucleotide sequence of the ddB-2, ddB-5 and ddB-47 fragments (accession numbers Z70214, Z70217 and Z71215, respectively). The primers used for the differential display reaction in each case are underlined.

Discussion

Here we report the isolation of several cDNA fragments representing *in planta* induced *B. cinerea* genes by applying the differential display method (Liang and Pardee, 1992). The procedure has demonstrated its utility for the analysis of differential gene expression in very different systems including plants (Sharma and Davis, 1995; van der Knaap and Kende, 1995; Wilson, 1963) and fungi (Appleyard *et al*., 1995). Analysis of a more complex system, like a plant-pathogen interaction, has been attempted but focussing on the study of the plant defence response (Bertioli *et al*., 1995; Goormachtig *et al*., 1995). To our knowledge this is the first report on DDRT-PCR analysis of an interaction system focussing on gene expression in the pathogen.

In our study we focussed on fungal genes whose expression is enhanced *in planta.* As starting material tomato leaves in which effective and synchronised infection by *B. cinerea* occurred were used. In this situation the limiting factor for fungal gene expression analysis is the low proportion of fungal RNA in the interaction material, since it is necessary to detect induced mRNAs within an RNA subpopulation that represents only 3-5 % in the total interaction RNA population. A reconstruction experiment demonstrated that the DDRT-PCR

method is sensitive enough to be applied in such a system. However, the system shows two inherent limitations that must be considered. First, *Botrytis* and plant cDNAs fragments of the same size are indistinguishable. Second, not all the bands detected in the *Botrytis in vitro* grown derived cDNA sample were detected in the reconstruction sample at the 1:30 dilution factor. The weakest bands detected in the *Botrytis in vitro* grown samples were not detected in the reconstruction sample, suggesting that fungal messengers expressed at a lower level *in planta* may be overlooked. Although differential display is only a qualitative method (Bauer *et al*., 1993) and the complexity of the template and of the band patterns obtained does not allow quantitative estimation of the abundance of specific mRNAs, it may be assumed that the detection limit in our DDRT-PCR system was much lower than in hybridisation-based methods.

Comparison of the gene expression patterns of the two organisms involved in an interaction by analysing the differential display patterns requires experimental conditions providing optimal resolution on the differential display gels. The level of complexity of the templates derived from an interaction RNA sample is intrinsically high because RNAs from two organisms are present. Therefore, the number of cDNA species synthesised with a particular anchored primer will be higher and consequently the band pattern obtained will be more complex. In the experimental conditions applied in this work, the complexity of the template was reduced by using two-nucleotide anchored primers for cDNA synthesis. The utilisation of one-nucleotide anchored primers for cDNA synthesis has been reported, allowing a reduction of the number of reverse transcription reactions needed for each RNA sample (Liang *et al*., 1994). However, these primers would have generated too complex templates in our experimental conditions. An additional advantage of using two-nucleotide anchored primers is that a reduction of the template complexity will contribute to increase the sensitivity of the DDRT-PCR for any particular cDNA, as suggested previously (Bertioli *et al*., 1995).

From our comparative analysis of gene expression in the *B. cinerea*-tomato interaction it is evident that the controls must be chosen very carefully. In the first set of experiments a number of interaction-specific cDNA were isolated and some of them detected on northern blot mRNAs induced at early stages during the interaction. However, none of them represented *B. cinerea* cDNAs. All turned out to be plant mRNAs displaying varying levels of induction. The *P. infestans*-tomato interaction was included in order to discriminate plant defence mRNAs induced in response to a stress situation caused by a pathogen but, as these results demonstrate, either specific defence mechanisms are activated by *B. cinerea* or the timing and extent of defence responses induced by the two pathogens are different. The detection of fragment ddT- 19 provides a representative example. Sequence analysis demonstrates that this fragment is derived from the mRNA encoding *trans*-cinnamate 4-monooxygenase, an enzyme of the general phenylpropanoid pathway (Hahlbrock and Scheel, 1989) involved in initial steps of phytoalexin and lignin production (Lamb *et al*., 1989). Enhanced expression of the tomato *trans*-cinnamate 4-monooxygenase encoding gene reflects that the phenylpropanoid pathway is activated by *B. cinerea* but not by *P. infestans,* at the time points considered in this study, shortly before symptom development in either of the two pathosystems. Eventually both pathogens develop necrotic lesions of equal dark appearance, indicative of the production of phenolic substances.

Fragment ddT-19 includes the two primers used for the initial differential display reaction, providing information about its orientation. According to the principle of the differential display method, the anchored primer should anneal to the poly(A) tail of the mRNA and, therefore, the amplified fragment would represent the 3' extreme of the messenger Liang and Pardee, 1992). However, the deduced amino acid sequence of ddT-19 shows high homology to an internal region of other *trans*-cinnamate 4-monooxygenase enzymes, indicating that an

oligo(A) stretch is located in an internal part of the tomato *trans*-cinnamate 4-monooxygenase mRNA and that the ddT-19 cDNA fragment does not represent the 3' extreme of a mRNA.

The defence responses induced in the plant by TNV, on the basis of visual observations, better resembled those induced by *B. cinerea* than does the defence response induced by *P. infestans*. Including both *P. infestans* and TNV control infections, the analysis performed yielded better results. Four out of 17 fragments tested individually were shown to be *B. cinerea* cDNA fragments. Only individual clones reproducing the hybridisation pattern obtained with the original differential display fragment on genomic Southern blots were used as probes on northern blots and sequenced. Three of these fragments, ddB-2, ddB-5 and ddB-47, detected fungal mRNAs showing different levels of induction *in planta,* while fragment ddB-12 did not give any detectable signal on the northern blot. Although the Southern hybridisation analysis has to be extended, the fact that these three fragments hybridised to single *Hin*dIII fragments in *B. cinerea* genomic DNA indicates that the three genes are probably present in a single copy in the *B. cinerea* genome.

ddB-2 and ddB-5 detected mRNAs accumulating only at late time points after inoculation: 120 h for ddB-2 and 96 h for ddB-5. Strikingly, ddB-5 detected an mRNA demonstrating transient expression since it was not detected at the last time point considered (120 h). Both mRNAs were not detected when the fungus is grown *in vitro,* but are induced *in planta* at a rather low level (it should be kept in mind that at late time points after inoculation the proportion of fungal RNA in the total interaction RNA population increases to 25 % at 96 h and more than 90 % at 120 h). ddB-2 and ddB-5 were displayed in the 16 h and 72 h interaction samples, but on the northern blots no signal was detected at these points, confirming that the DDRT-PCR method possesses a high level of sensitivity.

ddB-47 detects two mRNAs expressed *in vitro and in planta,* both showing enhanced expression *in planta,* already detectable at the first time point studied (16 h). The kinetics of accumulation of the two mRNAs was different and seemed to be complementary during the progress of the infection (evident at intermediate time points, 48, 72 and 96 h after inoculation). Interestingly, ddB-47 was displayed in the 72 h interaction sample, but not in the 16 h interaction sample and in the *Botrytis in vitro* cultured sample. However, on the northern blot the corresponding mRNAs are detected in the three samples and, for the 1.4 kb transcript, the intensities of the signals detected in the *Botrytis in vitro* sample and in the 72 h interaction sample are comparable. These observations indicate that the sensitivity of DDRT-PCR for a particular cDNA is conditioned by the composition and complexity of the template mixture, and can be explained considering some of the features of the technique. In DDRT-PCR the complexity of the template is enormous, the concentration of dNTPs is much lower than in standard PCR and mismatches, essential for the priming of most of the templates, are allowed by using low annealing temperatures (Liang and Pardee, 1992). Under these conditions competition for substrates by the many PCR products influences the sensitivity for a particular cDNA (Bertioli *et al*., 1995) and, for a given primer combination, the level of amplification of a specific cDNA is a function not only of its copy number in the template mixture but also of the level of complexity of the template and the priming capacity of the primers used in the template mixture analysed.

Sequence analysis and database searches revealed no significant homology of these fragments to any known sequence, neither at nucleotide nor at amino acid level. ddB-2 and ddB-47 (263 and 222 bp respectively) probably represent 3' mRNA extremes, mainly untranslated regions which are less conserved than the coding regions between homologous genes in different organisms. ddB-5 is a small 'RAPD' fragment amplified only with the 10-mer upstream primer. Its sequence shows characteristics of a coding region and three of the

six possible reading frames are uninterrupted by stop codons, hut no significant homology is detected to any sequence in databases.

Although the sequence data do not provide relevant information about the nature of the fragments isolated, they can be used as probes to isolate the full length cDNA and genomic copies. The expression patterns observed demonstrated that they detect fungal *in planta* induced genes of potential interest. Determining whether these genes play a role in pathogenesis will require further investigations.

There is controversy about the sensitivity of the DDRT-PCR procedure. Bertioli *et al.* (1995) presented experimental data suggesting that differential display shows a strong bias towards high-copy-number mRNAs and attributed to this fact their failure to detect differences in gene expression between uninfected plants and plants responding to pathogen attack. Our observations, in contrast, indicate that the DDRT-PCR technique possesses a high level of sensitivity since induced mRNAs within a subpopulation of mRNAs which constitutes 3-5 % of the interaction mRNA population have been detected. Furthermore, several plant mRNAs were detected which are specifically induced by *B. cinerea* but not by *P. infestans* infection. The finding that a large number of mRNAs, probably representing general plant defence genes induced in response to both pathogens, were detected in our comparative analysis, demonstrates the versatility of the procedure if appropriate controls are included. Thus, the differential display technique can be applied to the analysis of a plant-fungus interaction and it shows enough sensitivity to allow detection of fungal genes induced *in planta.* The analysis has been performed using only a limited set of primer combinations (two out of the twelve possible mRNA subpopulations have been studied). About 4000 *B. cinerea c*DNA fragments derived from genes expressed *in vitro* have been displayed, of which 700-800 were detected in interaction samples. The screening will be extended to obtain a survey of the *B. cinerea* genes whose expression is induced *in planta.* It provides an alternative unbiased strategy to the isolation of genes encoding defined enzymes and may lead to the isolation of pathogenicity genes that probably would not be detected by hybridisation-based procedures.

Experimental Procedures

Organisms and growth conditions

B. cinerea strain SAS 56 is a single ascospore isolate from a cross between two Vitis-derived field isolate (Faretra *et al*., 1988). It was grown on potato dextrose agar containing homogenated tomato leaves (25 % w/v) or in liquid Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) containing 10 mM sucrose and 10 mM KH_2P0_4 in shaking cultures. *P infestans* strain 88069 was cultured on rye-agar medium containing 2 % (w/v) sucrose (Caten and Jinks, 1968). TNV inoculum was obtained from dried tobacco leaves infected with TNV. *Escherichia coli* strain DH5α was used for cloning experiments and propagation of plasmids. It was grown under previously described conditions (Sambrook *et al*., 1989). A near-isogenic line of tomato cultivar Moneymaker, containing the *Cf4* resistance gene, was used in all the inoculation experiments.

Inoculation of B. cinerea *on detached tomato leaves*

Spores of *B. cinerea* were collected from 10-day old tomato agarplates with 5 ml of Tween (0.01 %), filtered through glasswool, washed three times with sterile distilled water and resuspended in B5 medium supplemented with 10 mM sucrose and 10 mM KH_2P0_4 at a

concentration of 10^6 spores per ml. The spore suspension was incubated at room temperature without shaking for 2 h. Inoculations were performed by spraying the spore suspension on the surface of detached leaves of 7-week old tomato plants (cv. Moneymaker genotype Cf4), inserted in florist's foam oasis. The inoculated leaves were allowed to dry for 30 min and then incubated at 20° C and high humidity under a 16 h photoperiod. Samples were taken at different time points, frozen immediately in liquid nitrogen and stored at -80° C.

Inoculation of P. infestans *on detached tomato leaves*

Tomato leaves (cv. Moneymaker genotype Cf4) were inoculated with sporangia collected with sterile distilled water from 2-week old rye-agar cultures. Leaves inserted into florist's foam oasis were inoculated by spraying the surfaces with a sporangia suspension (5 x 10^5 sporangia per ml). Leaves were then incubated at 18° C and high humidity conditions under a 16 h photoperiod.

Infection of tomato with tobacco necrosis virus

TNV infected tobacco leaves (1 g) were homogenised in 5 ml of 10 mM phosphate buffer (pH 7.0) with 1 mM EDTA. The sap was rubbed gently on the surface of tomato leaves (cv. Moneymaker genotype Cf4) dusted with carborundum. The plants were kept at 20° C under a 16 h photoperiod. An average of 25-50 lesions developed per leaflet.

Isolation of nucleic acids

Total RNA isolations from *B. cinerea,* healthy tomato leaves or infected tomato leaves (either with *B. cinerea, P. infestans* or TNV) were performed according to the method of Logemann *et al*. (1987). For DNA isolations from *B. cinerea* or tomato leaves the procedure described by Möller *et al.* (1992) was used. Poly(A)$^+$ mRNA was purified from total RNA by using the oligotex-dT mRNA purification kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. For *B. cinerea* nucleic acid preparation, mycelium was taken from 16 h old liquid cultures inoculated with 10^6 spores per ml.

Differential display of mRNA

For first-strand cDNA synthesis, 2 µl of poly(A)$^+$ mRNA (50 ng/µl) were mixed with 4 µl of the anchor primer (100 ng/µl) and 11 µl of dH_20. The mixture was heated to 70° C for 10 min and put immediately on ice. 8 µl of 5 x Reverse transcriptase buffer (250 mM Tris-HCI pH 8.3, 375 mM KCI, 15 mM $MgC1_2$), 4 µl of 0.1 M DTT, 8 µl of 0.1 mM dNTPs and 1 µl of RNasin (20 U/µ1) (Gibco/BRL, Life Technologies, Breda, The Netherlands) were then added and the reaction mixture was incubated at room temperature for 3 min. After addition of 2 µl of MMLV Reverse Transcriptase (200 U/µl) (Gibco/BRL) the mixture was incubated at room temperature for 8 min and then at 35° C for 1 h. The reaction was stopped by incubation at 95° C for 5 min, diluted to 100 µl final volume and stored at -20° C. The anchorprimers used were RT1 (T_{11}GG), RT2 (T_{11}GC), RT3 (T_{11}CC) and RT5 (T_{11}AA).

PCR amplification reactions were performed in 20 µl final volume reactions containing 1 µl of the diluted cDNA synthesis reaction, 10 mM Tris-HCI pH 8.3, 50 mM KCI, 1.5 mM $MgCl_2$, 1 µCi [α-^{33}P]dATP (specific activity 3000 Ci/mmol), 2.5 mM anchor primer, 0.5 mM RAPD primer, 2 µM dNTPs and 1 U AmpliTaq Polymerase (Perkin Elmer Cetus, Gouda, The Netherlands). Amplifications were performed for 40 cycles at 94 °C for 30 s, 42 °C for 1 min and 70 °C for 30 s and an additional extension period at 72 °C for 5 min in a 9600

thermal cycler (Perkin Elmer Cetus). As RAPD primers the 10-mer primers (26 primers) included in the differential display 10-mer kit from Operon Technologies (Alameda, CA) (sequences described by Bauer *et al.*, 1993) were used. For the reconstruction experiment primer P35 (5'-GACACGGACC) was used as the upstream primer.

PCR-amplified cDNA fragments were separated on non-denaturing 6 % polyacrylamide gels in Tris-borate buffer. The gel was blotted onto Whatman 3MM paper, dried without fixing and exposed for 48 h.

Recovery of bands

Gel and autoradiogram were oriented and bands of interest were cut out from the gel. DNA was recovered from the gel slices by boiling water as described by Zimmermann and Schultz (1994), precipitated with ethanol and glycogen as a carrier and resuspended in 8 µl dH_20. 4 µl of this solution were used for reamplification. PCR conditions were as indicated above except that the dNTPs concentration was 20 mM, primer (both anchored and 10-mer) concentrations were 1 µM and no [α-^{33}P]dATP was added. The amplified products were purified from 2 % agarose gels and used directly for labelling or cloning.

Northern and Southern blot analysis

The reamplified cDNA fragments were used as probes in northern and Southern blot analysis. They were labelled by PCR in 20 µl reaction mixtures containing 1 ng of the cDNA fragment as template, 1 µM anchor primer, 1 mM 10-mer primer, 10 mM Tris-HCI pH 8.3, 50 mM KCI, 1.5 mM $MgC1_2$, 50 µM µCTP, 50 µM dGTP, 50 µM dTTP, 0.5 µM dATP, 0.5 µM [α-^{32}P]dATP (specific activity 3000 Ci/mmol) and 1 U of AmpliTaq Polymerase (Perkin Elmer Cetus). The PCR profile was as indicated above but only 30 cycles were performed. The *B. cinerea* β-tubulin gene was labelled by random priming using the Random Primers DNA Labelling System (Gibco-BRL).

Dotblots containing tomato and *B. cinerea* undigested genomic DNA (2 µg per dot) were prepared with the BIO-DOT apparatus (BioRad Laboratories, Veenendaal, The Netherlands) as described by Sambrook *et al.* (1989). Southern blots containing 5 µg of total genomic DNA per lane were prepared following standard procedures (Sambrook *et al.*, 1989). For northern blot preparation, 20 µg of total RNA were separated under denaturing conditions on a 1.5 % agarose gel containing formaldehyde and transferred to Hybond N^+ membranes (Amersham International, UK) as described by Sambrook *et al.* (1989). The blots were stained with ethidiumbromide and equal loading was checked visually on the basis of the intensity of rRNA bands. Prehybridisations and hybridisation were performed according to Church and Gilbert (1984) under high-stringency conditions.

Fragments of interest selected on the basis of their hybridisation patterns were cloned in the pGEM-T vector (Promega, Madison, WI). Inserts derived from individual plasmids from each ligation were labelled and used to confirm the hybridisation pattern obtained with the originally reamplified product.

Sequence analysis

DNA sequencing was performed in an ABI 373A automated DNA sequencer (Applied Biosystems). Sequence analysis was carried out using the GCG software (Genetics Computer Group, Madison, WI) and the BLAST network services at the National Centre for Biotechnology Information.

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CHAPTER 3

Structure and *in planta* expression of *Botrytis cinerea* ubiquitin genes

Theo W. Prins, Lia Wagemakers and Jan A.L. van Kan

Summary

To identify genes of the necrotrophic pathogenic fungus *Botrytis cinerea* that are expressed during infection of tomato leaves, a differential screening of a genomic library with radioactively labelled cDNA from *B. cinerea* and *B. cinerea* in interaction with tomato was performed. This resulted in the identification of a *B. cinerea* gene denominated *Bcubi*4, encoding a precursor protein consisting of four identical head-to-tail repeats of a 76 amino acid ubiquitin unit. Subsequently a gene denominated *Bcubi*1CEP79, encoding a single ubiquitin unit joined to a Carboxyl Extension Protein of 79 amino acids, was isolated. The expression of the two ubiquitin genes was studied during pathogenesis of *B. cinerea* on tomato. *Bcubi*1CEP79 but not *Bcubi*4 mRNA was induced at 16 hours after inoculation, relative to t=0 and t=24 h. The increased expression of the *Bcubi*1CEP79 gene at this stage of pathogenesis might be required for enhanced ribosomal biogenesis.

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The nucleotide sequence data are in the EMBL and GENBANK databases under accession numbers AF060501 (*Bcubi*4) and AF060232 (*Bcubi*1CEP79).

Introduction

The ubiquitous plant pathogen *Botrytis cinerea* Pers.: ex. Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetz.), is a necrotrophic fungus with a broad host range (reviewed in Prins *et al.*, 2000: Chapter 1). The infection of a host plant by *B. cinerea* is accompanied by an oxidative burst (Deighton *et al*., 1999), and results in many different chemical defence responses triggered in the host. These include, among others, synthesis of phytoalexins and pathogenesis related (PR)-proteins, as well as lignification of the plant cell wall. These defence responses presumably impose a stress on the pathogen, resulting in a temporary inhibition of fungal growth (Benito *et al.*, 1998) and cellular malfunction. When fungistasis leads to protein misfunction, aberrant proteins may accumulate in the fungus and have to be removed. One way of removing non-functional proteins is by proteasomes through a process requiring ubiquination of proteins. This ubiquination enables degradation by the proteasome/proteolytic pathway (Ciechanover, 1994).

Ubiquitins are highly conserved among eukaryotes and are encoded by two classes of genes. Polyubiquitins consist of a 76 amino acid coding unit in multiple direct repeats. The C-terminal unit is usually followed by a single amino acid that is not conserved. The second class are ubiquitins with a single coding unit fused in frame with a stretch of unrelated amino acids at the C-terminal end, also known as the Carboxyl Extension Protein (CEP). Within the CEPs, two types can be identified: those of 52 or 76-80 amino acids (Monia *et al*., 1990). Once a target protein is ubiquinated by covalent attachment of ubiquitin units, the conjugate is transported to the proteasome were the target protein is proteolytically cleaved (Ciechanover, 1994). Besides ubiquination of cytoplasmatic proteins, ubiquitin can also be covalently attached to regulatory proteins, thereby fine-tuning cell processes like cell cycling (von Kampen *et al.*, 1996).

Ubiquitin has been studied most extensively in *Saccharomyces cerevisiae*. Polyubiquitin is suggested to be required for resistance of cells to high temperatures, starvation and other types of stress (Özkaynak *et al*, 1987; Finley *et al*., 1987). Furthermore, polyubiquitin is known to contribute to oxidative stress tolerance (Cheng *et al.*, 1994) and catabolite derepression (Watt and Piper, 1997). Ubiquitin has also been studied in a number of plant pathogens (Pieterse *et al*., 1991; McCafferty and Talbot, 1998; Loser and Weltring, 1998). Pieterse *et al*. (1991) observed that ubiquitin expression in the oomycete *Phytophthora infestans* was induced during colonisation of potato leaves. McCafferty and Talbot (1998) reported that one of three ubiquitin genes was induced during initial colonisation of the rice blast fungus *Magnaporthe grisea*. In *Gibberella pulicaris*, ubiquitin transcription is induced by the phytoalexins rishitin and lubimin and by heat shock (Loser and Weltring, 1998).

In this study, we have isolated mono- and polyubiquitin of *B. cinerea* and have analysed their expression during pathogenesis and during different nutritional conditions *in vitro*.

Results

Isolation of B. cinerea *ubiquitin genes*

A differential screening was carried out (as described by Pieterse *et al*., 1991) of *Botrytis cinerea* strain SAS56 genomic phage library in λEMBL3, using two batches of radioactively labelled cDNA. "Interaction cDNA" synthesised on RNA isolated from a *B. cinerea*-tomato interaction 16 hours post inoculation (h.p.i.) and "*In vitro* cDNA" synthesised on RNA from *B. cinerea* grown overnight in liquid culture (Benito *et al*., 1996: Chapter 2). Less than 5 % of *B. cinerea* RNA was present in the total infected plant RNA population that was used for

preparing the "Interaction cDNA" (results not shown). Using "interaction cDNA" against "*in vitro* cDNA", differentially hybridising phages were identified. One of the differentially hybridising phages was purified and a hybridising restriction fragment derived from this phage was cloned and sequenced. Sequence similarity was found with ubiquitin, a 76 amino acid protein that is highly conserved in all eukaryotes.

