Genetic variation and pathogenicity of *Botrytis cinerea*

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Genetic variation and pathogenicity of *Botrytis cinerea*

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Abstract

Botrytis cinerea is a fungal pathogen of more than 200 hosts including a wide variety of economically important crops. Although many ecological and physiological studies on this destructive pathogen have been reported, not much is known about the molecular basis of the interaction of this pathogen with its various host plants. This thesis describes the use of molecular techniques to study the genetic variation and pathogenicity of *B. cinerea*.

Genetic variation among ten strains of *B. cinerea* was studied by RAPD analysis. Strains appeared to be highly similar and could not be grouped according to their host, suggesting that host specialization does not occur in *B. cinerea*. Segregation analysis of RAPD markers in generative progeny collected from ordered ascospore octads, predominantly revealed segregation ratios of 1:1. Occasionally, a 1:3 segregation ratio, or the appearance and disapperance of RAPD markers were observed. These unexpected observations were explained by the heterokaryotic association of presumably polyploid nuclei.

An important role for cutinase was suggested in penetration of undamaged host tissue. To investigate the relevance of cutinase in more detail, cloning of the cutinase gene was an essential step. Molecular strategies such as heterologous screening and immunoscreening of libraries and a PCR based cloning strategy were employed without success. Purification of the enzyme, partial determination of its amino acid sequence and the design of cutinase specific primers finally led to the cloning and identification of the corresponding gene (*cutA*). A second putative esterase was co-purified and the encoding gene (*ekdA*) was isolated as well.

Using a *cutA* promoter - GUS reporter gene fusion, expression of the cutinase gene was studied *in planta*. Conidia of transformants harbouring the reporter construct and germinating on gerbera flowers and tomato fruits, contained GUS activity, indicating that the cutinase gene is expressed during penetration of host tissue by *B. cinerea*. However, disruption of the single copy *cutA* gene in a haploid strain of *B. cinerea*, showed that this cutinase is not required for successful infection of gerbera flowers and tomatoes. The ability of cutinase A-deficient mutants to infect and to develop disease was unaltered compared to the wild type strain. These results suggest that *B. cinerea* employs other strategies to penetrate undamaged host tissue. Production of other cutinases, generation of oxygen radicals by glucose oxidase or mechanical penetration by formation of appressoria might be involved.

Stellingen

1. Het feit dat cutinase A van *Botrytis cinerea* niet essentieel is voor succesvolle penetratie betekent niet dat penetratie louter een mechanisch proces is.

   Dit proefschrift.

2. Heterokaryons maskeren geslaagde gendisruptie in *Botrytis cinerea*.

   Dit proefschrift.

3. Voor de evaluatie van de rol van een specifiek cutinase van een plantepathogene schimmel in het infectieproces dienen gendisruptie-experimenten aangevuld te worden met analyses betreffende de cutine hydrolyserende activiteit en het tijdstip van enzymproduktie tijdens de interactie.

   Sweigard JA et al., 1992. Molecular and General Genetics 232: 174-182, 183-190.

4. De conclusie van Salinas dat cutinase essentieel is voor succesvolle infectie van gerbera bloemen door *Botrytis cinerea*, wordt onvoldoende ondersteund door controle experimenten.

   Salinas J, 1992. PhD-thesis, University of Utrecht, The Netherlands.

5. De tegenstrijdige opvattingen van Rogers *et al.* (1994) en Stahl *et al.* (1994) over de rol van cutinase tijdens infectie van erwtezaailingen door *Fusarium solani* f.sp. *pisi*, zouden wellicht niet bestaan indien de onderzoekers hun identieke cutinase deficiënte mutanten op een zelfde wijze geanalyseerd hadden.

   Rogers LM et al., 1994. Plant Cell 6: 935-945.
   Stahl DJ et al., 1994. Molecular Plant-Microbe Interactions 7: 713-725.

6. Gezien de aanzienlijke produktie van organochloorverbindingen door algemeen voorkomende basidiomyceten, dient de normstelling voor bodemverontreiniging met deze verbindingen herzien te worden.

   Verhagen FJM et al., 1996. Applied Microbiology and Biotechnology 45, in press.

7. Het benoemen van virussen slechts aan de hand van gedeeltelijke sequentiegegevens verdient geen navolging.

Sumi S et al., 1993. Journal of General Virology 74: 1879-1885.
Nagakubo T et al., 1994. Phytopathology 84: 640-645.
Tsuneyoshi T and Sumi S, 1996. Phytopathology 86: 253-259.

8. Het door Yamada *et al.* geïsoleerde arabinoxylo-oligosaccharide uit tarwebloem wordt door de auteurs ten onrechte nieuw genoemd.

Hoffmann RA et al., 1991. Carbohydrate Research 221: 63-81.
Gruppen H et al., 1992. Carbohydrate Research 233: 45-64.
Yamada H et al., 1994. Bioscience, Biotechnology and Biochemistry 58: 288-292.

9. Moleculair-biologische technieken kunnen ook een rem vormen op het verkrijgen van meer kennis over plant-pathogeen interacties.

10. Efficiënte bestrijding van rot in geoogste produkten veroorzaakt door *Botrytis cinerea* is mogelijk door tijdige consumptie.

11. 'Shot gun' onderzoek vereist geoefende scherpschutters.

12. Er dient zo spoedig mogelijk een kattenbelasting ingevoerd te worden.

13. Motorrijden is een filosofie.

Pirsig RM, 1974. Zen and the art of motorcycle maintenance.

Stellingen behorende bij het proefschrift
Genetic variation and pathogenicity of *Botrytis cinerea*

Wageningen, 12 juni 1996 Cécile van der Vlugt-Bergmans

Voorwoord

Als het proefschrift dan eindelijk zo goed als af is, blijft er toch nog één blanco pagina over met daarop slechts 'Voorwoord'. De meest gelezen bladzijde die het laatst geschreven wordt en moeilijk te vullen lijkt. Al peinzend blijkt dan dat hèt moment te zijn waarop de afgelopen jaren promotie-onderzoek nog eens aan je voorbij trekken. Hoewel het onderzoek, zoals het ook hoort, z'n dieptepunten heeft laten zien, is het een plezierig gevoel dat de leuke dingen toch de beste herinneringen vormen. Hiervoor wil ik graag een aantal mensen bedanken.