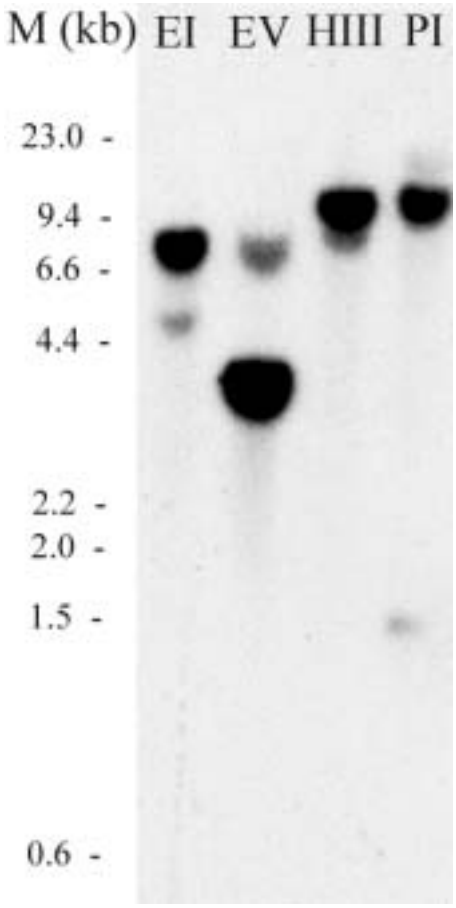

Figure 1. Autoradiograph of Southern blot containing 2 µg/lane *B. cinerea* B05.10 DNA digested with *Eco*RI(EI), *Eco*RV(EV), *Hin*dIII(HIII) and *Pst*I(PI), respectively, hybridised with the polyubiquitin probe.

Southern analysis of *B. cinerea* genomic DNA, digested with four restriction enzymes, indicated that the gene hybridised strongly to a single band in each digest and only weakly to a second band (Figure 1). In view of the presence of multiple ubiquitin genes in *Saccharomyces cerevisiae* and a number of plant pathogenic fungi (Özkaynak *et al*., 1987; McCafferty and Talbot, 1998; Loser and Weltring, 1998), the library was rescreened for additional *B. cinerea* ubiquitin genes. This resulted in the identification of a second type of phages, which also contained a gene encoding ubiquitin.

Structure of B. cinerea *ubiquitin genes*

The gene that was isolated first, designated *Bcubi*4 (Figure 2, GENBANK accession number AF060501), contains an ORF (interrupted by two introns of 125 and 56 nt.) encoding a

polyubiquitin precursor protein, consisting of four identical ubiquitin units. As in all polyubiquitin genes, the last unit is followed by an additional unique amino acid preceding the translation termination codon, in this case a glutamine residue. The promoter region contains elements that are homologous to conserved elements in promoters of stress-induced plant genes (Goldsbrough *et al.*, 1993). These are the four TCA-like sequences indicated in bold in Figure 2. The polyadenylation site, revealed by 3' sequence analysis of a polyubiquitin cDNA clone (Van der Vlugt-Bergmans, unpublished), is indicated bold and underlined in Figure 2.

```
gataacggtgagcaaagaaggcgcagcaaccgtacctgtga**ttcattcactcattctcact**aaactctaa          70
aaggtggcgtggcgctaagcgataaggcaagttcaatgcg**cttccgcttcctcctttca**atgcctactta          140
aacactccatcgcaccctcaaacg**tctccttctttcttcc**atcatacaactccatcaacgctctccacga          210
taa**cttcttttgattctc**cagaaatcatctacaatatcatctacttcatcaaaagattaacactacaaga          280
        ↓M1 Q  I  F  V  K  T                                                 7
acatcatcATGCAGATCTTCGTCAAGACTCgtaagtggtcacctttccgtttgccgcctgcgagcggtat          350
cgccacttcacttccagaagcagcttccatcaacccttttcatcaagtatgcatccaaaacatctgctaac          420
              L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D  N  V      26
atgtgcttgctacagTCACTGGAAAGACCATCACCCTCGAGGTTGAATCTTCCGATACCATCGACAACGT          490
  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K  Q      49
CAAGGCCAAGATCCAAGACAAGGAGGGAATCCCACCCGACCAACAGCGTTTGATTTTCGCCGGAAAGCAA          560
 L  E  D  G  R  T  L  S  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R       72
CTTGAGGATGGTCGCACCTTGAGTGATTACAACATCCAAAAGGAGTCTACTCTCCATCTCGTTCTCCGTC          630
L  R  G  G ↓M2 Q  I                                                         79
TTCGTGGTGGTATGCAAATCTgtaagttaaacaccattgccatcgcactacgccacaatttactgacatc          700
      F  V  K  T  L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D        100
atcatagTCGTCAAGACTCTTACTGGTAAGACTATCACCCTCGAGGTTGAGTCTTCCGATACCATCGACA          770
N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K      124
ACGTCAAGGCCAAGATCCAAGACAAGGAGGGAATCCCACCCGACCAACAGCGTTTGATTTTCGCTGGTAA          840
  Q  L  E  D  G  R  T  L  S  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L      147
ACAACTTGAGGATGGTCGTACCCTCTCCGATTACAACATTCAGAAGGAATCTACTCTCCACTTAGTTCTC          910
 R  L  R  G  G ↓M3 Q  I  F  V  K  T  L  T  G  K  T  I  T  L  E  V  E      170
AGACTTCGTGGTGGTATGCAAATCTTCGTTAAGACTCTCACTGGAAAGACAATCACTTTGGAGGTTGAGT          980
S  S  D  T  I  D  N  V  K  A  K  I  Q  D  K  E  G  I  P  P  D  Q  Q  R      194
CATCGGACACTATCGACAACGTGAAGGCTAAGATTCAAGACAAGGAGGGTATCCCACCCGATCAACAACG          1150
  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  S  D  Y  N  I  Q  K  E  S      217
TCTTATCTTTGCTGGTAAGCAACTTGAGGATGGCCGCACCCTCTCCGATTACAACATTCAAAAGGAATCA          1120
 T  L  H  L  V  L  R  L  R  G  G ↓M4 Q  I  F  V  K  T  L  T  G  K  T      240
ACATTACATTTGGTCCTTCGTCTACGTGGTGGTATGCAGATTTTCGTCAAGACTTTGACTGGAAAGACCA          1190
I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A  K  I  Q  D  K  E  G  I      264
TTACATTAGAAGTAGAATCATCAGATACTATTGATAACGTTAAGGCGAAGATCCAAGATAAGGAGGGTAT          1260
  P  P  D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R  T  L  S  D  Y      287
CCCACCCGACCAACAACGATTGATCTTTGCTGGAAAGCAGTTGGAGGACGGCAGAACCCTTTCGGATTAC          1330
 N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G  Q  *                  305
AACATTCAAAAGGAATCGACATTGCACTTGGTGCTCCGTCTTCGTGGTGGTCAATAAatgtttttgattc          1400
tatctgcacatatattctttaggcgttttacgatgacctgatccaatggtgttatgggggctgggcagca          1470
ggttctcatcatagtgcactggcatgtgccacattactgatagatagtattcaatagtcaaaactatatt          1540
tttgacattt**aa**ctcggtgctcttatgaatgtgatgttgaatatgatttagtatggaaacagttggtatc          1610
tcaatgcttaacttccttatcgtacctgaaatggagttggagataatgacggtgttatgctccactacct          1680
ccactcatgaagctaaagtgatgcgaggctgcag                                          1714
```

Figure 2. Nucleotide sequence of the *Botrytis cinerea* polyubiquitin *Bcubi*4 gene (accession number AF060501). The deduced amino acid sequence is shown below the open reading frame. The Methionine residue of each ubiquitin unit is numbered (M1 to M4). Putative stress-responsive elements (Goldsbrough *et al*., 1993) in the promoter are in bold. The polyadenylation site is underlined and was determined in a polyubiquitin cDNA clone (van der Vlugt-Bergmans, unpublished).

The second gene, designated *Bcubi*1CEP79 (Figure 3, GENBANK accession number AF060232), contains an ORF encoding a single ubiquitin unit joined to a Carboxyl Extension Protein of 79 aa (hereafter referred to as CEP79). The amino acid sequences of the five ubiquitin units in both *B. cinerea* genes are identical.

```
gataacggattttagcgcacaaccagactttcgaaattccctatcaattatctt**gaacttttcttttcga**          70
**ac**tctacaagacctttgaggaagacaatctaaaccgttgtccaggtatgttattactatctgaatgcgaa          140
                                                  M  Q  I  F  V  K  T     7
atcgcatattttagatatgattgaattctaacacctcttaaagatagtcATGCAGATCTTCGTCAAGACC          210
 L  T  G  K  T  I  T  L  E  V  E  S  S  D  T  I  D  N  V  K  A  K  I     30
CTCACGGGCAAAACCATCACCCTCGAGGTTGAGTCTTCGGACACCATCGACAATGTCAAGGCCAAGATCC          280
Q  D  K  E  G  I  P  P  D  Q  Q  R  L  I  F  A  G  K  Q  L  E  D  G  R     54
AAGACAAGGAGGGAATCCCACCCGACCAACAGCGATTGATCTTCGCCGGAAAGCAACTCGAAGATGGCCG          350
  T  L  S  D  Y  N  I  Q  K  E  S  T  L  H  L  V  L  R  L  R  G  G↓G     77
CACCCTCTCCGACTACAACATCCAAAAGGAGTCCACTCTTCACTTGGTGTTACGTCTCCGTGGTGGTGGC          420
 K  K  R  K  K  K  V  Y  T  T  P  K  K  I  K  H  K  R  K  K  T  K  L     100
AAGAAGCGCAAGAAGAAGGTCTACACTACCCCAAAGAAGATCAAGCACAAGCGCAAGAAGACCAAGCTCG          490
A  V  L  K  Y  Y  K  V  D  G  D  G  K  I  E  R  L  R  R  E  C  P  T  P     124
CTGTATTGAAGTACTACAAGGTTGATGGTGATGGAAAGATCGAGCGTCTCCGCAGAGAGTGCCCAACTCC          560
  D                                                          C  G  A  G     129
AGACgtaagttttcttttgatgagttgtatttttgggcgctactaacatatctacagTGTGGTGCCGGTG          630
V  F  M  A  A  M  H  D  R  Q  Y  C  G  R  C  H  L  T  Y  I  F  D  D  A     153
TTTTCATGGCTGCCATGCACGACCGTCAATACTGTGGACGCTGCCACTTGACCTACATCTTCGATGATGC          700
  N  K  *                                                                  155
CAACAAATAAattatcaccaatggataatcgtatatgaaggaatagattctcgcatgctatggtgtagtt          770
cagatattactcaatgctacgctgaatctatgtcaaatatgctcggttttttttaattattggctgtgaa          840
acatggattgtgctccgtgaatgtgtagaaaagtcctgcgtagagccttgaaatgattgaaaaatgaacc          910
tttatgatctctaatgacgaaagccagcagaaatctactatcagtactggcttcttacccgacgcataac          980
catgcaagacctagggctag                                                        1000
```

Figure 3. Nucleotide sequence of the *Botrytis cinerea* monoubiquitin *Bcubi*1CEP79 gene (accession number AF060232). The deduced amino acid sequence is shown below the open reading frame. The cleavage site for the 79 amino acid Carboxyl Extension Protein (CEP) is indicated by an arrow. A putative heat shock element (Fernandes *et al*., 1994) in the promoter is indicated in bold. A putative zinc-finger RNA binding site (Özkaynak *et al*., 1987) is underlined, with the cysteine residues at codons 121, 126, 141 and 144 (**C**) in bold. A putative nuclear localisation signal is thick underlined.

```
B. cinerea      MQIFVKTLTGKTITLEVESSDTIDNVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGG(Q)
N. crassa       ........................Q.............................................(F)
M. grisea       ........................S.............................................(.)
G. pulicaris    ........................S.............................................(.)
S. cerevisiae   ........................S.............................................(N)
C. albicans     ........................S.............................................(F)
P. chrysosp.    ..........................................................................(L)
P. infestans    .............D..P..S.....Q.............................................(N)
```

Figure 4. Alignment of *B. cinerea* ubiquitin units with homologues from other fungi. The amino acid sequences of BcUBI4 (4 identical units, accession AF060501) and BcUbi1CEP79 (1 unit, accession AF060232) were aligned with polyubiquitins from *Neurospora crassa* (X13140, 4 identical units; Taccioli *et al*., 1989), *Magnaporthe grisea* (AF056625, 5 identical units; McCafferty and Talbot, 1998), *Gibberella pulicaris* (AJ007936, 4 identical units; Loser and Weltring, 1998), *Saccharomyces cerevisiae* (X05731, 5 identical units; Özkaynak *et al*., 1987), *Candida albicans* (Z54197; unpublished), *Phanerochaete chrysosporium* (Z24723, 5 identical units; Escher, 1995), and *Phytophthora infestans* (X55717, 3 identical units; Pieterse *et al*., 1991). The additional C-terminal amino acid following the ultimate ubiquitin unit is indicated between brackets. A dot represents an amino acid residue that is identical to the *Botrytis cinerea* ubiquitin moiety.

Figure 4 shows the amino acid alignment of the *B. cinerea* ubiquitin units with homologues from other fungi. Ubiquitin of the basidiomycete *Phanerochaete chrysosporium* is identical to *B. cinerea*, whereas other ascomycetes each show one difference at residue 28, and the oomycete *P. infestans* ubiquitin has three additional differences.

Figure 5 shows that the extension protein CEP79 has homology to ubiquitin-CEPs from other fungi, especially in the N-terminus. A potential nuclear localisation signal and a

putative nucleic acid binding site (Özkaynak *et al.*, 1987) are present in the CEP79 moiety. Both motifs are conserved in all ubiquitin-CEPs as shown in the alignment in Figure 5. One of the homologues is the 76 amino acid UBI3 CEP of *S. cerevisiae* (Özkaynak *et al.*, 1987). This protein is cleaved from the ubiquitin moiety, and participates in ribosomal biogenesis (Finley *et al*., 1989).

```
B.c.   GKKRKKKVYTTPKKIKHKRKKTKLAVLKYYKVDGDGKIERLRRECPTPDCGAGVFMAAMHDRQYCGRCHLTYIFDDANK  79
A.n.   ..........................................S.E....I.....QN.....K.....V..ESK   78
N.c.   ..............................S............NET.........Q............V.EKSS   78
M.g.   ..................H..V.....STTRSTVTERLSASAAS..NET....I.....Q............V..KKD   78
S.c.   ..................H..V.....S.....AE..VTK.....SN.T......L.NHK..L...K..SV.KVNA     76
C.a.   ..................HR.......T.....NE.NV........A.T....I...N.K......K....LKAN      75
S.p.   .......T..........H..VE.........ED..SVK.......N--...ST...NHK..L........LKLEN      74
```

Figure 5. Alignment of the *B. cinerea* ubiquitin-CEP79 moiety with homologues from other fungi: *Aspergillus nidulans* (accession AF175521, Noventa-Jordão *et al*., 2000), *Neurospora crassa* (X15338, Taccioli *et al*., 1991), *Magnaporthe grisea* (AF056624; McCafferty and Talbot, 1998), *Saccharomyces cerevisiae* (X05730, Özkaynak *et al*., 1987), *Candida albicans* (Y15608; unpublished) and *Schizosaccharomyces pombe* (CAB11297, unpublished). A dot represents an amino acid residue that is identical to the *Botrytis cinerea* CEP79 protein. The nuclear localisation signal (positions 2-7) is underlined. The four cysteine residues that form part of the zinc binding site are indicated in bold. The number behind the sequence indicates the length of the CEP.

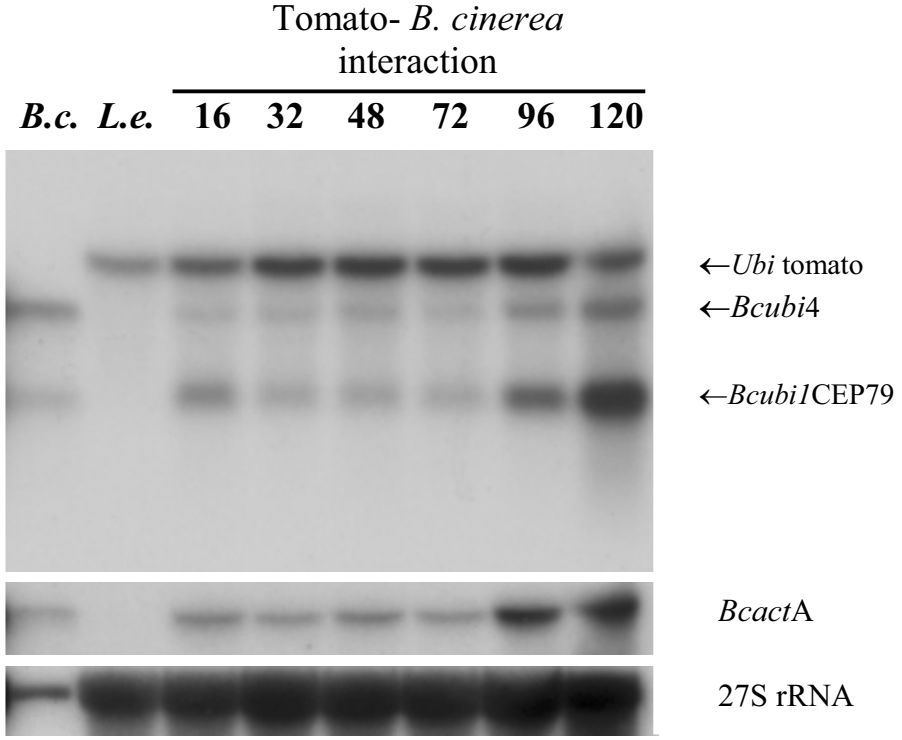

Figure 6. Expression analysis of *B. cinerea* ubiquitin genes *in planta*. Total RNA was isolated from a *B. cinerea* liquid culture (lane *B.c.*), from uninoculated tomato leaf (lane *L.e.)* and from *B. cinerea*-infected tomato leaves at different time points (numbers indicate h.p.i.), electrophoresed and blotted. In the left lane (*B.c.*) only 1/20th of the amount of RNA was loaded as compared to the other lanes, to approximate the amount of fungal RNA in the infected leaves at 16 h.p.i. (Chapter 2). The blot was hybridised with the *Bcubi4* probe (upper panel), the *B. cinerea* actin gene *Bcact*A (Benito *et al*., 1998; middle panel) and a *B. cinerea* 27S rDNA probe (Prins, unpublished; lower panel).

Expression of B. cinerea *ubiquitin genes*

In *S. cerevisiae*, ubiquitin gene expression is known to be regulated by carbon or nitrogen sources in the medium (Watt and Piper, 1997). *B. cinerea* was grown for various times in liquid culture in presence of different combinations of a carbon source (1 % glucose), a nitrogen source (100 mM NH_4Cl) and protein (1 % BSA). No obvious changes in ubiquitin mRNA levels were observed in the different media (results not shown).

The expression of the *B. cinerea* ubiquitin genes *in planta* was investigated. Detached tomato leaves were inoculated with a conidial suspension of *B. cinerea* strain SAS56 and harvested at different time points. RNA was extracted and electrophoresed. A blot containing total RNA isolated from infected tomato leaflets as well as from *in vitro* grown cultures of *B. cinerea* was hybridised with a ubiquitin probe, a *B. cinerea* actin probe (Benito *et al*., 1998) and a 27S rDNA probe (Prins, unpublished data). The rDNA probe detects both plant and fungal rRNAs and was used to visualise RNA loading in each lane. The results are shown in Figure 6. The actin mRNA level increased over time, especially at 96 and 120 h.p.i. This increase reflects the increase of fungal biomass in the infected tissue, which occurs biphasically. In the first phase until 16 h.p.i., primary necrotic lesions are formed by germinating conidia that penetrate the host tissue. These primary lesions remain quiescent for 2-3 days and only start to expand at 72 h.p.i. under the conditions used in this inoculation (Benito *et al*., 1998). The ubiquitin probe recognises two types of *B. cinerea* ubiquitin mRNAs, as well as the tomato hepta-ubiquitin (Rollfinke *et al.*, 1998). The level of *Bcubi*4 mRNA increased during the infection, and followed the expression pattern of the actin mRNA which reflects the increase of the fungal biomass during infection. *Bcubi*1CEP79 mRNA was transiently induced at 16 h.p.i., relative to t=0 and t=24 h, as compared to the actin messenger. At this timepoint the proportion of fungal RNA in the total infected leaf RNA was estimated to be 1-5 % (Chapter 2). We loaded 20 times less RNA in the *B. cinerea in vitro* control lane than in the other lanes, in order to obtain a hybridisation intensity of this sample, that would be equivalent to a proportion of 5 % *B. cinerea* RNA in the total infected leaf RNA. The *Bcubi*1CEP79 mRNA hybridisation signal in the interaction at 16 h.p.i. is clearly higher than that of the control sample, while the actin hybridisation signals are similar in both samples. After this timepoint, the *Bcubi*1CEP79 mRNA level decreased initially, but later increased again, following the pattern of the actin signal. The tomato hepta-ubiquitin mRNA level gradually increased during the infection by *B. cinerea*, possibly reflecting the stress triggered by the infection. The transient induction pattern of the *Bcubi*1CEP79 mRNA in early stages of the infection could be reproduced, not only in an infection assay at 20° C, but also at 4° C (results not shown).

Discussion

In search for *B. cinerea* genes that are involved in the infection of tomato, we performed a differential screening of a genomic library. This resulted in the identification of a ubiquitin gene family consisting of a tetraubiquitin and a monoubiquitin designated *Bcubi*4 and *Bcubi*1CEP79, respectively. *In planta* expression studies showed that *Bcubi*1CEP79 was transiently upregulated at 16 h.p.i., while *Bcubi*4 was not. However, also tomato ubiquitin mRNA was upregulated in comparison to the control (see Figure 6). Therefore, the isolated ubiquitin genes by a differential screen of a genomic library could have been the consequence of the increased levels of tomato heptaubiquitin mRNA, rather than the increased *Bcubi*1CEP79 level.

Ubiquitin is highly conserved among almost all eukaryotes and is involved in a number of cell processes like oxidative stress resistance (Cheng *et al*., 1994), selective protein degradation (Ciechanover, 1994), cell cycle regulation (King *et al*., 1996), p53-mediated apoptosis (Scheffner *et al*., 1993), NF-κB-induced apoptosis (Krappmann *et al*., 1996) and regulation of receptors (Strous *et al*., 1996). The function of ubiquitin in fungi has mainly been studied in *S. cerevisiae. S. cerevisiae* contains three genes designated *UBI1*, *UBI2* and *UBI3 that* encode monoubiquitins with CEPs of 52, 52 and 76 aa, respectively. In addition, the gene *UBI4* encodes a pentaubiquitin that is strongly induced during starvation, heat shock and other types of stress (Özkaynak *et al*., 1987). The *UBI*4 gene contributes to oxidative stress resistance (Cheng *et al*., 1994) and is subject to catabolite derepression control (Watt and Piper, 1997).