Pierre de Wit, hoewel je op een zekere afstand tot het Botrytis-onderzoek stond, wist je heel concreet punten van verbetering aan te wijzen. Zowel tijdens het onderzoek als later bij het schrijven, bleek dit van groot belang. Jan van Kan, de vrijheid die je me gegeven hebt om een eigen richting aan mijn promotie-onderzoek te geven, heb ik bijzonder gewaardeerd. En niet te vergeten, de reis naar Australië! John van 't Klooster en Lia Sibbel-Wagemakers, Botrytis-collega's van het eerste uur (er bestond dus wel degelijk meteen een Botrytis-groep!), jullie inzet, meedenken en gezelligheid was bijzonder. Ook dank aan Ernesto Pérez-Benito, Theo Prins, Arjen ten Have en Dianka Dees. Af en toe wat lenen, een uitgewerkt protocolletje overnemen of een aantal proeven uit handen geven, was zeer aangenaam. Elly Janssen en de studenten Bas Brandwagt en Bärbel Rudakoff wil ik bedanken voor hun bijdragen aan het onderzoek. Dank ook aan alle collega's van de vakgroep Fytopathologie voor de prettige sfeer en leuke samenwerking, en aan de mensen van het secretariaat, Duotone en de keuken voor hun ondersteunend werk.

Mijn ouders wil ik bedanken dat ze me op 5-jarige leeftijd het horlogemakers-gereedschap niet hebben afgenomen. Deze ervaring bleek later zeer van pas te komen bij het priegelen aan apothecia en asci van *Botrytis cinerea*. René, als de motivatie en inspiratie eens wat minder waren, bleek jij een enorme steun in de rug. Ook van jouw kritische opmerkingen en heldere formuleringen, die niet altijd even gewenst leken, heb ik veel geleerd.

Sommige boeken zijn ten onrechte vergeten;
aan geen enkel boek is de herinnering ten onrechte levend gebleven.

W.H. Auden (1962)

Contents

CHAPTER 1

General introduction

Botrytis cinerea

The genus *Botrytis* (from the Greek **βοτρυς** meaning a bunch of grapes) is one of the oldest genera of fungi, first described by Micheli in 1729. In the early 19th century, Persoon designated five species under the binominal system of Linnaeus and included one of Micheli's species, *B. cinerea*. In 1973 the genus *Botrytis* was redefined by Hennebert and it now comprises 22 species. Most species have a narrow host range, e.g. *B. aclada* on *Allium* spp. and *B. fabae* on *Leguminosae*. *B. cinerea*, however, attacks a wide range of plants in temperate regions and causes significant economic losses (Jarvis, 1977).

The most common symptoms induced by *B. cinerea* are decay or rot in all kinds of fleshy organs, flecking on leaves and small necrotic lesions (spots) on different plant parts. Spotting on tomato fruits is commonly called ghost spot (Verhoeff, 1970) and on gerbera it is denominated in Dutch 'smet' or 'pokken' (Bakker, 1986). Although *B. cinerea* is usually causing destructive plant diseases, there is one type of infection in grapevines which is both valued and encouraged. This is the infection known as *pourriture noble* in France and *Edelfaüle* in Germany. Under certain climatic conditions *B. cinerea* parasitizes the berry in such a way that the skin remains intact, but the content shrivels. In this process sugars become concentrated without increasing the acidity. Grapes affected by this noble rot are used in the production of certain high quality sweet wines. However, *B. cinerea* mostly causes a destructive disease in grapes, the *pourriture grise* or familiar grey mould (Ribéreau-Gayon *et al.*, 1980).

B. cinerea belongs to the class of Deuteromycetes (Fungi Imperfecti), order Moniliales and family Moniliaceae. Asexual spores, macroconidia and microconidia, and sclerotia, are frequently produced. Conidia are considered as the main fungal propagules for the dispersal of *B. cinerea*. The massive production of grey-brown clusters of macroconidia provides the fungus with the common name grey mould. Microconidia emerge from phialides on germ tubes, mature hyphae, sclerotia and sometimes within empty cells under harsh conditions (Jarvis, 1980). Only Brierley reported in 1918 that microconidia germinated in nutrient broth and grew out into mycelium. Since the studies of Drayton (1932) their sole function is supposed to be in spermatization. Sclerotia serve as survival structures for adverse conditions and germinate by forming conidiophores or, under special conditions, apothecia.

Besides the imperfect stage, the perfect stage, or teleomorph, is known as *Botryotinia fuckeliana* (de Bary) Whetz. belonging to the Ascomycetes, order Helotiales and the family Sclerotiniaceae (Korf, 1973). The connection between the anamorph *B. cinerea* and the teleomorph *B. fuckeliana* was first made by Groves and Drayton (1939), when they established the *in vitro* formation of apothecia by *B. cinerea*. The teleomorph consists of the reproductive body, the apothecium, containing ascospores in linear asci. It is generally recommended that for fungi the scientific name of the sexual stage should be preferred to that of the asexual stage. Therefore, *B. fuckeliana* should replace *B. cinerea* as the Latin binominal for the fungus causing the disease grey mould. However, the binominal

B. cinerea is recognized world-wide by numerous mycologists and plant pathologists. Reports on apothecia production in nature are rare (Kublitzkaya and Rjabtzeva, 1968; Polach and Abawi, 1975) and, therefore, it was decided to retain the binominal *B. cinerea* (X^{th} International *Botrytis* Symposium, Heraklion, Greece, 1992). The name of the anamorph is therefore used throughout this thesis.

Genetic variability in *B. cinerea*

B. cinerea has been described as a highly variable species. Variability in growth characteristics, production of conidia and sclerotia, enzyme production or pathogenicity have been reported among isolates of *B. cinerea* (Grindle, 1979; Di Lenna *et al.*, 1981; Lorenz, 1983; Leone, 1990; Movahedi and Heale, 1990b). This genetic variability is often attributed to the cytological condition of hyphal cells which are multinucleate and frequently heterokaryotic (first described by Hansen and Smith, 1932). From this mycelium, macroconidia originate which are also multinucleate and possibly heterokaryotic; a mean number of 4-6 nuclei per macroconidium has been reported by Shirane *et al.* (1988; 1989). Alternatively, the ploidy level of *B. cinerea* may play a role in variability. A study by Grindle (1979) showed that mutants were produced *in vitro* with very low frequency and were not stable. This was explained by the occurrence of diploidy in *B. cinerea*. Lorenz (1983) studied the phenotypic stability of monoascospore isolates. Since these isolates originate from homokaryotic ascospores (Lorenz and Eichhorn, 1983), variation due to heterokaryosis could be excluded. After repeated passage of monoascospore cultures variations in morphology were observed, for which aneuploidy was proposed as one possible explanation (Lorenz, 1983).

Two mechanisms by which variation in *B. cinerea* may be generated (Lorbeer, 1980) are proposed: anastomosis resulting in heterokaryons which contain more than one type of nucleus in the same cell or thallus of the fungus (asexual variability) and second, meiotic recombination and segregation of genes from two different nuclei (sexual variability). Both mechanisms are discussed below.

Asexual variability

The significance of heterokaryosis was first reported in studies on *B. cinerea* by Hansen and Smith (1932) and is now recognized as a major factor in variability of filamentous fungi in nature. They reported that nuclei of one strain of *B. cinerea* can migrate into the cells of another strain by anastomosis of neighbouring hyphae giving rise to heterokaryotic mycelium. From 47 field isolates subcultures were made by successive single-conidium transfers. After many such transfers, a wide range of morphologically distinct strains was obtained. Since anastomosis was common and hyphal cells and conidia were multinucleate, the conclusion was that conidia could act as heterokaryotic propagules. However, Jinks (1959) pointed out that the evidence for heterokaryosis does not preclude the possibility that such variation may be of cytoplasmic origin.

Later on, phenotypic variation of asexual progeny, observed by the variation in morphologic characteristics among monoconidial transfers, was also reported by Grindle (1979) and Lorenz (1983) and attributed to the multinucleate, heterokaryotic condition of hyphae and conidia of the fungus. Support for this conclusion came from studies of Akutsu *et al.* (1987, 1988) who demonstrated the frequent occurrence of hyphal anastomosis and the transfer of nuclei carrying fungicide resistance markers from one thallus to another.

Vegetative incompatibility, the phenomenon that hyphae of two strains cannot fuse or fail to form a viable heterokaryon, has been demonstrated for several plant-pathogenic fungi, e.g. *Ophiostoma ulmi* (Brasier, 1983), *Cryphonectria parasitica* (Anagnostakis, 1981), *Sclerotinia sclerotiorum* (Kohn *et al.*, 1990). Obviously, the existence of vegetative incompatibility may limit the extent of heterokaryon formation and thus restrict genetic variability. It was reported that vegetative incompatibility also occurs in *B. cinerea* (Beever and Parkes, 1993), but its impact on genetic variation in the fungal population remains to be determined.

The need for homokaryons for genetic analysis focussed attention on the microconidia and ascospores. Unfortunately, uninucleate microconidia do not germinate (Jarvis, 1980). Mature ascospores, although containing 3-4 nuclei, are considered homokaryotic since they originate from a young uninucleate ascospore (Lorenz and Eichhorn, 1983).

Sexual variability

Since the first report on the formation of apothecia by *B. cinerea* in 1939 (Groves and Drayton), detailed studies on the genetics of *Botrytis* have been hampered by the complexity of the crossing procedure and the long period needed for apothecial production in culture. More recently, production of apothecia under controlled environmental conditions has been described and improved by Faretra and Antonacci (1987) and Faretra *et al.* (1988a). To produce apothecia, sclerotia of one 'female' strain, formed at 15°C in the dark, were subjected to a cold period and subsequently spermatized with microconidia of the 'male' strain. After spermatization, apothecia arise after 6-15 weeks.

The work of Faretra *et al.* (1988b) revealed that sexual compatibility of *B. cinerea* is controlled by a single mating type locus with two alleles: *MAT1-1* and *MAT1-2*. The occurrence of field isolates containing both mating type alleles, designated *MAT1-1/2*, was ascribed to the heterokaryotic association of nuclei of opposite mating type (Lorenz and Eichhorn, 1983; Faretra *et al.*, 1988b). However, homothallism among strains derived from single ascospores was also observed, and this can not be explained by heterokaryosis. Therefore, further study is needed to explain this phenomenon (Faretra *et al.*, 1988b).

The mating type of new isolates is determined using reference strains of known mating type: the *MAT1-1* allele was assigned to strain SAS56 and the *MAT1-2* allele to strain SAS405 (Faretra *et al.*, 1988b). Both strains are now routinely used in mating type studies

(Beever and Parkes, 1993; Faretra and Pollastro, 1993; Van der Vlugt-Bergmans *et al.*, 1993).

The sexual stage of *B. cinerea* does not seem to play a prominent role in the fungal life cycle, since apothecia have rarely been found in nature (Grindle, 1979). However, determination of mating type alleles of field isolates in Italy (Faretra *et al.*, 1988b), New Zealand (Beever and Parkes, 1993) and nine other countries (Faretra and Pollastro, 1993) revealed that both mating types were equally distributed in populations from the same geographical districts. The lack of descriptions of apothecia occurring in the field is therefore not due to the paucity of potential mating partners in the same area.

Pathogenicity of *B. cinerea*

Disease occurrence

B. cinerea is a pathogen to at least 235 hosts (MacFarlane, 1968) including a wide variety of economically important plants, such as vegetables (tomato, cucumber), ornamentals (gerbera, rose), bulbs (onion, lily) and fruits (grape, strawberry). It is also a saprophyte on senescing, dead or already invaded plant material and causes post-harvest diseases, probably starting as a latent infection in the field. During transport and storage of agricultural products, external conditions or the ripening stage of harvested products favour the outgrowth of *B. cinerea*, causing post-harvest losses (McNicol *et al.*, 1985; Williamson *et al.*, 1987).