The induction of ubiquitin gene expression during infection of host plants has previously been described for other plant pathogens. Ubiquitin expression in the oomycete *P. infestans* (Pieterse *et al*., 1991) is induced upon colonisation of potato leaves, suggesting a role in pathogenicity. The rice blast fungus *M. grisea* contains two genes, *UEP1* and *UEP3*, encoding monoubiquitin joined to CEPs of 52 and 76 amino acids, respectively (McCafferty and Talbot, 1998). Both genes are highly expressed during active growth *in vitro*, and down regulated during starvation stress. *M. grisea* also contains a polyubiquitin gene *PUB4,* which is predominantly induced by environmental stress (McCafferty and Talbot, 1998). *UEP*1 is the only member of the *M. grisea* ubiquitin gene family that is expressed at detectable levels during plant colonisation (McCafferty and Talbot, 1998). A polyubiquitin gene was also cloned from the pathogenic fungus *G. pulicaris* (Loser and Weltring, 1998). Increased mRNA levels were detected when the fungus was grown at elevated temperature or in the presence of rishitin. Southern analysis suggested the presence of additional ubiquitin genes (Loser and Weltring, 1998). Expression of *G. pulicaris* ubiquitin genes was not studied *in planta*.

The CEP moieties of the three *S. cerevisiae* monoubiquitin gene products each have a nuclear localisation signal and a putative metal binding site that can assemble into a nucleic acid binding motif, suggesting that the CEP moieties bind to nucleic acids, either before or after cleavage from the ubiquitin moiety (Özkaynak *et al*., 1987). Targeted deletion of each of the three monoubiquitin-CEP genes resulted in mutants with severely slower growth rates. The mutants displayed a distortion of the stoichiometry of the ribosomal subunits (Finley *et al*., 1989). The growth defect caused by deletion of the *UBI*1, *UBI*2, or *UBI*3 gene could be relieved by complementation with gene constructs consisting of the CEP52 or CEP76 moiety alone, lacking the ubiquitin moiety (Finley *et al*., 1989). Tagging the CEP moiety with a twelve amino acid c-myc epitope revealed that the CEP52 and the CEP76 are incorporated in ribosomes. The CEP52 encoded by *UBI*1 and *UBI*2 is part of the large ribosomal subunit, whereas the *UBI*3-encoded CEP76 tail is part of the small ribosomal subunit (Finley *et al*., 1989). Incorporation of these CEP moieties into ribosomal subunits is thus essential for proper ribosome biogenesis.

The observation that the *B. cinerea* monoubiquitin gene, but not the polyubiquitin gene, is induced *in planta* at an early stage of infection, suggests that the need for increased production of the CEP79 moiety determines the induction of the *Bcubi*1CEP79 gene, rather than the need for ubiquitin. Since the *S. cerevisiae* CEP76 (homologue of the *B. cinerea* CEP79 moiety) is required for ribosomal biogenesis (Finley *et al*., 1989), the expression pattern probably reflects a need for a relatively high rate of ribosomal biogenesis in *B. cinerea* at the early stages of the infection process. A similar conclusion was drawn for the rice blast fungus, *M. grisea* (McCafferty and Talbot, 1998). This is in agreement with our observation that a differential display RT-PCR screening for *B. cinerea* genes that are differentially expressed *in planta* (Chapter 2), resulted in the isolation of a small number of

cDNA fragments derived from ribosomal protein-encoding genes (Prins, unpublished data). Initially this was considered to be an artefact of the DDRT-PCR procedure. In retrospect, this finding may be the consequence of enhanced ribosomal biogenesis in *B. cinerea* in early stages of pathogenesis, involving the induction of the *Bcubi*1CEP79 gene and other ribosomal protein encoding genes.

Experimental procedures

Fungal growth

B. cinerea strain SAS56 was cultured as described by Benito *et al.* (1998).

Screening of a genomic library

A genomic library of *B. cinerea* strain SAS56 in lambda EMBL3 was prepared according to the manufacturer's instructions (Promega). From this library, 15,000 plaque forming units (pfu) were plated representing five genome equivalents. Four replica filters (Hybond-N^+, Amersham) were prepared. For a differential screening, poly$(A)^+$ RNA was isolated from *B. cinerea* grown *in vitro* and a tomato-*B. cinerea* interaction (16 hours post inoculation (h.p.i.) (Chapter 2). This was reverse transcribed into cDNA in the presence of [α-^{32}P]dATP and hybridised to duplicate filters of the genomic library of *B. cinerea*. Since the fungal poly$(A)^+$ RNA in the interaction sample represents at maximum 5 % of the total (Chapter 2), the *in vitro* probe was supplemented with 19-fold excess poly $(A)^+$ RNA from uninfected tomato leaves. Filters were hybridised o/n at 65° C in 0.5 M sodium phosphate buffer pH 7.2; 7 % SDS (Church and Gilbert, 1984), washed down to 0.5x SSC; 0.5 % SDS at 65° C and exposed to Kodak X-OMAT AR film. Differentially hybridising plaques, confirmed with the replica filters to avoid false positives, were isolated and subjected to a second round of hybridisation. DNA of positive phages was isolated using the Qiagen phage DNA isolation kit (Qiagen Inc., USA). Southern analysis (Prins *et al*., 2000a: Chapter 4) indicated hybridising fragments, which were subsequently cloned into cloning vector pBluescript SK II(+) (Stratagene) as described in Sambrook *et al*. (1989).

Sequence analysis

DNA sequencing was performed with Applied Biosystems, 377 DNA Sequencer, using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit, with Amplitaq DNA Polymerase, and analysed using Lasergene software package (DNAstar Inc., Madison, USA). Nucleotide and amino acid homology analysis was performed using the Blast program (Gish and States, 1993; Altschul *et al.*, 1997).

Analysis of gene expression in planta

Tomato leaves (*Lycopersicon esculentum* cv. Money Maker genotype *Cf4*) were inoculated with conidia of *B. cinerea* according to Benito *et al.* (1998). Inoculated leaves were incubated at 4° C and 20° C. At 16, 32, 48, 72, 96 and 120 h.p.i., leaflets were harvested for RNA extraction.

Analysis of ubiquitin gene expression in liquid cultures

Liquid cultures of *B. cinerea* were grown overnight (B5 medium with additional 10 mM phosphate and 1 % sucrose) as described by Benito *et al.* (1998). At t=0, mycelium was filtered and transferred to liquid cultures containing different combinations of a carbon source (1 % glucose), a nitrogen source (100 mM NH_4Cl) and protein (1 % BSA). Two different pH values (4 and 7) were used in the presence of additional glucose. After 3 and 6 h, mycelia were harvested for RNA extraction.

RNA blot analysis

Total RNA from infected leaves and liquid grown mycelium was isolated according to Prins *et al*. (2000b: Chapter 4). As probes, the 400-bp *Xho*I/*Acc*I polyubiquitin fragment (nt. 741-1141), a fragment of the actin gene (Chapter 2) and a fragment of the 27S rDNA gene (Prins, unpublished) were used.

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CHAPTER 4

Cloning and characterisation of a glutathione S-transferase homologue from the plant pathogenic fungus *Botrytis cinerea*

Theo W. Prins, Lia Wagemakers, Alexander Schouten and Jan A.L. van Kan

Summary

A gene was cloned from *Botrytis cinerea* that encodes a protein homologous to glutathione S-transferase (GST). The gene, denominated *Bcgst*1, is present in a single copy and represents the first example of such a gene from a filamentous fungus. The biochemical function of GSTs is to conjugate toxic compounds to glutathione, thereby detoxifying the compound. In many other organisms GST plays a role in chemical stress tolerance. We anticipated that GST functions for *B. cinerea* as a potential virulence factor enabling the fungus to tolerate fungitoxic plant defence compounds. The expression of *Bcgst*1 mRNA under various presumably stressful conditions was investigated. *Bcgst*1 mRNA is expressed at a basal level in liquid cultures and it is induced upon addition of hydrogen peroxide to the medium. The level of *Bcgst*1 mRNA expression during infection of tomato leaves parallels the level of actin mRNA. The role of the *Bcgst*1 gene in virulence of *Botrytis cinerea* was evaluated by constructing gene disruption mutants. Three independent disruption mutants were obtained. The virulence of two mutants on tomato leaves was evaluated. Neither of the mutants showed a decrease in virulence, indicating that the *Bcgst*1 gene is not essential for virulence on tomato leaves under the conditions tested.

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The nucleotide sequence data are in the EMBL and GENBANK databases under accession number AF061253 (*Bcgst*1).

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional detoxification enzymes that are mainly cytosolic and catalyse the conjugation of a wide variety of xenobiotics to reduced glutathione (GSH) (Marrs, 1996). GSTs are best characterised in mammals and plants. In mammals, the cytosolic GSTs were classified in four distinct families on the basis of primary structures and substrate specificity (Mannervik and Danielson, 1988; Wilce *et al.*, 1995). All plant GSTs studied thus far belong to the theta-class, with the exception of carnation GST. Droog (1997) suggested further dividing plant GSTs in theta and tau classes. In plants, GSTs were first identified because of their ability to detoxify herbicides. Herbicide safeners (a.k.a. antidotes) can be used in crop protection before or during application of herbicides because they increase GST activity in crop plants. This application increases resistance in the crop to the herbicide (Riechers *et al.*, 1997).

Subsequent research indicated that GSTs are also involved in resistance toward drugs, antibiotics and insecticides (Cummins *et al.*, 1997; reviewed by Neuefeind *et al.*, 1997). In plants, the conjugation reaction is coupled to internal compartmentalisation, while in mammals the conjugate is catabolised and subsequently secreted (Neuefeind *et al.*, 1997). Besides detoxifying natural and xenobiotic toxic compounds by GSH conjugation, GSTs have been reported to be involved in auxin binding, as well as in cellular protection against H_2O_2, generated by the plant as a defence against invading pathogens during an oxidative burst. Hydrogen peroxide was found to induce the expression of GST genes (Ballas *et al.*, 1993; Levine *et al.*, 1994; Neuefeind *et al.*, 1997). Overexpression of a GST in transgenic tobacco enhances the growth of seedlings when stressed by chilling or salt (Roxas *et al.*, 1997). This observation supports the importance of GST to the general stress resistance in plants.

Besides in plants and animals, GST activity was detected in *Escherichia coli* (Shishido, 1981), yeasts (Kumagai *et al.*, 1988) and other lower eukaryotes (Wackett and Gibson, 1982; reviewed in Dowd *et al.*, 1997). The ability to detoxify xenobiotics has been reported in several fungi. The white-rot fungus *Phanerochaete chrysosporium* can detoxify a range of xenobiotic compounds and contains a highly labile protein with GST activity that uses 1,2-epoxy-3-(ρ-dinitrophenoxy)-propane as substrate (Dowd *et al.*, 1997). The protein was partially purified and the partial N-terminal amino acid sequence was determined. However, no evidence was provided that this particular protein is responsible for the detoxifying activity, nor was the enzyme classified. In the oomycete *Phytophthora infestans*, accumulation of oxidised glutathione (GSSG) was observed in liquid cultures after fungicide treatment, suggesting the involvement of the GSSG/GSH ratio as well as the total cellular glutathione content in resistance to the fungicide applied (Ellner, 1990). *Fusarium oxysporum* exposed to fungicides showed an initial reduction of the GSH level and GST activity, followed by a gradual increase. Remarkably, no significant differences were found between fungicide-tolerant and sensitive strains (Cohen *et al.*, 1986).

Botrytis cinerea Pers.: ex. Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetz.) is a necrotrophic fungus with a broad host range. It is the causal agent of grey mould and causes one of the most important post-harvest diseases on soft fruit, ornamentals and vegetables. Over 235 plant species have been reported to act as a host, implying that *B. cinerea* must possess an arsenal of (secreted) attack enzymes as well as an omnipotent capacity for detoxification of a spectrum of chemically diverse plant defence compounds (reviewed by Prins *et al.*, 2000a). In order to elucidate the molecular basis of pathogenesis of *B. cinerea*, we carried out differential gene expression studies in order to isolate fungal genes that play a role early in the infection process (Benito *et al.*, 1996). Here we describe that a subtractive hybridisation on RNA from *B. cinerea*-infected tomato leaves 16 hours post inoculation

(h.p.i.) resulted in the isolation of a gene encoding a glutathione S-transferase (GST) homologue. It is the first report of such a gene from a filamentous fungus. We have studied the effect of disruption of this gene on the virulence of *B. cinerea* on tomato leaves.

Results

Isolation and characterisation of the Bcgst*1 gene*

In an effort to isolate *B. cinerea* genes that are specifically induced during the infection of tomato leaves, a subtractive hybridisation RT-PCR was carried out, as described in the Experimental Procedures. The resulting PCR mixture was radioactively labelled and the labelled products were used to hybridise to filters of a genomic λ-EMBL3 library of *B. cinerea* strain SAS56. One of the hybridising phages was purified, the phage DNA extracted and the entire phage insert was amplified by long distance PCR with EMBL3-specific primers. The insert was digested with several enzymes and the hybridising region was mapped on the insert by hybridisation with the same probe. A strongly hybridising *Eco*RV fragment of 698 bp was cloned and sequenced. This fragment contained part of an ORF. Flanking regions on either side of the *Eco*RV fragment were sequenced by PCR on the phage insert, using gene-specific primers. Southern analysis of fungal genomic DNA revealed that the gene hybridised to a single band in each digest, even at intermediate stringency (55°C, 1x SSC, not shown).

The DNA sequence (Figure 1) contains an ORF of 254 amino acids with homology to GST genes from a range of organisms (Figure 2). The cloned gene was therefore designated as *Bcgst*1. The ORF is interrupted by two introns (55 bp and 138 bp), the position of which was confirmed by cloning and sequencing a RT-PCR fragment, synthesised on RNA isolated from mycelium grown in liquid culture. The amino acid sequence of BcGST1 was aligned to (parts of) six other GST-like genes (Figure 2). The majority of active site residues are located in the N-terminal domain, whereas the C-terminal domain is largely responsible for determining the substrate specificity of the different GST classes (Wilce *et al.*, 1995). Identity of BcGST1 with the other GST-like gene products ranges from 27 to 40 %, similarity is around 60 %.

The *Bcgst*1 gene contains a number of potential regulatory elements in the region upstream of the putative translation start codon: a stress responsive element (TCAGGGGG; Schüller *et al.*, 1994), core binding sites for nitrogen-responsive transcription factors (GATA; Marzluf, 1997) and a potential CREA binding site (GT/CGGGG; Ronne, 1995). Two possible TATA core promoter sequences (Gurr *et al.*, 1987) have been found, but no CAAT-motif was present. A putative polyadenylation signal (AATAAT) is present 38-43 bp downstream of the translation stop codon.

Expression of the Bcgst*1 gene in liquid cultures*

In *S. cerevisiae*, the expression of the GST/URE2 gene is subject to regulation by external nitrogen and carbon sources. In order to study whether a similar regulation occurs in *B. cinerea,* different combinations of carbon and nitrogen sources were added to liquid cultures. RNA was extracted and analysed by hybridisation. In all the cultures, *Bcgst*1 mRNA levels remained constant (results not shown).

```
ttttttcaggggggaggggcccacccaaaggatagctgttggcggttccaaatggggacg      60
acagaacatgataaccggcagggacttatttattgtcttcggttcaggtgtacatttcac      120
acaaaggggatttgtccgtcaaacagtcatgtgttgtggaaagaactgcctgtccggcat      180
ttcccgccaagcttcgttcccaaagagccgttttgaagcgtcagcttggtaagataaatg      240
gatcagcagtatgaacattttattccgtagatcgcatcatcgcaaggtgggtaggtgggg      300
taaagtagtaggaagtcacacatatgtgatgttcaagtacagacatattacaaatcaaag      360
agttcaagaattctttatatatctcactcattcccatttgcaaattcatatctatgaaca      420
accgtcttatacagttaaattctcatatcactcccaaaATGGCTTCCCAATCAGACATTC      480
                                        M  A  S  Q  S  D  I    7
ATCTCTACACAACCCAAACTCCTAATGGAATCAAGATATCGATCACTTTGGAGGAACTGG      540
H  L  Y  T  T  Q  T  P  N  G  I  K  I  S  I  T  L  E  E  L   27
GTCTTTCCTACGAGGTACACAAGATTGACATCTCTAAAAACACCCAAAAAGAGCCATGGT      600
G  L  S  Y  E  V  H  K  I  D  I  S  K  N  T  Q  K  E  P  W   47
TTCTTGAGATAAATCCCAATGGACGAATTCCCGCTTTGACCGATACATTCACGGATGGAA      660
F  L  E  I  N  P  N  G  R  I  P  A  L  T  D  T  F  T  D  G   67
AGAAGATCAATCTGTTTGAGAGTGGTAGCATTCAGCAATACCTCGTCGATAGATATGATA      720
K  K  I  N  L  F  E  S  G  S  I  Q  Q  Y  L  V  D  R  Y  D   87
CCGAGCATAAGATCTCATACCCAAAGGGTACAAGAGAGTACTATGAAGTCAACAACTGGg      780
T  E  H  K  I  S  Y  P  K  G  T  R  E  Y  Y  E  V  N  N  W  107
taaatgatgcaatgtttctagatctcaaaccaaacaagctgacatcatatctagCTCTTT      840
                                                    L  F  109
TTCCTCAATGCTGGTGTAGGTCCCATGCAAGGTCAAGCTAACCACTTCTCAAAATACGCC      900
 F  L  N  A  G  V  G  P  M  Q  G  Q  A  N  H  F  S  K  Y  A .129
CCCGAGAGGATCGAGTATGGTATCAATCGCTATACAAATGAGACCAGGCGTTTGTATTCT      960
 P  E  R  I  E  Y  G  I  N  R  Y  T  N  E  T  R  R  L  Y  S  149
GTCCTCAATACTCATCTAGAAAAATCAACCTCTGGTTATTgtaagtataactgactcctg     1020
 V  L  N  T  H  L  E  K  S  T  S  G  Y                       162
atacttgcttcgtgatcagccacttggtgcatatcttgcaatgcagttgttcaatttgct     1080
agggcatccctttccccttacccattttaaagaaatatcactaacaacatactttagTG      1140
                                                          L  163
GTTGGTGACAGATGTACAATCGCTGATATTGCTCATTGGGGTTGGGTAACTGCTGCCTTT     1200
 V  G  D  R  C  T  I  A  D  I  A  H  W  G  W  V  T  A  A  F  183
TACAGTGGAGTTGATATCGAGGAATTCCCAGCTCTCAAGGCATGGGATGAGAGAATGGAA     1260
 Y  S  G  V  D  I  E  E  F  P  A  L  K  A  W  D  E  R  M  E  203
AAGAGACCTGGAGTTGAGAAGGGCCGTCATGTACCAGATCCACACAACATCGGAGCCTTA     1320
 K  R  P  G  V  E  K  G  R  H  V  P  D  P  H  N  I  G  A  L  223
AAGAAAGACCCTGAACGTGAGGCAAAAATGAAGGCTGCGGCAGAGAAAGGTAGAGAATGG     1380
 K  K  D  P  E  R  E  A  K  M  K  A  A  A  E  K  G  R  E  W  243
ATCCAGGCAGGTATGAAGAGCGATGCCAAGAAATAAtttcttgacgagatcaagggtagc     1440
 I  Q  A  G  M  K  S  D  A  K  K  *                          254
agggctagagttcaataattgtcctacatttctgaatcaatcactgataagcgtttcttg     1500
aaatgataaaaattgcttttattgatatgttcgttcttttgtgctgtgaatctttactaa     1560
ctaagctcgaggctttgctctttcgcgatggatttcaatttgatcgcgcaattctaggcc     1620
actgacttatatttcagtgccggagaagtacatatctctaggtctaagtctaggcccaag     1680
tttgatttgacggaagctttgatacttgtagcaattttatgcaacaataaaccgtgtctg     1740
gtgtgacagacagttctcgggagatggtgaaaatataaagtgcgagcaattagttgatgt     1800
gaagggaaggaccatccatcttttctaatatgtgcatgcattcggaagtatcccacccat     1860
tggaactgccatgctgatccttttccttactacgttatc                          1899
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Figure 1. Nucleotide sequence of the *Botrytis cinerea Bcgst*1 gene (EMBL and GENBANK accession number AF061253). The deduced amino acid sequence is shown below the open reading frame. A potential stress-responsive element (TCAGGGGG, position 6-13) and a CREA binding site (GTGGGG, position 295-300) are underlined. Potential nitrogen-responsive (inverted) GATA/TATC boxes are double underlined. Two putative TATA boxes (positions 375-382 and 427-433) are indicated in bold.

```
B. cinerea      1       MASQSDIHLYTT.QTPNGIKISITLEELGLSYEVHK......IDISKNTQKEPWFLEINPNGRIPA
E. coli Yfcg    1       M.....IDLYFA.PTPNGHKITLFLEEAELDYRLIK......VDLGKGGQFRPEFLRISPNNKIPA
B. cepacia      1       M.....IELYTW.GTPNGRKVSIALEELGLAYNVHP......VDIMKGDQFTAGFLALNPNNKIPV
S. cerevisiae   123     ..............PNGFKVAIVLSELGFHYNTIF......LDFNLGEHRAPEFVSVNPNARVPA
E. coli Yghu    61      ......LQLYSL.GTPNGQKVTIMLEELLALGVTGAEYDAWLIRIGDGDQFSSGFVEVNPNSKIPA
S. pombe        1       MAH...FTLYSHAGGPNPWKVVLALKELNLSYEQIF......YDFQKGEQKCKEHLALNPNGRVPT
P. chrysosp.    1       MV....LKLYGNPMSTCTKRVATVLHEKGVPFELV   31

B. cinerea      60      LTDTFT.DG.KKINLFESGSIQQYLVDRYDTEHKISYPK..GTREYYEVNNWLFFLNAGVGPMQGQ
E. coli Yfcg            IVDHSPADGGEPLSLFESGAILLYLAEKT..GLFLSHE....TRERAATLQWLFWQVGGLGPMLGQ
B. cepacia              IKDSDSLDG.QPITLFESG.ILVYLADKTQRLLPVS.....GIAR.YETLQWLMFQMGGIGPMFGQ
S. cerevisiae           LIDH.GMDN...LSIWESGAILLHLVNKYYKETGNPLLWSDDLADQSQINAWLFFQTSGHAPMIGQ
E. coli Yghu            LRDHTHNPP...IRVFESGSILLYLAEKFGYFL......PQDLAKRTETMNWLFWLQGAAPFLGG
S. pombe                LVDHKNNDY....TIWESDAILIYLADKYDTDRKISLSF..DDPEYYKLIQYLFFQASGQGVIWGQ

B. cinerea      122     ANHFSKYAPERIEYGINRYTNETRRLYSVLNTHLEKSTSG.........................
E. coli Yfcg            NHHFNHAAPQTIPYAIERYQVETQRLYHVLNKRLENSP...........................
B. cepacia              THHFRRYAVQE.KYSLDRYTAETHRLYGVLNKRLAQSE...........................
S. cerevisiae           ALHFRYFHSQKIASAVERYTDEVRRVYGVVEMALAERREALVMELDTENAAAYSAGTTPMSQSRFF
E. coli Yghu            FGHFYHYAPVKIEYAINRFTMEAKRLLDVLDKQLAQHK...........................
S. pombe                AGWFNFFHHEPVVSAVTRYRNEIKRVLGVLEDILKDRD...........................

B. cinerea      162     ....YLVGDRCTIADIAHWGWVTAAFYSGV..............DIEEFPALKAWDERMEKRPGVE
E. coli Yfcg            ....WLGGENYSIADIACWPWVNAWTRQRI..............DLAMYPAVKNWHERIRSRPATG
B. cepacia              ....FVAGKEYSIADIAIYPWVSRFELHQL..............DWADVPHVRRWYDRAVGAPAVR
S. cerevisiae           DYPVWLVGDKLTIADLAFVPWNNVVDRIGIN.............IKIEFPEVYKWTKHMMRRPAV.
E. coli Yghu            ....FVAGDEYTIADMAIWPWFGNVVLGGVYDAAEFL.......DAGSYKHVQRWAKEVGERPAVK
S. pombe                ....YLVANKYTIADLSFIPWNYNLGGLFGEGKFSFKEEVPQLDFEKEFPKAYAWNQRLLARPAV.

B. cinerea      210     KGRHVPDPHNIGALKKDPEREAKMKAAAEKGREWIQAGMKSDAKK
E. coli Yfcg            ...........QALLK...........AQLGDE......RSDS
B. cepacia              RG........MGVLS
S. cerevisiae           ..........IKALRGE
E. coli Yghu            RGRIVNRTN..GPLN...EQLHERHDASFETNTEDKRQG
S. pombe                ..............KATFEELAKAKEQH
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Figure 2. Comparison of BcGST1 with GSTs from other organisms. *B. cinerea* GST1 (GENBANK AF061253, 254 amino acids), *E. coli* Yfcg (Blattner *et al*. 1997; GENBANK AE000319, 215 amino acids), *B. cepacia* ORF3 (Daubaras *et al*. 1995; GENBANK U19883, 205 amino acids), *S. cerevisiae* Ure2 (Coschigano and Magasanik 1991; GENBANK M35268, 354 amino acids), *E. coli* Yghu (G. Plunkett, unpublished results; GENBANK U28377, 304 amino acids), *S. pombe* ORF (V. Wood *et al*., unpublished results; GENBANK AL023590, 230 amino acids) and GST of *P. chrysosporium* (Dowd *et al*. 1997; 30 amino acids N-terminally sequenced). Bold amino acids are identical to BcGST1. Note that the first 122 amino acids of *S. cerevisiae* Ure2 and the first 60 amino acids of *E. coli* Yghu are not included in this alignment.

GSTs play a role in coping with chemical stress agents in several other organisms. Since the promoter region of the *Bcgst*1 gene contains a putative stress-responsive element, we investigated the expression of *Bcgst*1 under various stress conditions. Fungal cultures were exposed to UV, heat shock, osmotic shock, H_2O_2 and cycloheximide. The treatments were applied to mycelium from an overnight liquid culture and the mycelium was sampled after 0.5 and 3 h. Equal amounts of total RNA were electrophoresed and blotted. Hybridisation analysis was performed using probes for *Bcgst*1 and actin. The level of *Bcgst*1 mRNA increased in comparison with the appropriate control in mycelium that was treated either with high salt, H_2O_2 or cycloheximide (not shown). Some of the treatments (especially the heat treatment and 5 mM H_2O_2) reduced the ratio of the hybridisation signals of actin mRNA versus rRNA (not shown), probably as a consequence of mRNA turnover in response to the stress condition applied. A similar reduction of the ratio of actin mRNA versus rRNA was observed in liquid mycelium cultures that were depleted of nutrients (Wubben *et al*., 2000).

The response of *Bcgst*1 mRNA to H_2O_2 treatment was most pronounced and was investigated in more detail (Figure 3). Addition of 1 mM H_2O_2 transiently induced *Bcgst*1 mRNA to a maximal level at 1 h post application. Addition of 3 mM H_2O_2 resulted in a faster

transient induction and a higher level of *Bcgst*1 mRNA. Application of 5 mM H_2O_2 resulted in a continuously high transcript level during the entire course of the experiment (6 h; Figure 3). In the latter treatment the actin mRNA level was temporarily reduced, presumably as a consequence of mRNA turnover, but it recovered upon further incubation as a result of *de novo* transcription during growth resumption.

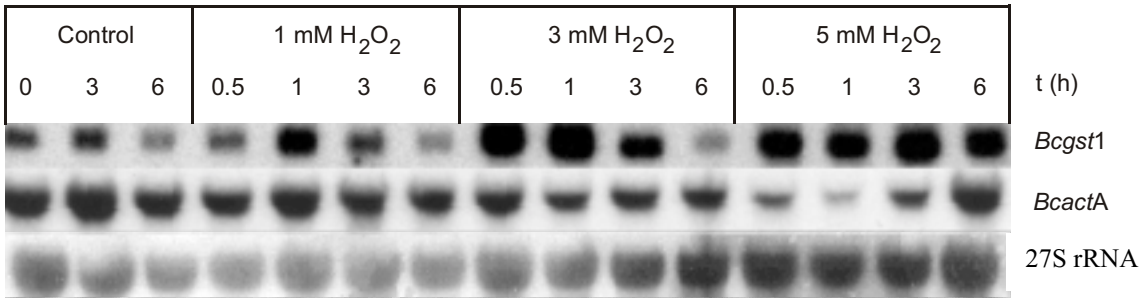

Figure 3. Hybridisation analysis of *Bcgst*1 expression in hydrogen peroxide-treated cultures of strain SAS56. Each lane contains 10 µg total RNA isolated from cultures treated with 0, 1, 3 or 5 mM H_2O_2, as indicated. Samples were taken at 0.5, 1, 3, and 6 h after addition of H_2O_2 as indicated. The blot was hybridised with the *Bcgst*1 probe, *B. cinerea* actin probe and the *B. cinerea* 27S rDNA probe, respectively.

In planta *expression of the* Bcgst*1 gene*

To analyse *Bcgst*1 expression during infection, detached tomato leaves were inoculated with a conidial suspension of *B. cinerea* strain SAS56, as described by van der Vlugt-Bergmans *et al.* (1997). A blot was made containing total RNA isolated from infected tomato leaflets as well as from *in vitro* cultures of *B. cinerea.* The proportion of fungal RNA in the total population of fungal and plant mRNAs is in the range of 1-5 % at 16 h.p.i. (Benito *et al.*, 1996), the time point at which RNA was harvested for performing the differential screening, that led to the isolation of the *Bcgst*1 gene. For a better comparison of hybridisation intensities, a lane was included in the blot in which RNA from an *in vitro* culture of *B. cinerea* was diluted 20x (lane 5 %). The blot was hybridised with a *Bcgst*1 probe, an actin probe (Figure 4) and a *B. cinerea* 27S rDNA probe (not shown). The actin mRNA signal increased over time, predominantly at 72 and 120 h.p.i. The 27S rDNA probe detects both plant and fungal rRNAs and was used to visualise equal loading of the blot (not shown). The level of *Bcgst*1 mRNA increases during the infection, and it follows the pattern of the actin mRNA. Thus, the *Bcgst*1 expression level remains more or less proportional to the fungal biomass during infection.

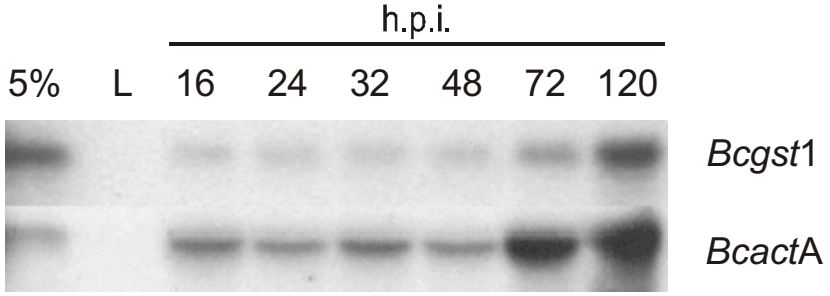

Figure 4. Hybridisation analysis of *Bcgst*1 expression *in planta*. Total RNA was isolated from liquid culture (lane 5 %), from uninoculated tomato leaf (L) and from *B. cinerea*-infected tomato leaves at different time points (numbers indicate h.p.i.), electrophoresed and blotted. In the left lane (marked 5 %), only one-twentieth of the amount of RNA was loaded as compared to the other lanes, to approximate the amount of fungal RNA in the infected leaves at 16 h.p.i. (Benito and van Kan, 1998; Benito *et al.*, 1996). The blot was hybridised with the *Bcgst*1 probe (upper panel) and the *B. cinerea* actin gene *Bcact*A (lower panel).

Targeted disruption of Bcgst*1*

Targeted disruption was carried out by a one-step insertion strategy, using the 698 bp *Eco*RV *Bcgst*1 fragment, which lacks 20 N-terminal and 66 C-terminal triplets of the ORF, cloned in a hygromycin selection marker plasmid (Figure 5A).

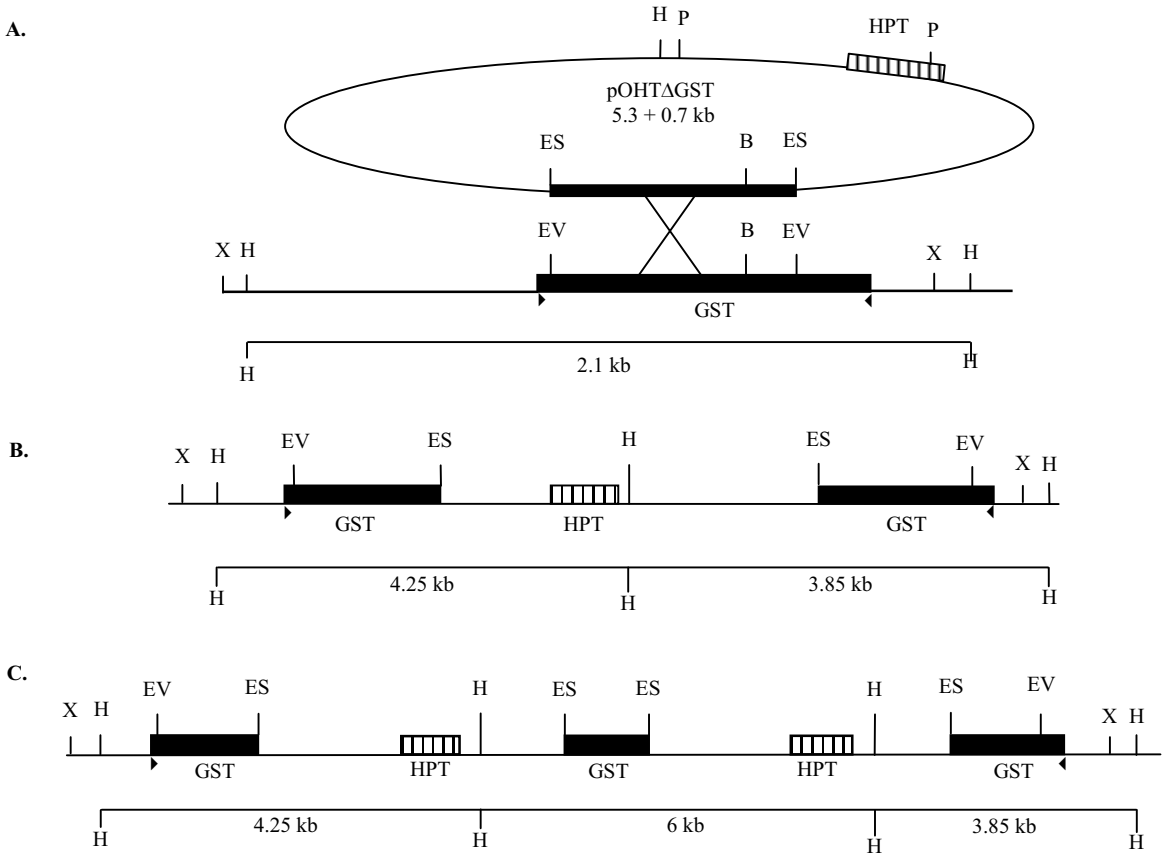

Figure 5. (A) pOHTΔGST was constructed by cloning the *Eco*RV fragment of *Bcgst*1 in the *Sma*I site of pOHT conferring resistance to hygromycin. Recombination occurred within the homologous *Eco*RV fragment. (B) Schematic representation of a single integration event. The hybridising *Xho*I fragment increases by 6.0 kb, whereas the *Hin*dIII digest in the recombinant results in two bands with the cumulative size of 8.1 kb. (C) *Hin*dIII digestion of a double integration event will yield an additional pOHTΔGST band, while *Xho*I will give a 13.4-kb band. B, *Bcl*I; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; X, *Xho*I; ES, site in which the *Eco*RV fragment is cloned into the *Sma*I site of pOHT; HPT, hygromycin phosphotransferase cassette; black bars represent the *Bcgst*1 ORF; hatched bars represent the HPT cassette; black triangles represent translation start and stop, respectively.

Transformants were subjected to Southern blot analysis to detect recombinants. Three independent transformants were identified that had a targeted insertion in the *Bcgst*1 gene. Digestions with *Hin*dIII and *Xho*I and hybridisation to the *Bcgst*1 probe (Figure 6) revealed that in one transformant a single integration had occurred, whereas in two transformants a double tandem integration had occurred. The configuration in the two types of homologous

recombinants is schematically represented in Figure 5 (panels B and C). Homologous recombination of pOHTΔGST leads to the disappearance of the 2.1 kb *Hin*dIII fragment in the wild type recipient strain. A single integration is predicted to result in two bands of 4.25 and 3.85 kb, while a double (tandem) integration should result in an additional third band of the transformation plasmid with a size of 6 kb.

Hybridisation of *Xho*I and *Hin*dIII-digests with the pOHT probe (not shown) confirmed these data. *Eco*RV digestion and subsequent hybridisation with an *Eco*RV-*Xho*I probe, downstream of the disruption fragment, confirmed that no rearrangements had taken place in this region (not shown).

RNA analysis of the wild type and the three transformants was carried out. Using comparable amounts of fungal RNA the wild type strain contained a discrete transcript of ± 900 nt., which was absent in the three transformants (not shown). Instead these transformants possessed a transcript of a much larger size resulting from read-through over the inserted hygromycin cassette, as was verified by using additional probes for the hygromycin cassette and for the 3' untranslated region of the *Bcgst*1 gene (not shown).

The virulence of two disruption mutants (1B2 and 1B4) was compared with the wild type recipient strain (B05.10) as well as with a control transformant with a single targeted integration of the pOHT plasmid in the *cut*A gene. The latter cutinase A-deficient transformant (T132) has a wild type level of virulence (van Kan *et al.*, 1997). No reduction of virulence was observed for the *Bcgst*1-deficient disruption mutants as compared to the controls.

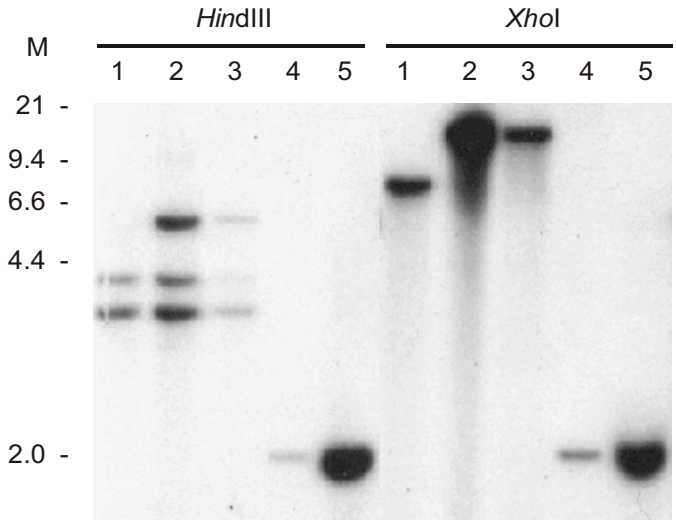

Figure 6. Southern blot analysis of the three independent *Bcgst*1 disruption mutants (1B2, 2B1, 3A3) and two wild type strains (SAS56 and B05.10). 1-3 µg genomic DNA was digested with *Hin*dIII and *Xho*I, respectively, electrophoresed and blotted. The blot was hybridised with the *Bcgst*1 probe. Lane 1, mutant 1B2; lane 2, mutant 2B1; lane 3, mutant 3A3; lane 4, wild type B05.10; lane 5, wild type SAS56. Lane M contains a size marker; sizes are indicated in the left margin in kilobasepairs.

Discussion

We have cloned a gene from *B. cinerea* encoding a glutathione S-transferase homologue. This is to our knowledge the first report of such a gene from a filamentous fungus. The gene was cloned in an attempt to isolate infection-specific fungal genes from a subtractive hybridisation RT-PCR screen. *Bcgst*1 occurs in a single copy in the genome of *B. cinerea* strain B05.10. The observation that *Bcgst*1 transcript levels are not specifically increased during the infection of tomato leaves as compared to expression during *in vitro* liquid cultures (see Figure 4), was unexpected and indicates that the cloning of this gene was a serendipity.

The genes aligned in Figure 2 show highest homology to *Bcgst*1. *E. coli* Yfcg and Yghu and the ORF from *S. pombe* have not been studied with respect to their GST activity. The Ure2 gene product from *S. cerevisiae* (Coschigano and Magasanik, 1991) is required for inactivation of glutamine synthetase upon addition of glutamine to cells growing on glutamate as the sole nitrogen source. The URE2 protein does not contain cysteine residues, lacks the arginine residue in the first conserved box proposed to be essential in the binding of glutathione (Ketterer *et al.*, 1988), and it has no detectable GST activity. Vuilleumier (1997) suggested that some GST homologues may encode proteins with a GSH-binding module, rather than being an active GST enzyme.

Whether the *Bcgst*1 gene product indeed possesses true GST activity remains to be determined. In extracts of *B. cinerea* grown under various stress conditions that induce GST activity in other organisms, we could hardly detect significant GST activity above background levels, using three different chromogenic substrates that are routinely used for quantifying GST activity (J. Diaz Varela and T.W.P., unpublished). In addition, no difference was observed in (the low levels of) GST activity between a wild type *B. cinerea* strain and the disruption mutants (results not shown). However, it might be that the enzyme is unstable, as was reported for GST from *Phanerochaete chrysosporium* (Dowd *et al.*, 1997).

The promoter region of the *Bcgst*1 gene contains several sequence elements that potentially regulate its expression. In spite of the presence of elements that match the fungal consensus for carbon and nitrogen-regulatory elements, *Bcgst*1 mRNA levels are not notably affected by external nitrogen and carbon sources, which questions the functionality of these elements.

Adding H_2O_2 to a mycelial suspension of *B. cinerea* resulted either in a transient (1 mM) or in a prolonged transcript accumulation (5 mM, see Figure 3). Possibly, this expression is mediated by the stress responsive element in the promoter region of the *Bcgst*1 gene. The addition of H_2O_2 to a mycelial culture partly mimics an oxidative stress that occurs *in planta* in *B. cinerea*-infected tissue during primary lesion formation (von Tiedemann, 1997). In the tomato leaf inoculation described here, necrotic lesion formation coincides with the induction of catalase gene expression by the host (van der Vlugt-Bergmans *et al.*, 1997). Moreover *B. cinerea* expresses an extracellular catalase during this stage of the infection (Schouten, unpublished), which possibly contributes to protecting the fungal hyphae from oxidative stress. We initially anticipated that this self-protection mechanism might also involve the *Bcgst*1 gene product described here, as well as ubiquitin, which is expressed to higher levels during early stages of the infection (Prins *et al.*, 2000b: Chapter 3). However, the observation that disruption of the *Bcgst*1 gene did not result in a decreased level of virulence suggests that the BcGST1 protein is not involved in the infection of tomato leaves.

It remains to be determined whether BcGST1 plays a role in the infection of one of the many other plant species that are hosts of *B. cinerea*. Preliminary experiments with grapevine (*Vitis vinifera*) leaves and broad bean (*Vicia faba*) leaves did not reveal a substantial reduction of virulence of the disruption mutants (T.W.P., unpublished). Further experiments with a range of other host plant species (e.g. cucumber or common bean, *Phaseolus vulgaris*)

or with different genotypes of a particular species remain to be carried out in order to establish whether the lack of phenotype is a general phenomenon, or specific for tomato.

It cannot be excluded that the *Bcgst*1 disruption mutants have a residual, yet unstable, enzyme activity due to translation of altered high molecular weight transcripts derived from the mutant *Bcgst*1 locus. Such a protein would be truncated, containing only 188 N-terminal amino acids followed by 26 amino acids encoded by the inverted *Aspergillus nidulans trp*C terminator. The hypothetical aberrant protein would retain the G-site responsible for GSH binding, residing in the N-terminus, but lack the H-site responsible for the binding of xenobiotic substrates, which is presumably situated near the C-terminus. In addition, there may be a gene in the *B. cinerea* genome that does not hybridise to *Bcgst*1, which functionally complements the GST activity in the *Bcgst*1 gene disruption mutants.

If BcGST1 would not be required for the ability of *B. cinerea* to infect host plants, what might its function be? Recent research in mammalian systems has suggested previously unexpected functions of the GSH pool (which is among others modulated by GST) in other cellular processes. Ward *et al.* (1998) reported that GSH and GSSG can irreversibly inactivate purified rat and human Protein Kinase C (PKC) by a non-redox mechanism. It was hypothesised that the inactivation of PKC can be relieved during oxidative stress as a consequence of GSH depletion. GSH may also act as a reversible inhibitor of neutral sphingomyelinase in human mammary carcinoma MCF7 cells (Liu *et al.*, 1998). GSH depletion may act as a trigger in the TNFα-induced cell death pathway. Pre-treatment of MCF7 cells with GSH inhibited TNFα-induced sphingomyelin hydrolysis, ceramide generation and cell death. A relation between GSH levels and death proteases was suggested since TNFα can activate caspases, which are involved in the initiation and execution of apoptosis (Liu *et al.*, 1998). Whether similar functions of the GSH pool and GST also occur in fungi in general, or more specifically in plant pathogens such as *B. cinerea*, remains to be investigated.

Experimental procedures

Fungal growth

B. cinerea was cultured as described by Benito *et al.* (1998: Chapter 2).

Subtractive hybridisation

Subtraction was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto, USA) according to the manufacturer's instructions. Tester poly A^+ RNA was derived from tomato leaves inoculated with *B. cinerea* strain SAS56, 16 h.p.i.. The proportion of fungal RNA in this sample of total RNA isolated from the infected leaf was estimated to be 5 %, as described by Benito *et al*. (1996) and Benito *et al*. (1998). Driver poly A^+ RNA consisted of a reconstituted mixture of 10 % RNA from a liquid culture of *B. cinerea* strain SAS56 and 90 % RNA from Tobacco Necrosis Virus-infected tomato leaves (Benito and Van Kan, 1998). The PCR mixture was run on a TAE-agarose gel (1 %). Several distinct bands were visualised, presumably enriched for differentially expressed cDNAs. One of the bands was excised, the DNA extracted by using GlassMax (Life Technologies) and labelled by random prime labelling. The labelling mixture was used to screen a genomic library of *B. cinerea* in EMBL3, constructed with the Packagene Lambda DNA packaging system (Promega) according to the manufacturer's instructions.

Long distance PCR

PCR amplification of long stretches of DNA (Barnes, 1994) was performed using the Advantage® Genomic Polymerase mix (Clontech) with proof-reading activity. LD-PCR was used to amplify phage inserts of the genomic library by using primers on the phage arms adjacent to the insert: EMBL-L (ATCATTATTTGATTTCAATTTTGTCCCACTCCC) and EMBL-R (GCGCACAAAACCATAGATTGCTCTTCTGTAAGG). Smaller fragments were obtained by using EMBL-L and EMBL-R primers in combination with internal GST gene specific primers.