Infection of plants can occur in various ways. Germ tubes of conidia easily invade wounded or dead plant tissue. They can occasionally also enter via stomata (Verhoeff, 1980) or directly penetrate unwounded healthy tissue, as has been observed on various hosts. Among these are grapes (McClellan and Hewitt, 1973) and tomato fruits (Verhoeff, 1970) in which rotting of the infected tissue occurs, or gerbera flowers (Salinas *et al.*, 1989; Salinas and Verhoeff, 1995) and French bean leaves (Van den Heuvel, 1981) on which localized necrotic lesions develop. From primary local lesions, spreading lesions may develop, depending on a number of external factors, as well as on the condition of the host plant.

Besides infection from germinating conidia, infection by mycelium spreading from dead, moribund or infected plant parts into healthy tissue, is very common (Jarvis, 1977). This can often be seen when infected flowers, acting as a nutrient source for the fungus, have fallen onto, or come into contact with leaves or fruits. In addition, fruit infections can develop from hyphal growth from senescent or necrotic flower parts during or just after bloom (Verhoeff, 1980).

Disease development

A high relative humidity or availability of free water on the surface of plants is often considered as the most important factor for germination of *Botrytis* conidia (Blakeman, 1980). Moreover, the infection of intact leaves of several plant species by *B. cinerea* is

stimulated by glucose and phosphates (Akutsu *et al.*, 1981b; Harper *et al.*, 1981; Van den Heuvel, 1981). Glucose was proposed to promote superficial growth and formation of prepenetration structures (Akutsu *et al.*, 1981a), whereas phosphates might be involved in stimulating the activity of pectolytic enzymes (Van den Heuvel and Waterreus, 1985). The presence of exogenous nutrients in the inoculum buffer might mimic the natural infection starting from mycelium growing in wounded or dead plant tissue. Following germination healthy, unwounded host tissue must be actively penetrated. This process is thought to occur either enzymatically or mechanically by the formation of appressoria or by a combination of enzymes and mechanical force. These penetration mechanisms have received much attention and are discussed below in more detail.

Subsequent invasion of the host is often preceded by cell death of host tissue. Movahedi and Heale (1990a, b) observed cell death in carrot root slices inoculated with *B. cinerea* which was attributed to the activities of an aspartic proteinase and endo-pectin lyase. It was suggested that the phytotoxic activity of both enzymes is indirectly caused by the toxic effect of cell wall components released by both enzymes. Death of the host cells allows invasion of *B. cinerea*. Since the infection was markedly reduced by pepstatin, an inhibitor of the aspartic proteinase, a primary role for this enzyme in pathogenesis was assumed (Movahedi and Heale, 1990b).

Cell wall degrading enzymes, such as polygalacturonases, pectin-lyase and pectin methyl esterase are secreted by *B. cinerea* (Verhoeff and Warren, 1972; Van den Heuvel and Waterreus, 1985; Leone *et al*, 1990; Reignault *et al.*, 1994), as well as oxalic and citric acid (Kamoen and Jamart, 1974). These acids were suggested to lower the pH in the plant tissue, thereby favouring the activity of the cell wall degrading enzymes (Verhoeff *et al.*, 1988). This concerted action causes extensive maceration and cell death. Citric acid alone was shown to cause a similar yellowing as a *B. cinerea* infection and was thought to act as a toxic compound to plant tissue on its own (Kamoen and Jamart, 1974). Regardless whether acids are toxic to host cells or contribute to creating optimal conditions for cell wall degrading enzymes, in both cases the fungus lives saprophytically on dying host tissue and continues to colonize tissues at the edge of the lesion.

Upon attack by *B. cinerea*, defence mechanisms like lignification and production of phytoalexins, are activated in the plant (Mansfield, 1980). Bar Nun and Mayer (1989; 1990) and Viterbo *et al.* (1993) have shown that cucurbitacins prevent formation of laccase in *B. cinerea*, and afford total protection against the invading fungus when applied to cucumber fruits or leaves. Host tissue treated with cucurbitacins had formed a lignified layer, preventing fungal penetration. In the absence of the cucurbitacins, lignification was apparently delayed and fungal penetration, accompanied by laccase secretion, occurred before the formation of a protective barrier. Laccase belongs to the group of polyphenol oxidases (Gigi *et al.*, 1980; Marbach *et al.*, 1983, 1984) and might be involved in inactivating host defences by disrupting lignin synthesis (Viterbo *et al.*, 1992). In addition to preventing the activation of the plant defence reactions, *B. cinerea* is known to detoxify plant defence compounds. The pathogenicity of isolates of *B. cinerea* towards French

bean was found to be correlated with their ability to metabolize the phytoalexin phaseollin (Van den Heuvel, 1976). Tomatine, present in the outer layers of young tomato fruits, is assumed to play a role in restricting fungal development in young fruits. The ability of *B. cinerea* to convert tomatine to tomatidine, which is not inhibitory, and the decrease in tomatine content during ripening might explain the outgrowth of *B. cinerea* in ripe fruits (Verhoeff and Liem, 1975). Pezet *et al.* (1991) demonstrated the ability of *B. cinerea* to oxidize pterostilbene and resveratrol by producing stilbene oxidase, an enzyme which is also a member of the group of polyphenol oxidases. A high level of pterostilbene is considered to be an important factor in the resistance of immature grapes to *B. cinerea*.

It is clear that *B. cinerea* possesses several mechanisms to infect host tissue and to inactivate or circumvent the plant defence response. Since research on the infection process has been performed on different hosts, it is not clear whether these mechanisms are employed in concert, or whether different mechanisms are applied for different hosts.

Disease control

Control of *B. cinerea* is hampered by its wide host range, its ability to infect various plant organs during plant development, and its fast life cycle of germination, colonization and sporulation in the field. Disease pressure is diminished by the removal of infested material and providing conditions for proper aeration and quick drying of plants or harvested products. Intensive pre- and postharvest control is needed to prevent expansion of the fungus from latent infections.

Chemical control is one of the major control methods for disease of *Botrytis* spp.. Benzimidazole and dicarboximide fungicides have been used extensively. However, soon after their introduction, rapid development of resistant *B. cinerea* populations occurred which limits their use (Grindle, 1981; Pommer and Lorenz, 1982; Gullino and Garibaldi, 1986; Elad, 1992).

Attempts have been undertaken to develop biological control methods by suppression of *B. cinerea* development and conidial production using antagonistic bacteria and fungi (Peng and Sutton, 1991; Elad *et al.*, 1994). Only recently, field experiments showed promising results (Köhl *et al.*, 1995), but the application of biological agents is not yet a major strategy in grey mould control.

Increased disease resistance to *B. cinerea* in transgenic tobacco plants has been achieved by introduction of a stilbene synthase gene from grapevine. When this gene is expressed in tobacco, the corresponding enzyme converts endogenous precursors into the phytoalexin resveratrol with fungitoxic potential (Hain *et al.*, 1993).

Penetration of undamaged host tissue by *B. cinerea*

The cuticle of aerial plant parts is the first barrier the fungus has to overcome for direct penetration of the epidermis. The structural component of the cuticle is cutin, a polymer mainly composed of C16 and C18 hydroxy fatty acids, predominantly linked by ester

bonds and some ether and peroxide bonds (Martin and Juniper, 1970).

The mechanism by which *B. cinerea* breaches the cuticle has been debated for a long time. Penetration was long thought to be mechanical (Blackman and Welsford, 1916; Brown and Harvey, 1927). The conidium, disseminated on the cuticle, germinates at high relative humidity and the tip of the germ tube is pressed against the cuticle, causing a slight indentation of the cuticle and the underlying epidermal cell wall. From this observation, it was concluded that the cuticle was ruptured mechanically by pressure of the tip of the germ tube. Later on, the formation of appressoria was observed from which a small penetration peg emerged that penetrated the cuticle (McKeen, 1974; Clark and Lorbeer, 1976). Appressoria were described as simple swollen tips of germ tubes, superficial hyphae or of branched structures (Akutsu *et al.*, 1981b).

After examining the penetration of bean leaves McKeen (1974) suggested that the cuticle was dissolved enzymatically, as holes in the cuticle appeared sharp and clean without curled edges. Furthermore, infection pegs did not cause an indentation of the cuticle or epidermal cell wall during penetration. Esterase activity (typical for cutinases, see below) was detected within the tips of germ tubes at the time of penetration. Studies by Rijkenberg *et al.* (1980) supported these observations; the cuticle of tomato fruits disappeared, without being ruptured, around penetrating germ tubes of conidia of *B. cinerea*.

Support for enzyme-mediated penetration was first provided by Linskens and Haage (1963) who found that *B. cinerea* could degrade potato leaf cutin *in vitro*. Shishiyama *et al.* (1970) described the purification of a cutinase from *B. cinerea* and showed an altered structure of cutin upon incubation with this enzyme. This finding suggested that cutinase could reduce the mechanical strength of the cuticle, thereby facilitating penetration. Further evidence supporting a role for cutinase in hydrolysis of the cutin layer and in penetration came from experiments using monoclonal antibodies raised against a cutinase of *B. cinerea*. Lesion formation was reduced by 80% when gerbera flowers were sprayed with this antibody before inoculation with *B. cinerea* (Salinas, 1992) and an important role for cutinase in the penetration process was claimed.

In artificial inoculations using *B. cinerea* conidia, the presence of glucose in the inoculum buffer is essential for successful infection. Glucose was proposed to promote superficial growth and formation of prepenetration structures (Akutsu *et al.*, 1981a, Clark and Lorbeer, 1976), facilitating the infection. Another hypothesis for the necessity of glucose in the inoculum buffer was postulated by Edlich *et al.* (1987, 1989) who studied the infection of broad bean (*Vicia faba*) by *B. cinerea* conidia. It was demonstrated that glucose does not serve as energy source, but rather as substrate for fungal glucose oxidases releasing H_2O_2 during the oxidation of glucose. H_2O_2 can be converted to O_2^- and $OH^\cdot$ radicals, which are capable of destroying relatively inert material, such as cutin and membrane lipids. H_2O_2 is also able to diffuse across the cell membrane and exert toxic effects on the interior of the plant cell. Weakening of plant tissue is thought to facilitate penetration by the fungus.

Fungal cutinases

The enzyme

Production of cutinase in *in vitro* cultures is reported for over 20 plant pathogenic fungi (Baker and Bateman, 1978; Ettinger *et al.*, 1987; Trail and Köller, 1990). Cutinase is produced in the presence of cutin, cutin hydrolysate or cutin monomers like 16-hydroxyhexadecanoic acid in the medium, but its production is repressed by glucose (Lin and Kolattukudy, 1978; Salinas, 1992).

Fungal cutinases are extracellular enzymes and consist of a single polypeptide with a molecular weight of approximately 22-24 kD. The cutinase of *B. cinerea* was reported to be 18 kD (Salinas, 1992). Inhibition of enzyme activity by diiospropylfluorophosphate (Purdy and Kolattukudy, 1975; Köller and Kolattukudy, 1982) indicated the involvement of a serine residue in the active centre of the enzyme. On the basis of this characteristic cutinase was assigned to the group of serine esterases that contain the catalytic triad serine, histidine and aspartate. In these enzymes the serine residue of the active centre is present in the consensus sequence glycine-X-serine-X-glycine (Martinez *et al.*, 1992).

Typical for serine esterases is their capacity to hydrolyze the model substrate para-nitrophenyl butyrate which is generally used to monitor cutinase activity (Purdy and Kolattukudy, 1975; Dickman *et al.*, 1982; Bonnen and Hammerschmidt, 1989; Trail and Köller; 1990; Salinas, 1992). However, to demonstrate specific cutinase activity, the enzyme should be tested on radioactively labelled cutin (Purdy and Kolattukudy, 1973, 1975). After hydrolytic cleavage of the ester bonds, fatty acids are released from the insoluble substrate into solution.