Southern blot analysis

Genomic DNA of *B. cinerea* was isolated according to Drenth *et al.* (1993). Digestion, blotting and radiolabeling was according to Sambrook *et al.* (1989) and specified in van der Vlugt-Bergmans *et al.* (1997). Restriction enzymes and the [α-^{32}P] dATP labelling kit were obtained from Life Technologies.

Cloning of cDNA

Total RNA was isolated from mycelium of an overnight *B. cinerea* B05.10 liquid culture, elicited with 1 mM H_2O_2 for 1 h. RT-PCR (as described by van der Vlugt-Bergmans *et al.* 1997) was performed in the following way. By PCR, a *Hin*dIII site (AAGCTT, underlined) was introduced in front of the ATG start codon (bold) using primer GST-6 (5'ATCGCGAAGCTTCAT**ATG**GCTTCCCAATCAGAC 3'), together with a *Kpn*I site (GGTACC, underlined) behind the stop codon using primer GST-8 (5'GCGCGCGGTACCGCCCTGCTACCCTTGATCTCG 3'). The amplified cDNA was digested with *Hin*dIII and *Kpn*I and cloned in the vector pFLAG®-ATS (Kodak). The clone was sequenced and compared with the genomic sequence to verify the correct removal of introns that were predicted.

Sequence analysis

DNA sequencing was performed with Applied Biosystems, 377 DNA Sequencer using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase. Sequences were analysed using Lasergene software (DNAstar Inc., Madison, USA). Nucleotide and amino acid homology analysis was performed using the Blast program (Gish and States 1993; Altschul *et al.* 1997).

In vitro *induction experiments*

Overnight liquid cultures of *B. cinerea* with equal amounts of mycelial suspensions were exposed to different environmental stress conditions: UV (312 nm, total UV intensity 0.09 J cm^{-2} in 15 s) and heat shock (20'at 42°C) with a recovery period of 30' and 3 h. Osmotic stress (400 mM NaCl) and H_2O_2 stress (1 mM and 5 mM) was applied at t=0, after which 0.5 and 3 h recovery time was allowed. After 0.5 and 3 h, mycelium was harvested. Further induction experiments were performed with 1, 3 and 5 mM H_2O_2 and mycelium was sampled at 0.5, 1, 3 and 6 h. Control samples consisted of the same starting material handled in an identical way, however without applying the stress treatment.

To analyse the expression of *Bcgst*1 in response to nutritional conditions, 100 ml aliquots of an overnight pre-culture were transferred to fresh medium supplemented with different

combinations of carbon source (1 % Glucose), nitrogen source (100 mM NH_4Cl) and protein (1 % BSA). After 3 and 6 h, mycelium was harvested.

RNA blot analysis

Total RNA from infected leaves and liquid grown mycelium was isolated by vortexing the freeze-dried mycelium in a tube with a metal spatula in liquid nitrogen and extracting the powder with 2 ml/g guanidine buffer (8.0 M guanidine hydrochloride; 20 mM MES; 20 mM EDTA; 50 mM β-mercaptoethanol pH 7.0). After phenol: chloroform: isoamylalcohol (25:24:1) extraction and centrifugation (45' 10,000 rpm at 4°C), a chloroform: isoamylalcohol (24:1) extraction was repeated twice. RNA was precipitated by addition of 1/3 vol. 8 M LiCl, incubation o/n at 4°C and centrifugation at 10,000 rpm. The resulting pellet was washed twice with 2 M LiCl, twice with 70 % EtOH, dried and dissolved in water. After measuring the OD_{260}, 10 µg of RNA was denatured with glyoxal/DMSO (Sambrook *et al.*, 1989), subjected to electrophoresis and blotted to Hybond N^+ membranes (Amersham). Blots were hybridised as described for Southern blots. As probes, the 698 bp *Eco*RV fragment of *Bcgst*1, a fragment of the actin gene (Benito *et al*., 1998) and a fragment of the *B. cinerea* 27S rDNA gene (Wubben *et al*., 2000) were used.

Bcgst*1 gene disruption in* Botrytis cinerea

An *Eco*RV fragment of 698 bp, situated in the middle of the *Bcgst*1 ORF, was cloned into the *Sma*I site of plasmid pOHT (van Kan *et al*., 1997), containing an *Aspergillus nidulans oli*C promoter (Turner *et al*., 1989), HPT (conferring resistance to Hygromycin) and an *A. nidulans trp*C terminator. The resulting plasmid, designated pOHTΔGST, was transferred to JM110. Plasmid DNA was isolated and digested with *Bcl*I at position 515 of the insert, since homologous recombination may be more efficient with linearised DNA (Bird and Bradshaw, 1997). A mixture of *Bcl*I-digested and undigested plasmid was transformed to *B. cinerea* as described by Hamada *et al.* (1994) and modified by van Kan *et al.* (1997) with minor adjustments. Transformants were selected on increasing hygromycin concentrations and subsequently transferred to non-selective medium for sporulation. Conidia were plated at low density on hygromycin and the emerging colonies propagated for Southern blot analysis, in order to detect homologous recombinants and to verify the absence of the wild type locus. A proportion of the correctly targeted homologous recombinants were heterokaryotic, as they still contained a small proportion of untransformed wild type nuclei (see also van Kan *et al*. 1997). Such recombinants were subjected to additional rounds of single conidia plating until Southern blot analysis demonstrated that they no longer contained wild type nuclei.

Virulence assay

Droplet inoculation (as described by Benito *et al.*, 1998) was performed on detached tomato leaves cv. Moneymaker-*Cf4* at 20° C with a 16-h photoperiod and at 4° C in the dark. Wild type strain B05.10 was applied on the left side and transformants on the right side of the leaflets. Eight droplets of 2 µl were applied on each side. After 3 days (20° C) or 7 days (4° C), the diameter of spreading lesions was measured. Statistical analysis was performed with the students' *t*-test (two-tailed distribution, two-sample unequal variance) on each leaflet and on accumulated data of multiple leaflets.

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CHAPTER 5

Aspartic protease BcAP1 is not a virulence factor for *Botrytis cinerea*

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Summary

A single copy gene *Bcap*1 was cloned from *Botrytis cinerea*, encoding a protein homologous to Aspartic Protease (AP). It has been suggested that AP functions as a potential virulence factor for *B. cinerea* (Movahedi and Heale, 1990a). The expression of *Bcap*1 was investigated under various nutritional conditions, *Bcap*1 is induced by exogenous protein at low free nitrogen concentrations. The *Bcap*1 transcript appears at 24 h post infection of tomato leaves with *B. cinerea* and steadily increases in time. The role of the *Bcap*1 gene in virulence of *Botrytis cinerea* was assayed by comparing *Bcap*1 gene disruption mutants with wild type *B. cinerea*. Two independent disruption mutants were carefully evaluated but neither of the mutants showed a decrease in virulence on tomato leaves.

The nucleotide sequence data are in the EMBL database under accession number AF121229.

Introduction

Botrytis cinerea Pers.: ex. Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is a typical necrotrophic phytopathogenic fungus with a broad host range. In early studies, fungal enzymes and secondary metabolites were analysed that are produced during pathogenesis (reviewed in Staples and Mayer, 1995). This was more recently extended to a functional molecular-genetic analysis (reviewed in Prins *et al.*, 2000b: Chapter 1). Fungal invasion initially leads to localised host cell death as a result of a hypersensitive response (Govrin and Levine, 2000). It was suggested that *B. cinerea* exploits the hypersensitive response by actively stimulating cell death and feeding on dead tissue (Govrin and Levine, 2000).

Proteases have frequently been suggested to function as a pathogenicity factor for *B. cinerea* (Karmona *et al.*, 1990; Movahedi and Heale, 1990a, 1990b; Touzani *et al.*, 1994; Lorito *et al.*, 1994; reviewed in Staples and Mayer, 1995). Proteases are subdivided into serine (EC 3.4.21), cysteine (EC 3.4.22), aspartic (EC 3.4.23) and metallo (EC 3.4.24) proteases, each with their own specific activity (Barrett, 1980). Most aspartic proteases are inhibited by the pentapeptide pepstatin, with some exceptions (van den Hombergh, 1996).

Aspartic protease genes have previously been cloned from the phytopathogenic fungi *Cryphonectria parasitica* (Razanamparany *et al.*, 1992) and *Glomerella cingulata* (Clark *et al.*, 1997), as well as from saprophytic fungi (Gente *et al.*, 1997; Paoletti *et al.*, 1998; van den Hombergh, 1996). The role of the genes from *C. parasitica* and *G. cingulata* in pathogenesis has not been investigated.

However, Movahedi and Heale (1990a, 1990b) observed that during early infection of carrot tissue by *B. cinerea*, extracellular secreted aspartic protease activity was detected before pectolytic activity. Furthermore, pre-treatment of conidia with the aspartic protease inhibitor pepstatin markedly reduced the infection. Also in *Sclerotinia sclerotiorum*, a close relative of *B. cinerea* with a similar infection strategy (see Chapter 1), aspartic protease activity was detected at early stages of infection (Poussereau *et al*., 1998; Poussereau *et al*., 2001). This suggests that aspartic proteases might play a role in virulence of *B. cinerea* and *S. sclerotiorum*. To validate this hypothesis at the molecular level, an aspartic protease gene (*Bcap*1) was isolated from *B. cinerea*. Its role in virulence was investigated by creating disruption mutants and comparing them with the wild-type strain in virulence assays.

Results

Isolation and characterisation of the Bcap*1 gene*

RT-PCR with degenerate primers was used to amplify aspartic protease genes of *B. cinerea*. Amplified cDNA fragments with the expected length were subsequently cloned. One of the cloned fragments showed homology to aspartic proteases (APs). This fragment was used as a probe to screen a genomic library of *B. cinerea* strain SAS56.

Southern analysis (Figure 1) was performed to determine the copy number with a 495 bp aspartic protease-specific fragment as probe at low stringency. The probe detected a single band in each lane, suggesting the presence of a single copy gene.

The DNA sequence (Figure 2) contains an ORF with homology to APs from a range of different organisms. The cloned gene was therefore designated *Bcap*1. The open reading frame is interrupted by two introns (52 bp and 56 bp, which was confirmed by sequencing a full-length cDNA clone of *Bcap*1). The encoded protein, denominated BcAP1, has a length of 432 amino acid residues with a predicted molecular weight of 46 kD and a pI of 4.5.

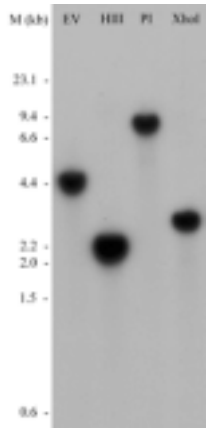

Figure 1. Autoradiogram of Southern blot.
2 µg/lane *B. cinerea* B05.10 DNA digested with *Eco*RV (EV), *Hin*dIII (HIII), *Pst*I (PI) and *Xho*I, hybridised with the BcAP1 probe. None of the restriction enzymes cuts in the *Bcap*1 probe.

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gatcatctgaaagctaggtatcttagcgggggaagggagctctggagataagagaaacttacaatgcttaa          70
cagcatttaaaggcagtgtttccctcaataaacttattctctcctaaaccatctatctctaaatcacatc          140
tacttacaaaagaattattattaactaaatttccatccaccttgatcaaattactgaacATGGCTTCTC          210
                                                          M  A  S       3
TCCAAGGCTTGTCAAAGATCAGACTGATTCCTAACAAGAACTACAAAAGATCCGGCACCAAATCATATGT          280
L  Q  G  L  S  K  I  R  L  I  P  N  K  N  Y  K  R  S  G  T  K  S  Y  V      27
ATATCTACTCAACAAATGGGGCTTTGAGCCCACTAAACCTGGACCATACTTCCAGATGAACAAGGCCACC          350
  Y  L  L  N  K  W  G  F  E  P  T  K  P  G  P  Y  F  Q  M  N  K  A  T      50
GCAACTTCAGCCAGCTTCCACAAGTTTGGCCATAAATCACAAACTCAACGTGTGTTGGCAAAGAAGACCG          420
 A  T  S  A  S  F  H  K  F  G  H  K  S  Q  T  Q  R  V  L  A  K  K  T      73
CTACCGGGGAGAACGGTGAAGTCCCAGCAGAAGACCAACAAAATGACTCTGAGTATCTCTGCCCTGTGCA          490
A  T  G  E  N  G  E  V  P  A  E  D  Q  Q  N  D  S  E  Y  L  C  P  V  Q      97
GATTGGTACGCCGGCACAAACTCTAATGCTAGACTTTGATACTGGATCGTCGGATTTATGGgtaggtaaa          560
  I  G  T  P  A  Q  T  L  M  L  D  F  D  T  G  S  S  D  L  W             117
caaatgtttggtttccgcgacgaaaactaacatgccgagatagGTCTGGTCCACAGAACTGCCCAAAGCC          630
                                              V  W  S  T  E  L  P  K  A     126
ACAACTTCCAATGCAACCGGCCACACAATTTTCGACCCAAAAAAGTCTTCCACTTTCAAAGCTGCCAAGA          700
 T  T  S  N  A  T  G  H  T  I  F  D  P  K  K  S  S  T  F  K  A  A  K      149
GTTCCAAGTGGCAAATCTCATATGGTGATTCCTCTTCTGCTTCAGGTACCGTTGGAACAGATACTGTTTC          770
S  S  K  W  Q  I  S  Y  G  D  S  S  S  A  S  G  T  V  G  T  D  T  V  S     173
TCTTGGTGGTCTGGCCATCAAAAACCAGGCAGTTGAGCTTGCAACCAAGTTGTCTGCTCAATTTGAACAG          840
  L  G  G  L  A  I  K  N  Q  A  V  E  L  A  T  K  L  S  A  Q  F  E  Q     196
GGTGCCGGCGATGGATTGCTTGGTCTCGCATGGGGTAGCATCAACACCGTCACACCCACACCAGTTGCTA          910
 G  A  G  D  G  L  L  G  L  A  W  G  S  I  N  T  V  T  P  T  P  V  A     219
CACCTGTTGAGAACATGATCAGCCAAGAAGACATCCCATCAGACGCCGAGCTTTTCACTGTAAATCTCGG          980
T  P  V  E  N  M  I  S  Q  E  D  I  P  S  D  A  E  L  F  T  V  N  L  G     243
TAGTTGGCGTGACGCCGACGAAGCCGACAAAGGTGCTAGTTTCTACACATTTGGCTACATCGACCAAGAC         1050
  S  W  R  D  A  D  E  A  D  K  G  A  S  F  Y  T  F  G  Y  I  D  Q  D     266
GTTGTTGGAAGCCAAGAAATTTACTACACTCCTGTGGACAACTCTCAAGGATTCTGGATGTTTGACTCCA         1120
 V  V  G  S  Q  E  I  Y  Y  T  P  V  D  N  S  Q  G  F  W  M  F  D  S     289
CATCTGCTACTGTCAATGGCAAGACTGTTGCGCAGACCGGTAACCAAGCCATTGCCGATACCGGAACTAC         1190
T  S  A  T  V  N  G  K  T  V  A  Q  T  G  N  Q  A  I  A  D  T  G  T  T     313
CCTTGCATTGGTCTCTGATGAAACTTGTCAAGCCATCTACGATGCAATCCCGGGATCTACTTATGATTCG         1260
  L  A  L  V  S  D  E  T  C  Q  A  I  Y  D  A  I  P  G  S  T  Y  D  S     336
GAACAACAAGGATACACATTCCCAAGTAACACCAGCGCTGACGATCTCCCTGTCGTCACTTTCGCTGTTG         1330
 E  Q  Q  G  Y  T  F  P  S  N  T  S  A  D  D  L  P  V  V  T  F  A  V     359
GCGGCAAGCAATTTGCAGTTCAAAAGGAAGATTTGGGATTTGCGGATGCGGGTAATGGTATGGTATATGG         1400
G  G  K  Q  F  A  V  Q  K  E  D  L  G  F  A  D  A  G  N  G  M  V  Y  G     383
CGGTATCCAATCCAGAGGCTCTATGACTTTTGATATTCTTGGTGACACTTTCCTCAAGGGAATTTATGCT         1470
  G  I  Q  S  R  G  S  M  T  F  D  I  L  G  D  T  F  L  K  G  I  Y  A     406
gtaagcttcatcccccactcatttctgatcgtacgaatcactaacattttctctagATCTTTGATCAAGG         1540
                                                    I  F  D  Q  G     411
TAACACCCGATTTGGAGCCGTTCAAAGAACCGAAGCTACACAGAACACAGCCGCTCCCCCAGCTtagatt         1610
  N  T  R  F  G  A  V  Q  R  T  E  A  T  Q  N  T  A  A  P  P  A  *      432
attttcaggcatggagtatggttttggaactttgtttatttgggaggatttgtcaggctatgggtttggt         1680
tgggagtgttcgggtcataggcaacaggggcacatcttgttgttggatgtgttgtttctttgggctttat         1750
cataacctagggttagaatcgattattcttccttcttgatttcttgttcatgcgatggtattgtgaagat         1820
gagatgatttgatgaattgcatacgtcatgataacgaatcctgaatttcggcgagatgtcccaagtatgt         1890
tacctctagtgagtctgctagattgaattaaaatctgcgtcctggattgaatacttgagtaaatgactag         1960
acaagaaaaaacaaatgtctatagagatattgaagatggcatttctactcattgaaacttcattgtgcca         2030
cctactttgccttgcttgcagcatcttcgtcagcaagtaaccctaa                                 2076
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Figure 2. Nucleotide sequence of the *B. cinerea Bcap*1 gene
The deduced amino acid sequence is shown below the open reading frame. DTG motifs and the putative GG motif necessary for psi-loops are indicated by a dotted line. Putative *CreA* and AREA-homologue binding sites in the promoter are indicated in bold.

BcAP1 is classified as an aspartic protease (EC 3.4.23) since it contains the conserved DTG motifs (amino acid positions 110 and 309). Enzymes belonging to the pepsin family show a bilobed structure where the active site cleft is located between the lobes (Rao *et al.* 1998), and both DTG motifs contribute to the catalytic mechanism. Characteristic sequence motifs known as psi-loops, consisting of a "hydrophobic-hydrophobic-G" motif are usually located 60 amino acid residues downstream of the DTG motif (Hill and Phylip, 1997) and are also present in BcAP1. BcAP1 contains three N-glycosylation signals (consensus N-X-S/T) at amino acid residues 88, 130 and 346. N-glycosylation is common for pepsin type aspartic proteases (Paoletti *et al.*, 1998; North, 1982; van den Hombergh, 1996). BcAP1 contains two cysteine residues, but they do not align with conserved cysteine residues in other aspartic proteases (Clark *et al.*, 1997) where they are known to form a disulfide bond (Cutfield *et al.*, 1995). At the N-terminus of BcAP1, no signal sequence (Nielsen *et al.*, 1997) was found, suggesting that the protein is not secreted.