A role for cutinase in penetration

The importance of cutinase for successful fungal penetration of host tissue has received support from various studies involving different pathogens. Firstly, cutinase of *Fusarium solani* f.sp. *pisi* was detected at the site of penetration using immuno electron microscopy (Shaykh *et al.*, 1977). Secondly, inhibition of cutinase by chemical inhibitors or antibodies prevented infection of host tissue by *F. solani* (Maiti and Kolattukudy, 1979), *Colletotrichum gloeosporioides* (Dickman *et al.*, 1982) and *Venturia inaequalis* (Köller *et al*, 1991). Thirdly, strains of *F. solani* (Dantzig *et al.*, 1986) and *Alternaria alternata* (Tanabe *et al.*, 1988) with reduced cutinase activity exhibited reduced pathogenicity in bioassays. Fourthly, the obligate wound parasite *Mycosphaerella* spp. transformed with the cutinase gene of *F. solani* f.sp. *pisi*, could penetrate an intact cuticle and cause disease. This infection could be prevented by antibodies against *F. solani* cutinase (Dickman *et al.*, 1989).

Final proof to demonstrate a role for cutinase during the penetration process was expected from the construction of cutinase-deficient mutants by means of gene disruption. Mutants of *Magnaporthe grisea* (Sweigard *et al.*, 1992b) and *A. brassicicola* (Yao and Köller, 1995) with a disrupted cutinase gene retained their pathogenicity, but also still exhibited some cutinase activity. In *M. grisea* mechanical penetration of the cuticle by

melanized appressoria prevails over enzymatic penetration mediated by cutinase (Chumley and Valent, 1990). Disruption mutants of *F. solani* f.sp. *pisi* (Stahl and Schäfer, 1992) had lost cutinase activity, but showed no altered pathogenicity to pea (Stahl *et al.*, 1994). However, Rogers *et al.* (1994) reported that in their experiments the same mutants of *F. solani* were significantly reduced in pathogenicity, since they rather penetrated through stomata than through the cuticle. Obviously, no unequivocal role for cutinase during penetration of plant tissue can be assigned and evaluation of a role for cutinase in pathogenicity is needed for every specific host-pathogen interaction.

A role for cutinase in adhesion

Another role for cutinase activity in host-pathogen interactions was recently presented by Deising *et al.* (1992). Before penetration, spores of the obligate rust fungus *Uromyces viciae-fabae* form adhesion pads on bean leaves. A cutinase and two other serine esterases were detected on the surface of these spores, and they contribute to the adhesion of the spores to the plant cuticle. Adhesion is greatly reduced after washing the spores or after adding a serine esterase inhibitor. In this system cutinase seems to promote adhesion of the fungal spore to the hydrophobic plant surface rather than penetration, since uredospore germtubes usually penetrate through the stomatal pore (Deising *et al.*, 1992).

It remains to be seen whether adhesion is also important for saprophytic fungi like *B. cinerea*. Doss *et al.* (1993) demonstrated that the adhesion of *B. cinerea* conidia on the plant surface is exclusively based on hydrophobic interactions.

Outline of the thesis

Due to its widespread distribution and often destructive effects, *B. cinerea* has been subject of many studies dealing with parasitism, physiology, biochemistry and epidemiology. Despite this wealth of information, many basic molecular and genetic techniques which have been applied to plant pathogenic fungi in the 1980s were not yet used for the analysis of *B. cinerea* (Van Kan *et al.*, 1992). Applying molecular techniques towards understanding genetic variability and pathogenicity of *B. cinerea* was the basis for this PhD research.

RAPD analysis is a powerful tool for studying differences among strains of which no genomic sequence information is available. This technique was used to study DNA polymorphisms among eight Dutch strains and two Italian monoascospore strains (Chapter 2). Crosses were performed to determine the mating type of the Dutch strains, and to study the segregation of DNA polymorphisms, fungicide resistance and phenotypic variation in progeny collected from ordered sets of ascospores.

The presumed involvement of cutinase in the penetration of host tissue by *B. cinerea* incited cloning of the gene to study the expression and requirement of cutinase during penetration. Molecular approaches to isolate the gene encoding cutinase, such as

heterologous screening, immunoscreening and PCR based gene cloning, are described in Chapter 3. Chapter 4 describes a biochemical approach that finally led to cloning of the gene. Cutinase and a second putative esterase were purified, the corresponding genes (*cutA* and *ekdA*) were cloned by reverse genetics and their expression was analyzed *in vitro* and *in planta*. The limited fungal biomass during the early steps of the infection hampered the detection of gene expression by northern blotting. By the use of a cutinase promoter - GUS reporter gene fusion, *cutA* gene expression could be visualized during penetration of host tissue in single penetrating germ tubes (Chapter 5). To unequivocally determine the biological relevance of cutinase during penetration of its host, cutinase A-deficient *B. cinerea* mutants were constructed by means of gene disruption. Transformants lacking a functional cutinase gene were assayed for their ability to infect gerbera and tomato tissue (Chapter 5).

An alternative hypothesis regarding the penetration of host tissue by *B. cinerea*, describes the release of H_2O_2 by fungal glucose oxidase (Edlich *et al.*, 1987, 1989). The toxic effects of H_2O_2 and active oxygen species derived from it, are thought to weaken the plant tissue, thus facilitating infection. Catalase could function in protecting the fungus from toxic effects of H_2O_2. A full length cDNA clone was isolated and its expression was studied *in vitro* and *in planta* (Chapter 6).

CHAPTER 2

Genetic variation and segregation of DNA polymorphisms in *Botrytis cinerea*

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SUMMARY. Genetic variation among eight Dutch strains and two Italian mono-ascospore strains of *Botrytis cinerea* has been studied by RAPD analysis. Of the 139 RAPD markers scored, 74 markers were present in all strains, while 65 markers showed polymorphisms. Computer analysis of these data showed a high similarity (ranging from 0.83 to 0.94) for each pair of strains. A similarity coefficient of 0.99 was found for two Dutch strains isolated from different hosts in 1986 and 1990.