Alignment of BcAP1 with sequences in the database revealed homology to fungal secreted APs (Figure 3). Highest identity was found with SAP of *G. cingulata* (Clark *et al.*, 1997; 41 % identity), pepA of *Aspergillus oryzae* (Gomi *et al.*, 1993; 40 % identity) and eapA of *C. parasitica* (Razanamparany *et al.*, 1992; 39 % identity). These genes all contain a predicted signal peptide of 16-20 amino acids and a potential pro-protein processing site (Figure 3).

```
BcAP1   1       ------MASLQGLSKIRLIPNKNYKRSGTKSYVYLLN---KWGFEPTKPGPYFQMNKATA
SAP             ------MTLITALTAGLALAS---SVIGAPTNNANEK---RFTVDQIKN-PRYIRNGPLA
pepA            ---MVILSKVAAVAVGLSTVASALPTGPSHSPHARRGFTINQITRQTRAVGPKTASFPAI
eapA            MSSPLKNALVTAMLAGGALSSPTKQHVGIPVNASPEVGPGKYSFKQVRN-PNYKFNGPLS

BcAP1   44      TSASFHKFGHKSQ----------TQRVLAKKTATGENGEVPAEDQQNDSEYLCPVQIGTP
SAP             LAKAYRKYGKALPEDLSRVVANITSTGATKR-ATG---SVAATPQDYDVEYLSPVQIGTP
pepA            YSRALAKYGGTVP----------AHLKSA--VASGH-GTVVTSPEPNDIEYLTPVNIGG-
eapA            VKKTYLKYGVPIPAWLEDAVQNSTSG-LAER-STGSATTTPIDSL--DDAYITPVQIGTP

BcAP1   95      AQTLMLDFDTGSSDLWVWSTELPKATTSNATGHTIFDPKKSSTFKAAKSSKWQISYGDSS
SAP             AQTLTLDFDTGSSDLWVFSTSTP---SSQRNGQTVYDPSKSSTASRLTGATWSISYGDGS
pepA            -TTLNLDFDTGSADLWVFSEELPK---SEQTGHDVYKPSGNASK--IAGASWDISYGDGS
eapA            AQTLNLDFDTGSSDLWVFSSE---TTASEVDGQTIYTPSKSTTAKLLSGATWSISYGDGS

BcAP1   155     SASGTVGTDTVSLGGLAIKNQAVELATKLSAQF-EQGAG-DGLLGLAWGSINTVTPTPVA
SAP             SSSGIVYKDTVSVGSLSVTGQAVEAASKVSSSFSEESDL-DGLLGLGFSSINTVSPTQQK
pepA            SASGDVYQDTVTVGGVTAQGQAVEAASKISDQF-VQDKNNDGLLGLAFSSINTVKPKPQT
eapA            SSSGDVYTDTVSVGGLTVTGQAVESAKKVSSSFTEDSTI-DGLLGLAFSTLNTVSPTQQK

BcAP1   213     TPVENMISQEDIPSDAELFTVNLGSWRDADEADKGASFYTFGYIDQDVVGSQEIYYTPVD
SAP             TFFETAKSKLDAY----LFTADLK--------HNTPGKYNFGYIDSSAYTG-AITYVSID
pepA            TFFDTVKDQLDAP----LFAVTLK--------YHAPGSYDFGFIDKSKFTG-ELAYADVD
eapA            TFFDNAKASLDSP----VFTADLG--------YHAPGTYNFGFIDTTAYTG-SITYTAVS

BcAP1   272     NSQGFWMFDSTSATVNGKTVAQTGNQAIADTGTTLALVSDETCQAIYDAIPGSTYDSEQQ
SAP             NSDGWWQFTSSGYSVGSASFTSTSLNGIADTGTTLLLLPQSVVTAYYAKISGAKYDSSQG
pepA            DSQGFWQFTADGYSVGKGDAQKAPISGIADTGTTLVMLDDEIVDAYYKQVQGAKNDASAG
eapA            TKQGFWEWTSTGYAVGSGTFKSTSIDGIADTGTTLLYLPATVVSAYWAQVSGAKSSSSVG

BcAP1   332     GYTFPSNTSADDLPVVTFAVGGKQFAVQKEDLGFADAGNG--MVYGGIQSRGSMTFDILG
SAP             GYTFPC---SATVPSFTFGVGSARVTIPASYMNYAPVST--STCFGGLQSSSGIGINIFG
pepA            GYVFPC---ETELPEFTVVIGSYNAVIPGKHINYAPLQEGSSTCVGGIQSNSGLGLSILG
eapA            GYVFPC---SATLPSFTFGVGSARIVIPGDYIDFGPISTGSSSCFGGIQSSAGIGINIFG

BcAP1   390     DTFLKGIYAIFDQGNTRFGAVQRTEATQNTAAPPA          432
SAP             DVALKAAFVVFDGASNRLGWAAKTLS                   407
pepA            DVFLKSQYVVFDSQGPRLGFAAQA                     404
eapA            DVALKAAFVVFNGATTPTLGFASK                     419
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Figure 3. Comparison of BcAP1 with homologous aspartic proteases.
B. cinerea AP1 (this paper, EMBL AF121229, 432 amino acids), *Glomerella cingulata* SAP (Clark *et al*., 1997, EMBL U43775, 407 amino acids, secreted), *Aspergillus oryzae* pepA (Gomi *et al*., 1993, EMBL D13894, 404 amino acids, extracellular), *Cryphonectria parasitica* eapA (Razanamparany *et al*., 1992, EMBL X63351, 419 amino acids, extracellular). The predicted N-terminal signal peptides are underlined. The propeptide processing sites are double underlined. Amino acids identical to BcAP1 are indicated in bold. The two DTG motifs are boxed.

In planta *and* in vitro *expression of* Bcap*1*

*Bcap*1 gene expression was monitored with RNA that was isolated from tomato leaves infected with *B. cinerea* strain SAS56. Northern blot analysis of total RNA using Bcap1 as a probe is shown in Figure 4. Hybridisation with the actin gene (Benito *et al.*, 1998) reflects the proportion of fungal mRNA in the total population of plant and fungal RNAs. The 27 S *B. cinerea* ribosomal probe (Prins, unpublished) was used to visualise equal loading of the blot. *Bcap*1 transcript is first detected at 24 h.p.i. and steadily increases in time.

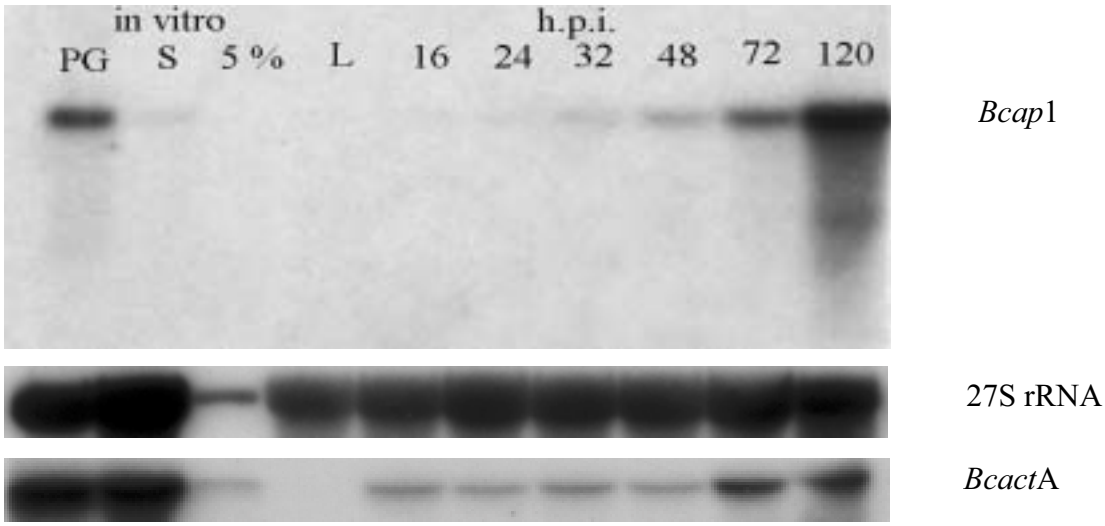

Figure 4. Expression of *Bcap*1 mRNA *in planta*.
Total RNA isolated from liquid cultures. PG: with 2 % polygalacturonic acid and 10 mM phosphate; S: with 10 mM sucrose and 10 mM phosphate; 5 %: sample identical to S, but only 1/20th of the amount of RNA, from uninoculated tomato leaves (L) and from *B. cinerea* infected tomato leaves at different time points (numbers indicate hours post inoculation). The blot was hybridised with the *Bcap1* probe (top panel) actin (*Bcact*A bottom panel) and a ribosomal probe (27 S rDNA middle panel).

In fungi, aspartic protease expression is often induced by exogenous protein and repressed by ammonium salts. In *Aspergillus nidulans*, the CREA protein represses the expression of genes that are involved in the use of less-favoured carbon sources (van den Hombergh, 1996). CREA binds to the consensus sequence 5'-SYGGGG-3' (Ronne, 1995). In *Bcap*1, a putative binding site for a CREA-homologue is present at –169 bp (GCGGGG). AREA, implicated in mediating nitrogen metabolite repression in *A. nidulans*, binds to the consensus sequence GATA (van den Hombergh, 1996). In the *Bcap*1 promoter region, two putative high affinity binding sites for AREA-homologues (Marzluf, 1997) have been found within 30 bp distance at –151 and –179 bp.

Because of the presence of the CREA consensus sequence and the GATA core sequences in the promoter region of the *Bcap*1 gene, expression of the gene was investigated under different carbon and nitrogen regiments. Liquid cultures of strain B05.10 were supplemented with different combinations of glucose, ammonium and exogenous protein (BSA). RNA was isolated at various timepoints and analysed by northern blot hybridisation. Figure 5 demonstrates that the level of *Bcap*1 transcript was not influenced by different carbon regimes, but it was induced when BSA was the sole nitrogen source. The *Bcap*1 transcript level was low when NH_4Cl was added alone or together with BSA, indicating that an excess of ammonium represses expression of the gene. In the lower panel of Figure 5, hybridisation with the actin probe is depicted to visualise loading of the gel.

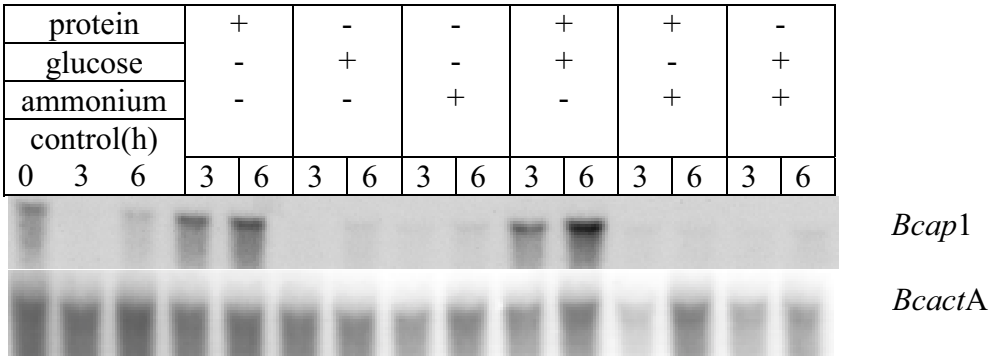

Figure 5. Expression of *Bcap*1 mRNA under different nutrient regimes.
A preculture grown overnight in B5 medium was supplemented with glucose (1 % w/v), ammonium chloride (100 mM) and protein (BSA 1 % w/v) as indicated. Each lane contains 10 µg fungal RNA. The blot was hybridised with the *Bcap*1 probe (top panel) and the actin probe (*Bcact*A, bottom panel).

Disruption of Bcap*1*

Targeted disruption was carried out by a one-step insertion strategy. A 495 bp PCR fragment that encodes amino acid residues 118-267 was cloned in a hygromycin selection marker plasmid. In Figure 6, the possible recombination products are depicted. In case of a single integration of the vector (6B), the fragment produced by adjacent *Xho*I sites will increase by 5.8 kb while *Hin*dIII will yield a double band, resulting from disruption of the *Bcap*1 ORF. *Hin*dIII-digestion of a tandem integration event (6C) will yield the same bands as in B, plus an additional pOHTΔAP of 5.8 kb; the hybridising *Xho*I fragment will increase by 11.6 kb.

Transformants were subjected to Southern analysis (Figure 7). DNA from two independent transformants was digested with *Hin*dIII and hybridised with the *Bcap*1 probe. Transformant 43 appeared to have a single and transformant 5B a double integration of pOHTΔAP. The 2.2 kb wild type band was absent in both transformants (Figure 7a). *Hin*dIII-digestions hybridised with the pOHT probe (Figure 7b) visualise the tandem or single integration of the plasmid. *Xho*I-digestions and hybridisation with *Bcap*1 and pOHT probes (not shown) confirmed these data. DNA of the transformants was digested with *Eco*RV and Southern analysis with probes adjacent to the disruption fragment (data not shown) confirmed that no rearrangements had occurred in the flanking regions.

RNA of the recipient strain B05.10 and of transformants 43 and 5B was hybridised with the *Bcap*1 probe (Figure 8a). The transformants did not show a transcript of wild type size, as expected. However, they still produced *Bcap*1 transcripts much larger than the wild type, due to read-through over the inserted plasmid. An actin probe was used to show mRNA levels on the blot (Figure 8b). The 27S ribosomal probe (Figure 8c) was used to visualise equal loading of the gel. Transcript levels of 27S (Figure 8c) are comparable for both B05.10 and transformants while transformants show an increase in actin transcript levels (Figure 8b).

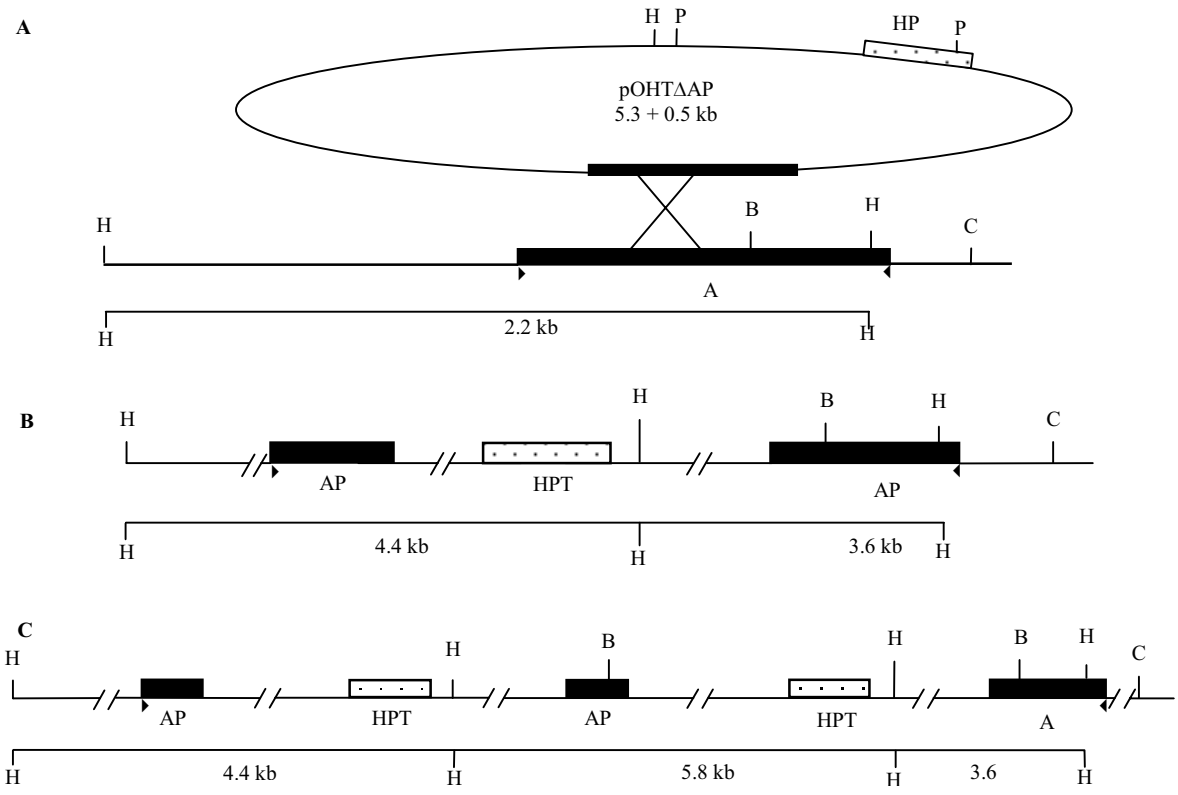

Figure 6. Schematic representation of disruption events.
pOHTΔAP was constructed by cloning the BcAP1-BcAP6 PCR fragment of *Bcap1* in the *Sma*I site of pOHT. Recombination occurred within the homologous PCR fragment. B. Single integration. C. Tandem integration.
B = *Bcl*I, C = *Cla*I, H = *Hind*III, P = *Pst*I, HPT = Hygromycin Phosphotransferase cassette, black bars represent BcAP1 ORF, dotted bars represent HPT cassette, black triangles represent translation start and stop, respectively.

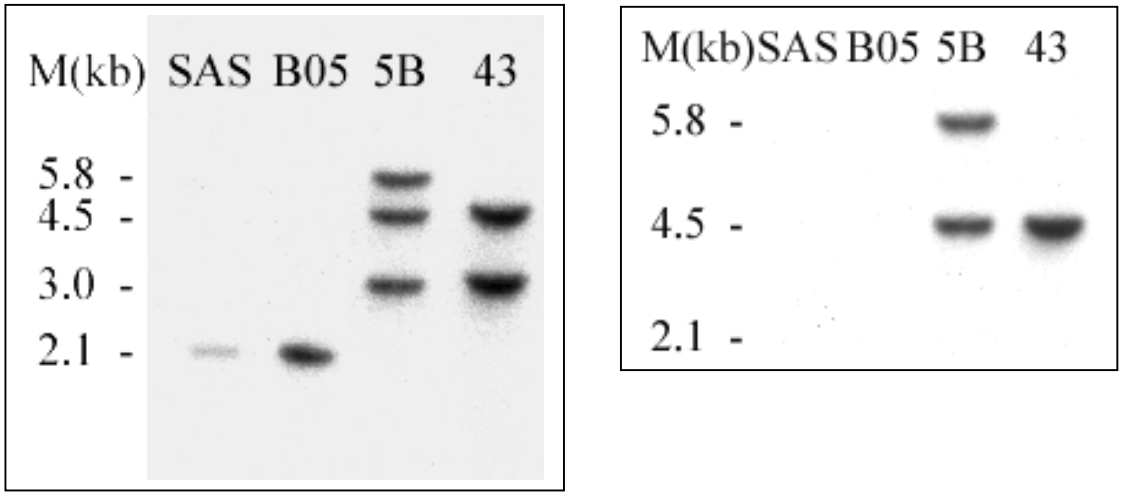

Figure 7. Southern blot analysis of DNA isolated from wild type SAS56, B05.10 and aspartic protease disruption mutants 5B and 43.
One µg of DNA was digested with *Hin*dIII. The left panel is hybridised with the *Bcap*1 probe and the right panel with a pOHT probe to confirm tandem and single integration (see Figure 6 for reference).

Phenotype of Bcap*1-deficient mutants*

B. cinerea conidia were inoculated onto detached leaves of tomato and broad bean and the leaves were incubated at 20° C or 4° C. Mutants 5B and 43 were compared with *B. cinerea* wild type strains SAS56 and B05.10. T132, a cutinase A-deficient mutant (van Kan *et al*., 1997) containing the hygromycin resistance cassette, was included as vector control. Several experiments were conducted to compare the virulence of the *Bcap*1 disruption mutants with the controls. Primary lesions, indicative for a successful infection, and secondary infection, i.e. outgrowth of the primary lesion into a spreading lesion, were scored for both number and diameter. In none of the experiments a statistically significant difference was observed between the controls and the *Bcap*1 disruption mutants (data not shown). No differences in lesion morphology, rate of lesion development or growth rate on maltose broth were observed between the *Bcap*1 disruption mutants and controls. A protease plate assay was performed on agar containing casein or skimmed milk by measuring the diameter of the halos around the colonies, resulting from protein breakdown by secreted proteases. No difference in halo formation was observed between the *Bcap*1 disruption mutants and controls. Thus, no discernible phenotype could be found for the *Bcap*1 disruption mutants under the conditions tested.

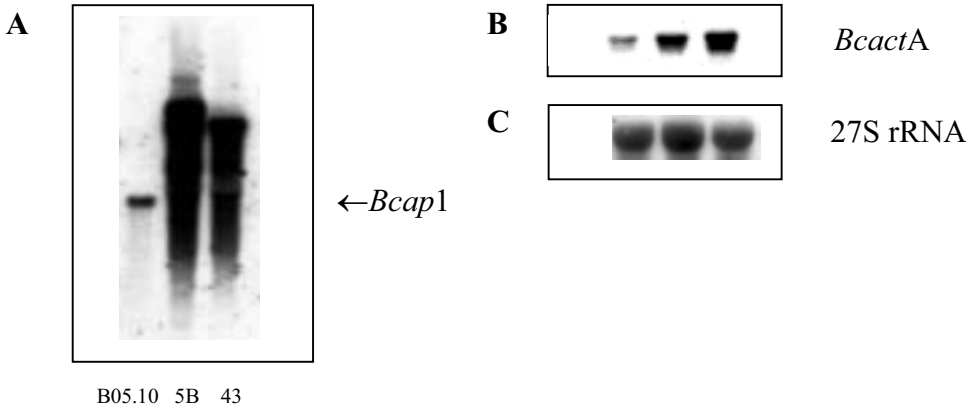

Figure 8. Northern blot analysis of wild type and *Bcap*1 disruption mutants.
Northern blot containing 10 µg of RNA from wild type B05.10 and *Bcap*1 disruption mutants 5B and 43. The blot was hybridised with the *Bcap*1-probe (panel A), with the Actin probe (panel B) and with the 27S ribosomal probe (panel C).

Discussion

BcAP1 has homology to extracellular fungal aspartic proteases

Sequence analysis of the *B. cinerea* gene *Bcap*1 revealed significant homology to extracellular fungal aspartic proteases. We studied *Bcap*1 because of the possible involvement of aspartic protease activity in pathogenicity, as suggested by Movahedi and Heale (1990a).

Since BcAP1 does not appear to have a signal peptide, it is presumably located intracellularly. Most aspartic proteases are zymogens: (pre)-propeptides capable to autocatalytically cleave off the propeptide of approximately 80 amino acids at low pH (van den Hombergh, 1996). This post-transcriptional regulatory system makes them less sensitive

to proteolytic degradation. In fungi, the processing usually occurs at dibasic cleavage sites (KR or KK) although monobasic cleavage sites have also been observed (van den Hombergh, 1996). It remains to be determined whether BcAP1 is also autocatalytically cleaved behind residue 72 (KK) (see Figure 3).

Lam *et al*. (1996) amplified two *B. cinerea* AP gene fragments of which one presumably originates from *Bcap*1. Recently, an EST library that was constructed under deprivation of nitrogen was sequenced and it contained two ESTs (GENBANK AL113910: 660 bp and AL111951: 540 bp) that are identical to *Bcap*1 (Bitton *et al*., 1999).

Bcap*1 is induced by exogenous protein at low free nitrogen concentrations*

Expression of some fungal proteases is regulated by the availability of carbon and nitrogen sources. The *A. nidulans* transcription factors CREA and AREA regulate gene expression by binding to specific sites in the promoter of target genes. These consensus sequences are also present in the promoter region of *Bcap*1, and might be involved in its regulation. Under conditions in which free nitrogen is limiting and in the presence of BSA as sole nitrogen source, transcript levels are induced. This would suggest that AREA-like transcription factors control the regulation of *Bcap*1. This expression pattern is in agreement with the presence of *Bcap*1 ESTs in a *B. cinerea* EST library that was constructed from mRNA isolated from a *B. cinerea* liquid culture under nitrogen deprivation (Bitton *et al*., 1999).

Expression of the most related fungal aspartic protease *GcSAP* (*G. cingulata*; Clark *et al.*, 1997) is induced by exogenous protein and repressed by ammonium salts, as was also found for *Bcap*1. This is in contrast to *pap*A from *P. anserina* (Paoletti *et al.*, 1998) of which the expression is induced during carbon limitation but not during nitrogen limitation.

BcAP1 gene disruption mutants

Disruption mutants of the *Bcap*1 gene have been obtained as could be confirmed by Southern (Figure 7) and northern (Figure 8) hybridisation. The *Bcap*1 transcript of wild type size is absent in both disruption mutants. The mutants do, however, produce a very abundant transcript of much larger size. Such abundant levels of transcripts with aberrant size were also observed in glutathione S-transferase (GST) mutants of *B. cinerea* (Prins *et al*., 2000a: Chapter 4). Possibly the plasmid pOHT, used as a selection marker, contains an enhancer element that could explain the observed transcript levels. The presence of aberrant transcripts has made it difficult to demonstrate loss of function in both *Bcgst*1 and *Bcap*1 disruption mutants, especially since enzyme activity was not detectable in the recipient strain. In future experiments it is recommended to use a gene replacement rather than a gene disruptant strategy.

Virulence assays have been used to study possible reduction in virulence of the *Bcap*1 disruption mutants, but no discernible phenotype was observed. Therefore, we conclude that *Bcap*1 does not play a significant role in the virulence of *B. cinerea* on the host plants tested. Disruption of *alp*1 in *Cochliobolus carbonum* (Murphy and Walton, 1996) led to reduction in total protease activity, but no decrease in virulence was observed. It is unlikely that *Bcap*1 encodes the extracellular aspartic protease described by Movahedi and Heale (1990a) since BcAP1 lacks a signal peptide and *Bcap*1 transcripts are not detected at early stages of infection. Since *B. cinerea* contains additional aspartic protease genes, it is important to clone these genes and investigate their role in virulence.

Experimental procedures

Fungal growth

Wild type strain SAS56 of *B. cinerea* (van der Vlugt-Bergmans *et al.* 1993) and the haploid strain B05.10 (derivative of SAS56, a gift from P. Büttner and P. Tudzynski, Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany) were grown and cultured as described in Prins *et al.* (2000a: Chapter 4). Inoculation was performed as described by van der Vlugt-Bergmans *et al.* (1997) and Benito *et al.* (1998).

Cloning of Bcap*1*

RNA was isolated from *B. cinerea* strain SAS56 grown in a liquid culture and reverse transcribed into cDNA that was used as template. AP-specific degenerate primers (forward (5'TGGMGHGAYGCHGAYGARGC 3') and reverse (5'ARDGTDGTDCCDGTRTCDGCR AT 3')) designed on conserved regions were used to amplify a fragment of aspartic protease by PCR on the *B. cinerea* cDNA. PCR products of the expected size of 208 bp were isolated from gel, cloned in pZERO (Invitrogen, USA) and sequenced. One of the clones contained a nucleotide sequence encoding an ORF with homology to aspartic proteases, flanked by the primers. A genomic library of *B. cinerea* strain SAS56 was subsequently screened with the cloned and labelled fragment. Hybridising plaques were purified, phage DNA was isolated and a *Cla*I-fragment was cloned in *Cla*I-digested pBluescript SK^+ (Stratagene).

In vitro *induction experiments*

Conidia were collected (Benito *et al*., 1998), pre-incubated for 2 h at RT in Gamborg's B5 medium supplemented with 10 mM potassium phosphate buffer (pH=6.5) and 10 mM sucrose and subsequently grown in the dark in a rotary shaker (180 rpm, 20°C). 100 ml aliquots with equal amounts of mycelial suspensions were supplemented with combinations of carbon (1 % glucose), nitrogen (100 mM NH_4Cl) and protein (1 % BSA) as described in Figure 5. After 3 and 6 h, mycelium was harvested and RNA was isolated as described.

Analysis of gene expression in vitro

Total RNA from infected leaves and liquid grown mycelium was isolated as described by Prins *et al.* (2000a: Chapter 4). After blotting to Hybond N^+ membranes (Amersham), blots were hybridised as described for Southern blots. A fragment of the *Bcact*A gene (Benito *et al.*, 1998) was radio labelled and used as a probe to determine fungal biomass, while a 27S ribosomal probe (Prins, unpublished) was used to confirm equal loading of the gel.

Southern blot analysis

Genomic DNA was isolated according to Drenth *et al.* (1993) with minor adjustments (Prins *et al.*, 2000a). Digestion and blotting was according to Sambrook *et al.* (1989) and specified in van der Vlugt-Bergmans *et al.* (1997). Restriction enzymes and the [α-^{32}P]dATP labelling kit were obtained from Life Technologies. As a probe, a PCR fragment of 495 bp was amplified using primers BcAP6 (5'AACAAATGTTTGGTTTCCGCG 3') and BcAP1 (5'CAACGTCTTGGTCGATGTAGCC 3'), indicated by a solid bar in pOHTΔAP in Figure 6A.

Construction of transformation vector

The disruption vector pOHTΔAP was constructed in the following way. Primer BcAP6 and BcAP1 were used to PCR a fragment of 495 bp, situated in the middle of the *Bcap*1 ORF. This fragment was cloned into the *Sma*I site of plasmid pOHT (van Kan *et al*., 1997), containing an *Aspergillus nidulans oli*C promoter (Turner *et al*., 1989), *hph* (conferring resistance to Hygromycin) and an *A. nidulans trp*C terminator. The resulting plasmid designated pOHTΔAP was transferred to JM110 and isolated. Prior to transformation, 1 μg of the plasmid was partially digested with *Bcl*I at position 367 of the insert, in order to increase the transformation efficiency (Bird and Bradshaw, 1997).

Transformation of Botrytis cinerea

Transformation was performed as described by Prins *et al.* (2000a: Chapter 4). The haploid wild type B05.10 was used as recipient strain. Southern blot analysis was used to verify that the transformants did not possess any wild-type nuclei. Primary transformants were subjected to single spore isolation.

Bioassay

Droplet inoculation (as described by Benito *et al.*, 1998) was performed on detached tomato leaves cv. Moneymaker *Cf4* and broad bean at 20°C with a 16 h photoperiod and at 4°C in the dark under high relative humidity. Wild type B05.10 was applied on the left side and transformants on the right side of the leaflets, 6 droplets of 1 μl ($1 \cdot 10^6$ conidia/ml in B5 medium, supplemented with 10 mM potassium phosphate buffer (pH=6.5) and 10 mM sucrose) as described by Prins *et al.* (2000a: Chapter 4).

Protease plate assay

Plates containing either 1 % casein (BDH, Poole, England) or skimmed milkpowder in water agar (1 % purified agar, Oxoid) were used to assay the radial growth of *B. cinerea*. Non-sporulating plugs of plates containing SAS56, B05.10, 5B or 43 were applied to study protease secretion. Production of extracellular proteases result in a typical halo around the inoculum, which is indicative for the protease secretion.

Long distance PCR

Using the Advantage® Genomic Polymerase mix (Clontech) with proof-reading activity, PCR amplification of long stretches of DNA (Barnes, 1994) was performed to amplify phage inserts as described by Prins *et al.* (2000a: Chapter 4).

Cloning of cDNA

Total RNA was isolated from mycelium of an overnight *B. cinerea* B05.10 liquid culture. RT-PCR (as described by van der Vlugt-Bergmans *et al.*, 1997) was performed to introduce an *Xho*I site (CTCGAG, underlined) in front of the ATG start codon (bold) using primer BcAP-8 (5'GCGCG<u>CTCGAG</u>ATGGCTTCTCTCCAAGGC3'). A *Bam*HI site (GGATCC, underlined) was introduced behind the stop codon using primer BcAP-9 (5'CGCGC <u>GGATCC</u>TAAGCTGGGGGAGCGGC 3'). The amplified cDNA was digested with *Xho*I and *Bam*HI and cloned in the vector pFLAG®-ATS (Kodak).

Sequence analysis

DNA sequencing was performed with Applied Biosystems, 377 DNA Sequencer using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA Polymerase. Sequences were analysed using Lasergene software (DNAstar Inc., Madison, USA). Nucleotide and amino acid homology analysis was performed using the Blast program (Gish and States, 1993; Altschul *et al.*, 1997).

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CHAPTER 6

General discussion

Introduction

In this thesis we have used four methods to identify genes that are putatively involved in the virulence of *B. cinerea*. Three methods follow a non-biased approach. They are Differential Display Reverse Transcriptase PCR technique (DDRT-PCR: Chapter 2), differential screening of a genomic library (Chapter 3) and a subtractive hybridisation (Chapter 4). The fourth method describes a biased approach in which a specific gene (aspartic protease: Chapter 5) is isolated with a presumed function in virulence. The transcript levels of the isolated genes have been analysed in wild type *B. cinerea*. In chapters 4 and 5 the isolated genes have been disrupted and the virulence of the disruption mutants was compared to wild type. In this general discussion the four methods used will be discussed and compared with the following non-biased approaches that have not been applied in our study.