To study the segregation of polymorphic markers in the progeny, crosses were performed between Dutch strains and Italian strains. These crosses revealed the presence of either one or both mating types in the Dutch strains. Of each successful cross, the total progeny was collected from ten complete asci of which the ascospores were released in an ordered way. Morphological characteristics and fungicide resistance in cultures of such an ordered collection of progeny regularly revealed an aberration in spore order within an ascus. RAPD analysis of the progeny emphasized this phenomenon.

In the segregation analysis of DNA polymorphisms in the progeny, cross SAS405 x Bc7 was analyzed. Most of the RAPD markers tested segregated in a normal Mendelian ratio of 1:1. Several unexpected phenomena were observed: two markers, derived from parent SAS405, segregated in a 1:0 ratio; three markers, originating from parent Bc7, were absent in the progeny; and two markers, not scored in the parental strains, segregated in a 1:1 ratio in the progeny. These observations are discussed in view of the multinucleate, heterokaryotic nature of *B.cinerea* and its unknown ploidy level.

Introduction

Botrytis cinerea Pers.:Fr. causes grey mould on economically important crops. Due to its widespread distribution and often destructive effects the fungus has been subject to many studies dealing with parasitism, physiology, biochemistry, chemical and biological control (Coley-Smith *et al.*, 1980). However, genetic knowledge about this fungus is scarce.

Somatic variation among different isolates has been shown for many characteristics like pathogenicity, growth patterns, (Grindle, 1979) and enzyme production *in vitro* and *in vivo* (Salinas and Schot, 1987; Leone, 1990; Movahedi and Heale, 1990b). Asexual progeny of individual cultures were sometimes phenotypically diverse, probably due to the multinucleate and heterokaryotic nature of the fungus (Hansen and Smith, 1932; Grindle, 1979; Lorenz, 1983). Sexual segregation of phenotypical characteristics has hardly been studied, although *in vitro* production of the teleomorph, identified as *Botryotinia fuckeliana* (de Bary) Whertz. has been described (Groves and Loveland, 1953; Lorenz and Eichhorn, 1983; Faretra and Antonacci, 1987). Recently, Faretra *et al.* (1988a) have optimized production of apothecia of *Botryotinia fuckeliana in vitro* and subsequent studies resulted in some fundamental genetic knowledge. Crosses revealed that sexual compatibility is controlled by a single mating type locus with two alleles (*MAT1-1* and *MAT1-2*). The occurrence of field isolates containing both mating type alleles was explained by the heterokaryotic association of nuclei of opposite mating type (Lorenz and Eichhorn, 1983; Faretra *et al.*, 1988b).

The scarce knowledge on genotypic variation in *B. cinerea* has prompted us to study DNA polymorphisms in different strains of *B. cinerea* by RAPD analysis (Williams *et al.*,

1990). Using this method DNA polymorphisms were generated by enzymatic amplification of genomic DNA fragments using the polymerase chain reaction (PCR) with arbitrary oligonucleotide primers. The segregation of some DNA polymorphisms in the progeny has also been studied. Thus far, genetic studies have mostly been performed using random ascospore strains collected from squashed apothecia (Faretra *et al.*, 1988b, Faretra and Pollastro, 1991). In contrast, we have analyzed ordered octads. To this end we have crossed eight Dutch strains with two Italian strains containing known, opposite mating types and collected the progeny in ordered sets of ascospores. Analysis of the segregation of DNA polymorphisms in one cross has revealed interesting fundamental information about meiotic events in *B. cinerea.*

Materials and Methods

Strains. Eight strains of *Botrytis cinerea* (Table 1) collected from different hosts in The Netherlands were donated by Dr J. Salinas (Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands). Two monoascospore strains SAS56 and SAS405 containing mating type allele *MAT1-1* and *MAT1-2*, respectively, were provided by Dr F. Faretra (University of Bari, Italy) and used as reference strains in sexual crosses. Both strains were progeny obtained from crosses of field isolates. Furthermore SAS405 contained resistance genes *Mbc1HR* and *Daf1LR* conferring resistance to benzimidazoles and dicarboximides (Faretra & Pollastro, 1991). All strains were maintained by mass-transfer and stored as conidial suspensions in a 10% glycerol solution, at -70°C.

Sexual crosses. Dutch strains were crossed with Italian strains SAS56 and SAS405 according to a method described by Faretra *et al.* (1988a). Strains producing both 'male' (microconidia) and 'female' (sclerotia) structures were mated in reciprocal crosses. Controls included unspermatized and self-fertilized sclerotia. Each cross was repeated five times, using a total of 15 sclerotia. Apothecia were collected during 2-5 months after spermatization and directly used for collection of ordered sets of ascospores or stored in a 10% glycerol solution at -70°C.

Collection of ordered sets of ascospores. Fully developed asci were collected from ripe apothecia by squashing the apothecial head in water. Asci were separately incubated in a droplet of 5 mg/ml Novozym (buffered in 10 mM MES, pH 6.0) on water agar for 30 seconds at 37°C and subsequently rinsed in a droplet of water. By weakening the natural opening of the ascus in this way, the ascospores could now be released by forcing them

Table 1: Dutch strains of *B. cinerea*, their host and year of isolation.

Strain	Host	Year of isolation
Bc7	tomato	1970
Bc12	gerbera	1986
Bc16	gerbera	1986
Bc18	gerbera	1986
Bc21	rose	1990
Bc25	rose	1990
Bc26	rose	1990
Bc29	gerbera	1991

in an ordered way from the ascus. The eight ascospores of each ascus were separated on water agar and incubated for 2 days. Young colonies were subsequently transferred to tPDA (potato dextrose agar containing 300 g homogenized tomato leaves per litre) and cultured up to conidiation. The cultures were scored for morphological characteristics, like colony diameter and amount of aerial hyphae four to five days after isolation. Conidia of each monoascospore culture were collected and stored in 10% glycerol at -70°C. In most cases ascospores from ten asci of five different apothecia from each cross were separately isolated resulting in a collection of 80 monoascospore strains per cross.