Random insertional mutagenesis can be used to create a large collection of tagged transformants and followed by screening the individuals for reduced virulence. The mutant locus can subsequently be isolated by means of the tag sequence. Methods for random insertional mutagenesis involve Restriction Enzyme Mediated Integration (REMI), *Agrobacterium tumefaciens* mediated insertion (de Groot *et al.*, 1998), or transposon tagging with a heterologous transposable element (Hua-Van *et al*., 1998; Hua-Van *et al*., 2001; Villalba *et al*., 2001). These methods require a substantial investment in the construction of the mutant collection and require an enormous effort to screen the mutant collection for a phenotype of interest, especially when it is for reduced virulence. It is important to note that *B. cinerea* often forms heterokaryons containing both transformed and untransformed nuclei. Such a heterokaryon would not likely display any loss of function even if it has an insertion in an important gene.

Amplified restriction Fragment Length Polymorphisms (AFLP®) and Expressed Sequence Tag (EST) analysis can be combined with high throughput functional genome analysis to compare and analyse complete genomes. The availability of an increasing amount of sequence data will make it possible to study and annotate orthologous genes of different genomes.

We will describe the principles of several methods, including their advantages and disadvantages. For applying these methods to analyse of the infection process of *B. cinerea* we frequently had to adapt the initially described basal method because of constraints of the biological system. We will discuss the considerations leading to the modifications.

Differential Display Reverse Transcription PCR (DDRT-PCR)

Since the development of the Polymerase Chain Reaction (PCR) technique (Mullis and Faloona, 1987), many variations have been added. DDRT-PCR is a variation of RT-PCR and is used to identify differences in mRNA expression patterns between two cell lines. Liang and Pardee (1992) first described the differential display method, which was later improved by Bauer *et al.* (1993) and named DDRT-PCR. cDNA is primed using a set of primers

anchoring to the poly(A)-tail of different mRNA populations. The oligo-dT primers carry two additional nucleotides at the junction between the poly(A)-tail and the 3'-end of the mRNA. The two nucleotides allow a reduction of the complexity of the population of cDNAs that is reverse transcribed, since they will only anneal to a subset of mRNA molecules. After first cDNA strand synthesis the amplification of the single strand cDNA is continued in the presence of 10-mer primers. Separation of the PCR mixture by gel electrophoresis allows comparison of the different banding patterns and identifies new or lacking bands. Liang *et al*. (1994) optimised the differential screening by using three one-base anchored oligo-dT primers that provide further selectivity in subdividing cDNA into three populations. The method is qualitative rather than quantitative as it only considers the presence or absence of bands. The DDRT-PCR procedure has been used to screen in a non-biased way for pathogenicity genes of *B. cinerea* (Chapter 2). We were the first to apply this method to identify fungal genes that are differentially expressed during interaction with a host plant. Several control reactions were included to select for truly newly emerging bands (Benito and van Kan, 1998). These included mRNAs of *B. cinerea* and tomato, respectively, to identify housekeeping genes of both interacting partners, as well as mRNA of tomato induced by a different necrotising pathogen such as *Phytophthora infestans* to identify plant defence genes that are generally induced upon infection by pathogens with a similar mode of action. Subtraction of bands appearing in these control lanes enabled to specifically detect cDNA fragments derived from *B. cinerea* genes expressed during infection of tomato.

Due to the sensitivity of the PCR-based techniques artefacts can show up. Slight contaminations can disturb the banding patterns. A number of fragments that we cloned in the course of our experiments appeared to be of human origin (unpublished). Also the presence of epiphytic microorganisms on the plant surfaces may influence the results. Thus the extreme sensitivity may introduce a rather high number of 'false positives'. The use of anchor oligo-dT primers implies amplification of sequences immediately upstream of the poly(A)-tail. Therefore, cDNAs obtained by DDRT-PCR represents only ~100-300 bp of the extreme 3' ends of the mRNAs and therefore often represent untranscribed regions. Since the sequences are often not present in sequence databases and vary greatly between organisms, Sompayrac *et al.* (1995) adapted the DDRT-PCR method to allow 'walking' along the mRNA towards its 5' end in steps of 1 kb. In short, reverse transcriptase and a primer located near the 5' end of the known sequence are used to amplify cDNA on mRNA from a cell in which the gene of interest is expressed. After second strand copy the ends of the cDNA are ligated to form circles. PCR primers are used to amplify the sequences that are 5' to the known sequences.

A potential disadvantage of DDRT-PCR, however, is the under-representation of particular mRNA species. Bertoli *et al*. (1995) reported that differential display shows a strong bias towards highly abundant mRNAs. Our results presented in Chapter 2, however, have convincingly shown that the method can be extremely sensitive when the experiments are carried out accurately and the proper controls are included. Several of the *B. cinerea* mRNAs that we identified by DDRT-PCR as being expressed during the infection of tomato leaves, were hardly detectable by northern blot analysis (e.g. ddB2, ddB5, ddB47; see Figure 5 in Chapter 2). Further studies on the role in virulence of corresponding genes is in progress (Benito, pers. comm.).

Differential hybridisation screening followed by isolation of genes from a genomic library

A differential hybridisation procedure with two complex, radioactively labelled cDNA probes was developed by Pieterse *et al*. (1991) for the isolation of virulence genes of *P. infestans*. For a typical differential hybridisation, a genomic library was differentially screened with two labelled first strand cDNA probes. One was synthesised on RNA isolated from *B. cinerea*-infected tomato leaves (interaction probe) and the other was synthesised on RNA isolated from the fungus grown in liquid culture (*in vitro* probe). This strategy relies on the assumption that transcripts of virulence genes occur not or are absent during growth in liquid culture, but are (transiently) induced during infection. The advantage of using a genomic library to isolate the induced genes rather than an interaction-based cDNA library is that the eventually cloned genes are truly fungal genes. The concentration of phage DNA on the hybridisation membranes is in excess over the concentration of probes used to screen the library. This implies that the hybridisation intensity of a particular phage plaque reflects the concentration of the probe molecule in the cDNA pool if the gene represents a single copy gene. A disadvantage is that the average insert size of the genomic phage library is much larger (~16 kb) than that of a cDNA library (~ 500 bp). Also one insert may contain non-coding or repetitive DNA, and most probably several functional genes. This requires additional rounds of analysis to identify the gene(s) of interest. The method is in principle semi-quantitative.

The time point of harvesting the biological material for RNA isolation is crucial since it reflects the population of the pool of mRNAs that will be subjected to the differential screening. In the screening as described in Chapter 3, we chose as time point 16 hours post inoculation (h.p.i.), which just precedes the time point of primary lesion formation (18 h.p.i.). Mycelium of a *B. cinerea* culture grown for 16 h in liquid culture was used as reference.

In this procedure it was anticipated that certain phages would show a stronger signal with the interaction probe than with the *in vitro* probe. These phages were assumed to contain *B. cinerea* genes that are preferentially transcribed during infection. A disadvantage of this screening method is that tomato genes orthologous to *B. cinerea* genes will also hybridise to the *B. cinerea* library, masking the actual expression level of the fungal gene. This problem is clearly illustrated in Chapter 3. The differential hybridisation of a phage containing a *B. cinerea* ubiquitin gene may have been the consequence of increased expression of either the *Bcubi*1CEP79 gene, the *Bcubi*4 gene or the tomato heptaubiquitin gene.

It is striking that several attempts of hybridisation screenings by different research groups to identify differentially expressed genes in plant pathogens have all resulted in the isolation of ubiquitin genes. Pieterse *et al*. (1991) performed a screening for potential virulence genes of the oomycete *P. infestans* using *P. infestans* interaction probes in combination with a genomic library and isolated, amongst others, a polyubiquitin gene. McCafferty and Talbot (1998) used a cDNA library and identified genes that were expressed during the infection of rice leaves by *Magnaporthe grisea*. One of the genes they identified was a monoubiquitin gene. Loser and Weltring (1998) isolated a polyubiquitin gene by screening a cDNA library of *Gibberella pulicaris* after induction by the phytoalexins rishitin and lubimin.

With the increasing possibilities of PCR-based screening methods, it is expected that these more sensitive methods can complement the differential hybridisation technique.

Hybridisation screening with subtractive RT-PCR products

The procedure of genomic subtraction has first been described by Wieland *et al*. (1990) to isolate unique target sequences present in a genomic DNA population (tester) that was deprived of common sequences present in another (driver) population. The method compares two genomic populations in which genes are differentially induced. Many research groups have attempted to improve the subtractive hybridisation procedure to overcome specific problems arising in the method. PCR-coupled subtraction of genomic DNA by Representational Difference Analysis (RDA) has been described by Lisitsyn *et al.* (1993). It has been adapted for cDNA by Hubank and Schatz (1994) and was found to be fast, sensitive, reproducible and predominantly suppressing the occurrence of false positives. Identification of rare transcripts by RDA has been optimised by O'Neill *et al.* (1997). Zeng *et al*. (1994) developed Enzymatic Degrading Subtraction (EDS) in which they constructed subtractive libraries from PCR amplified cDNA. EDS facilitates the tester DNA to be blocked by thionucleotide incorporation and that driver cDNA and hybrid molecules to be removed by digestion with exonucleases. Hampson *et al*. (1996) developed Degenerate Oligonucleotide primed PCR (DOP) into a Directional Random Oligonucleotide Primed (DROP) synthesis of cDNA. DROP achieves directional PCR amplification of total cDNA and provides, in combination with Chemical Cross Linking Subtraction (CCLS), a solution to the problem of cloning differentially expressed cDNAs from limited cell numbers. To determine subtle changes in gene expression profiles combined with a high throughput screening for rarely transcribed differentially expressed genes, von Stein *et al*. (1997) combined the Subtraction Suppression Hybridisation (SSH: combining high subtraction efficiency with equalised representation of differentially expressed sequences) with a high throughput differential screening. The authors claim that this method excludes virtually all false positive and false negative clones.

Our search for induced genes of *B. cinerea* during infection of tomato (Chapter 4), however, involves both tomato transcripts and *B. cinerea* transcripts, which made the subtraction more complicated. Hence, the protocol was adapted to avoid disturbance by the presence of the tomato transcripts. mRNAs isolated from tomato leaf tissue infected by *B. cinerea* (tester) was compared with mRNA isolated from *B. cinerea* grown in liquid culture, diluted with a 9-fold excess of mRNAs isolated from TNV-infected tomato leaf (driver). This dilution was introduced to correct for low level of fungal biomass in the *B. cinerea*-tomato leaf interaction at early stages of infection (estimated to be 1-5 %, see Chapter 2 and Benito *et al*., 1998). Two tester populations were created with different adapters, whereas driver cDNA had no adapters. After hybridisation, nested primers would be able to amplify unique tester cDNA sequences, while cDNA fragments present in both pools would not be amplified. The entire mixture of PCR products was then radioactively labelled and hybridised to a genomic library of *B. cinerea* to identify the unique genes.

We initially considered cloning and sequencing of the PCR products obtained by the subtractive RT-PCR procedure. This would, however, also result in the unwanted cloning of plant genes. Therefore, the complete PCR mixture was used to screen a genomic library of *B. cinerea*. However, as described before, it is still possible that orthologous plant genes cross-hybridise with genes in the *B. cinerea* library. As described in Chapter 4, the subtractive RT-PCR procedure has resulted in the isolation of a glutathione S-transferase gene. The expression of the gene was induced by exogenous hydrogen peroxide (see Figure 3 Chapter 4) and the gene appears to function as a second line of defence against oxidative stress (A. Schouten, unpublished). Disruption of *Bcgst*1 did not result in decreased virulence. The role in controlling oxidative stress was not anticipated. Therefore, we can conclude that a non-

biased approach using the hybridisation screening procedure with subtractive RT-PCR products is indeed successful to identify genes of *B. cinerea* with novel functions.

Restriction Enzyme Mediated Integration (REMI)

REMI is a procedure for random mutagenesis of fungi, first developed for *Saccharomyces cerevisiae* (Schiestl and Petes, 1991) and thoroughly reviewed by Kahmann and Basse (1999). The principle of REMI is based on linearised plasmid DNA that is transformed into the target organism in the presence of a compatible restriction enzyme. The plasmid contains a bacterial transformation marker and a fungal transformation marker. Random insertion of the plasmid is likely to occur when no sequence homology with the target organism is present. The use of a restriction enzyme in the procedure enhances the transformation efficiency in some fungi and/or reduces the number of integrated plasmid copies (Shi *et al*., 1995). Integration in promoter regions or the reading frame of functional genes results in loss of gene function. A prerequisite is that the target pathogen is haploid since insertion events in diploids probably mask phenotypic alterations.

REMI transformants must be selected for an aberrant phenotype of interest. Subsequently Southern blot analysis is performed on DNA from mutant strains, using the introduced plasmid as probe, to determine the number of insertions. One should determine whether the mutation event is correlated with the presence of the integrated plasmid, to rule out a possible rearrangement or other mutation due to the use of the restriction enzyme in the REMI procedure. Especially uncontrolled rearrangements are a major disadvantage in the procedure since they may result in a mutant phenotype, which is not tagged by a plasmid (B. Tudzynski, pers. comm.).

Identification of mutated genes can be facilitated by plasmid rescue; digestion of genomic DNA by a restriction enzyme that does not cut in the integrated plasmid excises it together with its flanking sequences. The plasmid can subsequently be religated and transformed to *E. coli*. Sequence analysis of the additional flanking sequences reveals the site of integration of the plasmid, and thereby the gene that is disturbed by the REMI procedure.

REMI has proven to be successful for a number of phytopathogenic fungi: *Cochliobolus heterostrophus* (Lu *et al*., 1994), *Ustilago maydis* (Kahmann and Basse, 1999), *Magnaporthe grisea* (Shi *et al*., 1995; Sweigard *et al*., 1998). For *B. cinerea* this approach has been unsuccessful and was abandoned (Stefanato, Schouten and van Kan, unpublished).

Another option to tag genes *in situ* is the use of transposons. *Impala*, a Tc1–mariner transposable element from *F. oxysporum*, was introduced into the rice blast fungus *M. grisea* to develop transposon-based insertional mutagenesis (Villalba *et al*., 1999; 2001). An autonomous *impala* copy was inserted in the promoter of *nia*D and introduced by transformation into a *M. grisea* nitrate reductase-deficient mutant. *Impala* could be excised and reintegrated by a functional *impala* transposase, resulting in the tagging of a pathogenicity gene (Villalba *et al*., 2001).

The method could also be applied to *B. cinerea* using either *impala* or another heterologous transposon, such as *Restless* from *Tolypocladium inflatum* (Kempken *et al.*, 1998; Kempken and Kück, 1998).

Amplified restriction Fragment Length Polymorphism (AFLP®)

The AFLP® DNA fingerprinting technique (Vos *et al*., 1995) enables the simultaneous amplification of large numbers of restriction fragments from a single template which are

separated by electrophoresis and visualised. The method can detect polymorphisms between individuals, either in restriction sites or flanking nucleotides, leading to polymorphisms in lengths of restriction fragments. Selective amplification is achieved by using additional nucleotides 3' of the primers, thereby reducing the number of bands (Vos *et al*., 1995).

The cDNA-AFLP technology is an adaptation of AFLP® and uses cDNA reverse transcribed from mRNA as starting material (Bachem *et al*., 1996). The method is highly sensitive in identifying developmentally regulated genes, requires low amounts of starting material and allows detection of low abundant transcripts. It was also reported that the method is quantitatively profiling the transcripts, as intensity of the PCR bands represents the level of expression (Bachem *et al*., 1996).

High throughput functional genome analysis

By AFLP and other 'high throughput' analyses, sequence data obtained can be used to construct specific micro arrays (Duggan *et al*., 1999) as has been performed for *Arabidopsis thaliana* (Desprez *et al*., 1998). This sensitive technology enables spotting minute amounts of cDNA in large densities *in duplo* on glass chips that can be screened differentially with two or more different fluorescently labelled probes. Computer analysis aligns the fluorescence data and subtracts common housekeeping genes from unique and differentially expressed signals. This type of analysis adds a new dimension to the previously described techniques. The only limiting step will be the cost factor and the availability of the equipment.

The emerging availability of bio-informatics and the use of EMBL and GENBANK database search engines like BLAST (Gish and States, 1993; Altschul *et al.*, 1997), together with the increasing amount of sequences in these databases, enable to make predictions about the function(s) of the gene studied. Milestones in genomics are the recent completions of the *Saccharomyces cerevisiae* (Hudson *et al.*, 1997), *Arabidopsis thaliana* (Kaul *et al.*, 2000) and human (Lander *et al.*, 2000; McPherson *et al.*, 2000) genome sequencing projects. The genome sequences of filamentous fungi (e.g. *Aspergillus* species) will soon follow. Although the genomes of *S. cerevisiae* and *Aspergillus* spp. are probably not entirely paralogous to that of *B. cinerea,* they will be useful with respect to annotation, intron/exon splice site prediction and promoter analysis by applying comparative genome analysis.

Sequence information, proteomics and modelling will contribute to the understanding of newly discovered *B. cinerea* sequences. Gene prediction can be very helpful in research but one should be very careful as to what annotation is used. Nevertheless, the most reliable methods are the most laborious ones: sequencing genomic and cDNA, 5' and 3' RACE, protein expression and analysis.

Until recently, most sequences of *B. cinerea* genes were isolated by using heterologous probes or degenerate primers. An Expressed Sequence Tag (EST) library has now become available that contains sequences of cDNA clones constructed on mRNA isolated from *B. cinerea* grown under deprivation of nitrogen (Bitton *et al*., 1999). This EST library contains approximately 5000 cDNA entries of *B. cinerea* that are accessible via GENBANK. EST libraries contribute to the study of genes since they are indicative for the expression pattern of the genes under specific conditions and they reveal the intron/exon splice sites. Although random sequencing of ESTs is not very specific, the method can be considered as nonbiased for a particular condition.

Concluding remarks

Here we have discussed several methods that can be used to perform a non-biased screening for differentially expressed genes. Each of the methods has advantages and drawbacks, depending on the organism studied and the application. Differential display RT-PCR (Chapter 2), differential screening (Chapter 3) and hybridisation with subtractive RT-PCR products (Chapter 4) have been used to screen for *B. cinerea* genes that could be involved in pathogenesis.

The genes that were described in Chapters 3 (UBI), 4 (GST) and 5 (AP) all presumably play a non-essential role in pathogenesis. The gene fragments that were described in Chapter 2 await further analysis. Recent results have learned that the model presented in Figure 5 of Chapter 1 should be slightly adapted. BcGST1 is more likely to contribute to tolerance to oxidative stress than to the evasion of host defence responses.

It is recommended to pursue a high throughput sequencing of ESTs in an EST library enriched for fungal genes that are expressed at different stages of pathogenesis. These sequences may provide additional genes that might play a role in pathogenesis.

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Summary

In the past decade many new methods have been developed that improved the unbiased search for differentially expressed genes. The fine-tuning towards high throughput analysis with increased sensitivity for genes that are expressed at a low level makes these new methods very powerful.

This thesis focuses on the isolation and characterisation of novel pathogenicity genes of *Botrytis cinerea.* They were isolated based on the assumption that such genes are preferentially expressed during interaction of the pathogen with its host plant. Tomato was chosen as a host plant. Differential Display RT-PCR (DDRT-PCR: chapter 2), differential hybridisation screening of a genomic library of *B. cinerea* (chapter 3) and hybridisation with subtractive RT-PCR products (chapter 4) have been performed to isolate novel genes that are potentially involved in pathogenesis. Some of these methods are, however, error-prone and relatively insensitive. We faced the difficulty of searching for *B. cinerea* genes that are induced upon infection of a host, at a timepoint where the majority of the isolated RNAs (>95 %) is of plant origin. This implies that expression of fungal genes *per se* is difficult to detect. Small differences in expression levels of fungal genes often remain undetected and expression of tomato genes may blur interpretation of the screening. Although many successful studies have been reported showing differences in gene expression with a single organism grown in two different environments, the methods that we applied on two interacting organisms were not always successful.

At the start of this project, we developed a synchronised infection method and observed that the infection of tomato leaves by *B. cinerea* occurs in three phases (chapter 2; Benito *et al*., 1998), namely: 1) In the first 18 h after *B. cinerea* conidia are sprayed on plant tissue, no clear visual response of the plant can be observed. 2) In the period between 18 h and 72 h, lesions are visible that do not expand. 3) After 72 h, lesions are spreading and at a later stage *B. cinerea* is infesting the whole tissue. With the use of the DDRT-PCR method (chapter 2) we performed an unbiased screening for induced genes involved in the infection process. It was necessary to adjust the method to our needs. Several gene fragments were cloned and analysed, but none of them displays any sequence homology to sequences present in the public databases. DDRT-PCR, however, is a very sensitive technique that yields fragments of novel, unidentified genes. The lack of sequence homology of these fragments requires isolation of the complete genes to reveal the gene products and subsequent mutational analysis to evaluate their role in pathogenesis. The emerging sequence information of whole genomes may circumvent the first step in the future.

In parallel we chose for a straightforward differential screening of a genomic library of *B. cinerea* (chapter 3) as was successfully performed by Pieterse *et al*. (1991) for *Phytophthora infestans*. The screening resulted in the identification of two genes encoding polyubiquitin and monoubiquitin, respectively. Ubiquitin is a protein predominantly involved in labelling misfolded or damaged proteins for degradation. Induction of ubiquitin gene expression in *B. cinerea* during infection of a host plant might suggest that the fungus experiences a stress situation, presumably caused by oxidative processes occurring in the lesion. This would result in malfunctioning of proteins and protein degradation would be imperative. This might explain the need for increased levels of ubiquitin. However, the monoubiquitin gene also encodes a carboxyl extension protein that is required for ribosome synthesis. The enhanced expression of the monoubiquitin gene may also reflect the need for increased ribosome synthesis.

A glutathione-S-transferase gene (GST) was identified by a hybridisation with subtractive RT-PCR products (chapter 4). Disruption of the *Bcgst*1 gene did not result in decreased virulence. Expression of *Bcgst*1 was induced by adding hydrogen peroxide to liquid cultures,

suggesting that the (presumably cytosolic) enzyme may be involved in protection of *B. cinerea* against oxidative damage. Under the same conditions an extracellular catalase gene is also induced (Schouten, unpublished). This catalase probably serves as a first extracellular line of defence against hydrogen peroxide, while GST serves as intracellular backup for removing residual hydrogen peroxide that has not efficiently been removed by the catalase. Therefore, catalase could mask the action of GST as no phenotype is observed for a GST-deficient mutant.

As Movahedi and Heale (1990) claimed a role for aspartic protease in the infection process, we set out for a targeted approach to isolate an aspartic protease of *B. cinerea* (*Bcap*1: Chapter 5). *Bcap*1 was isolated using degenerate primers for PCR. BcAP1 is not secreted and disruption of *Bcap*1 as described in Chapter 5 does not have any detectable phenotype on the infection process. Other aspartic proteases must be responsible for the action described by Movahedi and Heale (1990). Recently, other aspartic protease gene sequences have been discovered (ten Have and van Kan, pers. comm.; Bitton *et al*., 1999), suggesting a small gene family of aspartic proteases. These aspartic protease genes should be investigated (either apart or as double mutants) to determine the significance of their role during pathogenesis.

The results described in this thesis and recently published data extend our knowledge of the infection strategy of *B. cinerea*. Since *B. cinerea* is a necrotrophic fungus, its infection strategy must be substantially different from that of biotrophic fungi. The infection of host plants by *B. cinerea* and the related necrotroph *Sclerotinia sclerotiorum* is not necessarily subtle. *B. cinerea* is often referred to as an opportunistic pathogen that can grow on weakened or senescent tissue. Although *B. cinerea* has a broad host range (Jarvis, 1977), a vast number of mainly monocotyledonous species cannot be invaded. The reason for this is yet unclear. Possible explanations could be that some species do not react to *B. cinerea* with a necrotic response, thereby depriving the fungus of dead tissue serving as substrate. It is also conceivable that non-hosts produce toxic compounds, which cannot be degraded by *B. cinerea*.

Essentially, three basic features can contribute to the infection strategy of a necrotroph:

- Initiation of infection by stimulating host cell death at the site of infection. This can be facilitated by an active process caused by the necrotroph (Movahedi and Heale, 1990; von Tiedemann, 1997; Liu *et al*., 1998), a defence-related cell-death (hypersensitive response) of the host plant (Malolepsza and Urbanek, 2000) incited by the necrotroph, or a combination.
- Coinciding with a hypersensitive response, the host produces active oxygen species (AOS) and other defence compounds. Therefore, the necrotroph needs sufficient protection against, and/or neutralisation of these AOS (Cessna *et al*., 2000) and defence compounds (Quidde *et al*., 1998). GST and ubiquitin may play a role in this period of consolidation; GST is presumably able to diminish the oxidative stress while ubiquitin can selectively remove defective proteins.
- After this period of consolidation at the primary site of infection, *B. cinerea* can exploit its hydrolytic enzymes to degrade the host tissue in order to infest and sporulate (reviewed in Prins *et al*., Chapter 1; ten Have, 2000 and references therein). These three steps are repeated to extend the area of necrotic tissue.

Samenvatting

In de afgelopen tien jaar zijn veel nieuwe technieken ontwikkeld die de isolatie hebben verbeterd van differentieel tot expressie gebrachte genen. De mogelijkheid om op grote schaal en met hoge gevoeligheid genen te analyseren die op een laag niveau tot expressie komen, maken deze technieken erg krachtig.

Dit proefschrift richt zich op de isolatie en karakterisatie van nieuwe genen die betrokken kunnen zijn bij de pathogenese van *Botrytis cinerea*. De genen zijn geïsoleerd in de veronderstelling dat ze preferentieel tot expressie komen tijdens de interactie van het pathogeen met zijn waardplant. In deze studie is de tomaat gekozen als waardplant. "Differential Display" RT-PCR (DDRT-PCR: hoofdstuk 2), differentiële hybridisatie analyse van een genomische bank van *B. cinerea* (hoofdstuk 3) en hybridisatie met een selectief verrijkte probe van RT-PCR producten (hoofdstuk 4) zijn uitgevoerd om deze genen te isoleren. Sommige van deze methoden zijn echter aan fouten onderhevig en relatief ongevoelig. Tevens deed zich de moeilijkheid voor dat gezocht werd naar *B. cinerea* genen, die geïnduceerd worden tijdens het infectieproces, op een tijdstip waarop meer dan 95 % van de geïsoleerde RNA's van de waardplant afkomstig zijn. Dit impliceert dat de expressie van de *B. cinerea* genen moeilijk is te detecteren. Kleine verschillen in expressieniveaus van schimmelgenen blijven vaak onopgemerkt en de expressie van tomatengenen kunnen de interpretatie vertroebelen. In de literatuur zijn veel studies beschreven over de analyse van genexpressie in één organisme, opgekweekt onder twee verschillende condities. Het bleek echter niet eenvoudig dit toe te passen op een interactie tussen twee organismen.

Aan het begin van dit project is een methode ontwikkeld om een gesynchroniseerde infectie te bewerkstelligen. Waargenomen werd dat de infectie van tomatenbladeren door *B. cinerea* in drie fasen verloopt is (hoofdstuk 2; Benito *et al.*, 1998). 1) In de eerste 18 uur nadat conidia van *B. cinerea* op tomatenblad werden gesproeid kon geen zichtbare reactie van de plant worden waargenomen. 2) In de periode hierop volgend werden lesies zichtbaar die zich niet verder ontwikkelden. 3) 72 uur na inoculatie begonnen deze lesies zich verder te ontwikkelen totdat uiteindelijk het hele blad werd geïnfecteerd. Met de toepassing van DDRT-PCR (hoofdstuk 2) is een onbevooroordeelde screening gebruikt om nieuwe schimmelgenen te isoleren, die geïnduceerd werden tijdens het infectieproces. Hierbij was het nodig om de bestaande methode aan te passen aan het biologisch systeem. Diverse genfragmenten werden gekloneerd en geanalyseerd, maar geen van de fragmenten heeft homologie met sequenties aanwezig in de publieke databestanden. Desalniettemin is DDRT-PCR een erg gevoelige techniek waarmee fragmenten van tot nu toe onbekende genen zijn geïsoleerd. Het ontbreken van enige sequentiehomologie vereist dat de complete genen geïsoleerd dienen te worden en deze vervolgens uit te schakelen om de eventuele rol tijdens de pathogenese te bepalen. De toenemende hoeveelheid sequentiedata voortkomend uit de sequentieanalyse van verschillende genomen zou in de toekomst de eerste stap kunnen omzeilen.

Parallel aan deze benadering is gekozen voor een differentiële hybridisatie analyse van een genomische bank van *B. cinerea* (hoofdstuk 3), zoals die ook met succes is uitgevoerd door Pieterse *et al.* (1991) voor *Phytophthora infestans*. Deze analyse resulteerde in de identificatie van monoubiquitine en polyubiquitine genen. Ubiquitine is een eiwit dat voornamelijk betrokken is bij de afbraak van verkeerd gevouwen of beschadigde eiwitten. Inductie van ubiquitine genexpressie in *B. cinerea* tijdens de infectie van de waardplant zou kunnen suggereren dat de schimmel aan stress wordt blootgesteld, waarschijnlijk door oxidatieve processen tijdens het ontstaan van de lesie. Dit zou tot gevolg kunnen hebben dat bepaalde eiwitten niet meer functioneren en dientengevolge voor afbraak in aanmerking

komen. Dit zou de toename in ubiquitine mRNA kunnen verklaren. Verder heeft het monoubiquitine een carboxyl extensie eiwit dat nodig is voor de synthese van ribosomen. De toename van monoubiquitine kan daarom ook verklaard worden door een verhoogde behoefte aan ribosoomsynthese.

Een glutathione S-transferase (GST) werd geïdentificeerd na hybridisatie van een genomische bank van *B. cinerea* met een probe die verkregen was door verrijking van RT-PCR producten (hoofdstuk 4). Disruptie van dit *Bcgst*1 gen resulteerde niet in een verminderde virulentie. Expressie van *Bcgst*1 kon worden geïnduceerd door het toevoegen van waterstof peroxide aan vloeistofcultures, hetgeen suggereert dat het (vermoedelijk cytosolisch gelokaliseerde) enzym betrokken is bij de bescherming van *B. cinerea* tegen oxidatieve schade. Onder identieke condities werd ook een extracellulair catalase geïnduceerd (Schouten, niet gepubliceerd). Dit catalase dient waarschijnlijk als een primaire extracellulaire verdediging tegen waterstof peroxide, terwijl GST waterstof peroxide kan afbreken dat desondanks doordringt in het cytoplasma. Het is mogelijk dat het catalase de werking van GST maskeert in de GST-deficiënte mutant.

Omdat Movahedi en Heale (1990) veronderstelden dat aspartyl protease een rol speelt in het infectieproces, werd een gerichte benadering gekozen voor de isolatie van een aspartyl protease gen van *B. cinerea* (*Bcap*1: hoofdstuk 5). *Bcap*1 is geïsoleerd met PCR met gebruikmaking van gedegenereerde primers. BcAP1 wordt niet uitgescheiden en de disruptie zoals beschreven in hoofdstuk 5 leidt niet tot een waarneembaar afwijkend fenotype tijdens het infectieproces. Het is daarom waarschijnlijk dat andere proteases verantwoordelijk zijn voor de functie die gesuggereerd is door Movahedi en Heale (1990). Recentelijk zijn sequenties van andere aspartyl proteases bekend geworden (ten Have en van Kan, pers. mededeling; Bitton *et al.*, 1999), hetgeen de aanwezigheid van een kleine genfamilie suggereert. Deze aspartyl proteases moeten worden onderzocht, als enkele of dubbele mutanten, om de rol van elk van deze proteases tijdens het infectieproces vast te stellen.

De resultaten die zijn beschreven in dit proefschrift en in de recentelijk gepubliceerde literatuur vergroten het inzicht in de infectie strategie van *B. cinerea*. Daar *B. cinerea* een necrotrofe schimmel is, zal de infectie strategie sterk verschillen van die van biotrofe schimmels. De infectie van waardplanten door *B. cinerea* en vergelijkbare necrotrofe schimmels als *Sclerotinia sclerotiorum* hoeft niet noodzakelijkerwijs subtiel te zijn. *B. cinerea* wordt ook wel een opportune schimmel genoemd omdat hij in staat is te groeien op verzwakt of afstervend weefsel. Hoewel *B. cinerea* een brede waardreeks heeft (Jarvis, 1977) kan een groot aantal gewassen, waaronder vele monocotylen, niet worden geïnfecteerd. De reden hiervoor is nog onduidelijk. Mogelijke verklaringen zouden kunnen zijn dat deze planten niet reageren op *B. cinerea* met een necrotische respons. Hierdoor is er geen dood weefsel aanwezig dat als substraat kan dienen. Het is ook mogelijk dat deze niet-waardplanten toxische stoffen produceren die niet door *B. cinerea* kunnen worden afgebroken.

In het kort zijn er drie processen die van belang zijn bij de infectie strategie van een necrotroof:

- Initiatie van infectie door lokale afsterving van waardweefsel te stimuleren. Dit kan worden veroorzaakt door een actief proces vanuit de necrotroof (Movahedi en Heale, 1990; von Tiedemann, 1997; Liu *et al.*, 1998), door een verdedigingsmechanisme (overgevoeligheidsreactie) van de waardplant (Malolepsza en Urbanek, 2000) als reactie op de necrotroof, of een combinatie van factoren.
- Het opwekken van een overgevoeligheidsreactie door de waardplant gaat gepaard met de productie van actieve zuurstof (AOS) en andere verdedigingscomponenten. Hiervoor moet de necrotroof mechanismen bezitten om zichzelf te beschermen of om deze componenten onschadelijk te maken (Cessna *et al.*, 2000; Quidde *et al.*, 1998). GST en

ubiquitine kunnen een rol spelen in dit proces van consolidatie; GST is waarschijnlijk in staat om bij te dragen aan de vermindering van de oxidatieve stres terwijl ubiquitine de afbraak van defectieve eiwitten bevordert.

- Na deze periode van consolidatie rond de primaire infectie kan *B. cinerea* een spectrum van hydrolytische enzymen gebruiken om cellen van de waardplant af te breken en zich hiermee te voeden, vervolgens de plant verder binnen te dringen en uiteindelijk te sporuleren (overzicht in Prins *et al.*, hoofdstuk 1; ten Have *et al.*, 2000 en referenties). Deze drie stappen worden herhaald om het oppervlak van necrotisch weefsel te vergroten.

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Nawoord

And now, the end is near, and so I face the final curtain...

Het is af. Ik heb het voor mijzelf gedaan, maar gelukkig niet alleen. Het geeft een goed gevoel te weten dat je op mensen kunt steunen en vertrouwen. In wetenschappelijke zin, op persoonlijk vlak of beide. Ik grijp deze gelegenheid graag aan om een aantal van jullie te bedanken voor de rol die jullie gespeeld hebben in de totstandkoming van dit proefschrift.

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Dank aan de *Botrytis*-groep waarvan ik 5 jaar deel heb uitgemaakt. Jan, jij was het die mij in staat stelde om eerst als vrijwilliger, en later als OIO aan *Botrytis* te werken. Je stond altijd klaar om mij te helpen en een duw in de goede richting te geven. Ook in de periode waarin het minder voortvarend ging, kon je toch begrip opbrengen voor de situatie. We hebben veel gesproken over de inhoud van het proefschrift en je hebt me erg kritisch leren lezen en schrijven. Het is fijn dat je zo'n gedreven co-promotor voor me was. Pierre, bedankt dat je mijn promotor wilde zijn en dat je toch nog tijd vrij kon maken om mijn concepten kritisch door te nemen.

Ernesto, my companion in the VMT lab and great example of how I would like to be in science. You were always very critical about your statements and thorough in your work. Almost every line you started with 'In my hands...', and this allowed you to put things into perspective. Your ability to see hybridising bands on films that nobody else could see still amazes me.

Als basis voor mijn werk heb ik veel gebruik mogen maken van het eerdere werk dat Cécile, John en Lia hebben verricht aan *Botrytis*. Onder andere de transformatie is door jullie met veel zorg opgezet en daar heb ik dankbaar gebruik van gemaakt. Lia, het was erg plezierig om samen met jou aan het project te werken. Ik ben je erg dankbaar voor de rust en realiteitszin die je op mij overbracht en je kennis van zaken op het lab; we konden niet alleen praten over de wetenschap, maar ook over de dingen des levens. Arjen, jij hebt de klus al geklaard. Sander, je kwam halverwege bij de groep en ik heb veel kunnen leren van je nieuwsgierigheid en ervaring. Ik hoop dat jullie samen het aspartic protease en glutathione S-transferase werk naar een hoger plan brengen. Samen met Wendy, Lute-Harm, Alan en Henk-jan hebben we vele uren op het lab doorgebracht en een ieder heeft op zijn eigen manier een bijdrage geleverd aan een op zijn minst gevarieerde discussie.

De verfrissende rookpauzes waren een rustpunt en uitlaatklep tussen de experimenten door. Buiten op de trap de koude trotserend, of met Willem in zijn kantoor. Het heeft me erg goed gedaan. Ook heb ik veel te danken aan Paul en John. Door jullie kennis en ervaring ben ik een stuk wijzer geworden op het lab. Het is erg waardevol voor een vakgroep om mensen als jullie te kunnen raadplegen zodat het wiel niet steeds opnieuw uitgevonden hoeft te worden.

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Naast het werk was natuurlijk ook tijd voor ontspanning. De vrijdagmiddagsessies in Loburg waren altijd een fijn moment om de week te besluiten. Ik heb er met veel (verschillende) vrienden menige Koninck gedronken.

Ook op de werkplekken na fytopathologie heb ik het prima naar mijn zin gehad. Op MGIM werden Marco en Steph en Peter meer dan alleen collega's. De wekelijkse borrels van virologie in De Zaaier waren erg gezellig en de Whisky Vodka Waits-avonden met Ingeborg en Marjolein echt onvergetelijk. Het is fijn te weten dat je leuke mensen op de werkvloer hebt waarmee je naast de wetenschap ook nog eens over andere dingen kan praten!

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Dick, samen met jou heb ik me de laatste paar jaar flink kunnen afreageren met squash. Het is echt zalig om je in een uur helemaal lek te slaan en dan na het douchen een pils of twee te doen. Bedankt voor wat Annemarie en jij voor mij gedaan hebben en ik hoop dat je nog lang mijn squashpartner en Vriend wilt zijn.

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Curriculum Vitae

Theo Prins werd geboren op 26 april 1965 te Nijkerk. In 1982 behaalde ik het MAVO diploma aan de christelijke MAVO te Nijkerk. Hierna heb ik in 1984 mijn HAVO diploma behaald aan het Farel College te Amersfoort. Na de propedeuse HLO aan de Amersfoortse Laboratorium School heb ik voor de botanische richting gekozen, welke ik in 1988 met een diploma heb afgesloten aan de Rijks Hogere en Middelbare Laboratorium School te Wageningen. Tijdens deze studie werden een stage bij het toenmalige ITAL (dr. Hans Helsper) en een afstudeervak bij het toenmalige IVT (dr. Tini Colijn) voltooid. Vervolgens heb ik mijn dienstplicht vervuld bij het Korps Rijdende Artillerie als Wachtmeester Voorwaarts Waarnemer.
Eind 1989 ben ik gaan studeren aan de Landbouwuniversiteit Wageningen in de richting Plantenveredeling met moleculaire oriëntatie. Tijdens deze studie heb ik twee afstudeervakken voltooid bij de vakgroepen Virologie (ir. Frank van Poelwijk) en Moleculaire Biologie (July Mylona MSc). In augustus 1992 ben ik afgestudeerd in de Levenswetenschappen.
Na het behalen van mijn academische graad ben ik in 1992 voor zeven maanden als toegevoegd onderzoeker gaan werken op de vakgroep Fytopathologie (LUW) aan een door het NRLO gefinancierd literatuuronderzoek naar de horizontale genoverdracht bij planten onder begeleiding van prof. dr. ir. J.-C. Zadoks. Ook werkte ik zes maanden op de vakgroep Fytopathologie (LUW) als vrijwilliger aan de karyotypering van *Botrytis cinerea* (dr. Jan van Kan).
In september 1994 begon ik als onderzoeker in opleiding (OIO) bij de vakgroep Fytopathologie (LUW) aan een door de SLW gefinancierd project. Dit project werd uitgevoerd in de onderzoeksschool Experimentele Plantenwetenschappen onder begeleiding van dr. Jan van Kan en heeft geleid tot dit proefschrift.
Van februari tot juli 1999 werkte ik als postdoc op de sectie Moleculaire Genetica van Industriële Micro-organismen (MGIM, LUW, dr. ir. Jaap Visser) aan de klonering van selectiemarkers en virulentiegenen van *B. cinerea*.
Van september 1999 tot oktober 2001 was ik werkzaam als postdoc bij de vakgroep Virologie (Wageningen Universiteit, prof. dr. Rob Goldbach). In dit door STW gefinancierde onderzoek heb ik mij bezig gehouden met de klonering en karakterisering van het *Sw5* resistentiegen uit tomaat tegen het tomato spotted wilt virus (TSWV) en het mogelijke avirulentiegen.
Per oktober 2001 ben ik werkzaam als wetenschappelijk onderzoeker bij de businessunit Genetica en Veredeling in het cluster Ornamental Non Food Crops van Plant Research International.

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