Screening for fungicide resistance. Agar plugs of young mycelial colonies were transferred to potato dextrose agar (PDA) containing 5 μg/ml benomyl (benzimidazole) or 1 μg/ml vinclozolin (dicarboximide). Cultures were incubated at 18°C and scored for growth after 2 days.

DNA isolation. Conidial suspensions of parental strains or monoascospore strains in potato dextrose broth (PDB), were incubated overnight under rotation at 18°C. The resulting mycelium was collected and freeze-dried. 0.1 g mycelium of the parental strains was used for DNA isolation according to a method of Van Kan *et al.* (1991) with some minor modifications. Only 10 mg mycelium of the monoascospore strains was used in a similar, small scale procedure yielding enough DNA for 10-15 PCR assays.

RAPD analysis. Primers used in PCR assays were obtained from Operon Technologies Inc., Alameda CA, USA (RAPD primer sets B and D, each containing 20 primers) or they were home-made (primer R1 to R11, Dept. Molecular Biology, Wageningen Agricultural University, Klein-Lankhorst *et al.*, 1991). Amplification reactions were performed in 40 μl volumes containing 50 ng fungal DNA, 37.5 ng of a single primer, 100 μM dNTPs and 0.3 units *Tth* polymerase (HT Biotechnology, Cambridge, UK) in the prescribed buffer. The reaction mixtures were overlaid with one drop of mineral oil. The PCR assay used an initial denaturation at 95°C for 4 minutes, followed by 40 cycles of 1 min at 95°C, 2 min at 38°C and 2 min at 72°C. All assays were performed on a Perkin Elmer Cetus DNA Thermal Cycler (type 480). As a control PCR assays were run without adding fungal template DNA.

Size separation of the amplification products was by agarose gel electrophoresis on a 2% agarose gel (Sambrook *et al.*, 1989). Half of the PCR mix was loaded on the gel. Fragments were named after the primer with which they were amplified and their size. For example, B13-0.8 is a RAPD marker of 0.8 Kb and amplified with primer B13.

To calculate the genetic similarity among the strains, based on the degree of DNA polymorphisms, the mathematical model of Nei and Li (1979) has been used in the CLUSTAN3.2 VAX-VMS program.

Results

DNA polymorphisms in parental strains. The genetic variation of eight Dutch strains and two Italian monoascospore strains was studied by RAPD analysis (Williams *et al.*, 1990) using 50 different primers. All primers generated 1 to 10 amplification products per primer ranging from 3 to 0.4 Kb in size (Fig. 1). Since the intensity of amplification products on gel could vary slightly between two different DNA samples of the same strain and even between two identical reactions run on different days, one should only consider the absence or presence of intense bands on gel. Only these amplification products were scored as RAPD markers.

Among ten strains tested, the number of different RAPD profiles obtained per primer ranged from one (Fig. 1A, B) to six (Fig. 1D), but three or four different RAPD profiles per primer (Fig. 1C) were most prevalent. No single primer could be used to distinguish all *B. cinerea* strains. However, by combining RAPD profiles from several primers, it was evident that all ten strains showed DNA polymorphisms. To calculate the genetic similarity among the strains, all RAPD markers obtained with 50 primers were scored. Of 139 markers scored, 74 were present in all strains, while 65 showed polymorphisms.

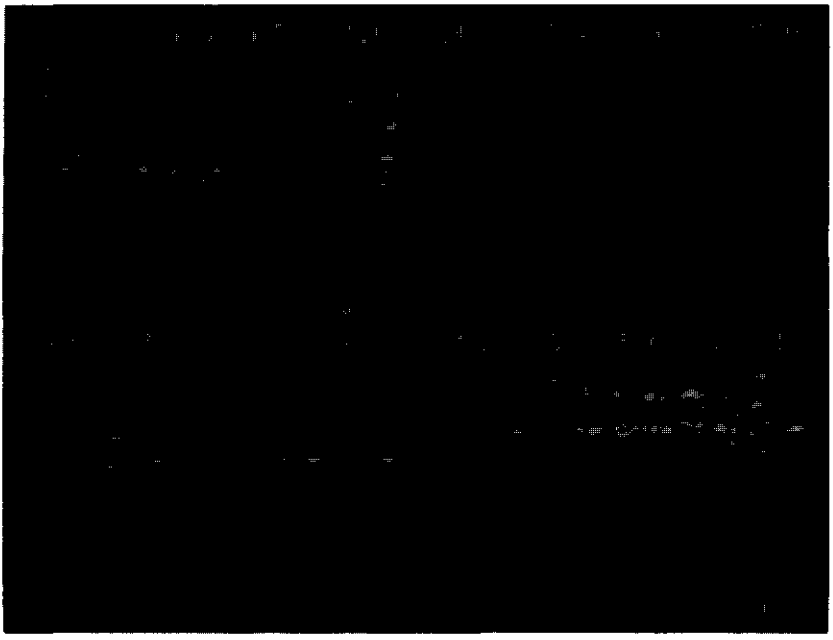

Figure 1: RAPD profiles of ten *B.cinerea* strains. **A** and **B**: universal RAPD profiles obtained by using primer B1 (A) or D6 (B). **C**: set of 4 different RAPD profiles obtained by using primer B6. **D**: set of 6 different RAPD profiles obtained by using primer B20. Lanes numbered 1 to 10 represent strain SAS56, SAS405, Bc7, Bc12, Bc16, Bc18, Bc21, Bc25, Bc26, Bc29, respectively, and lane M shows a marker. Sizes of the marker fragments are 2605, 1605, 1198, 676, 517, 460, 396, 350 and 222 bp.

Table 2: Similarity coefficients for every pair of *B. cinerea* strains calculated according to the mathematical model of Nei & Li (1979). The coefficient of the pair Bc16 and Bc21 is underlined.

	SAS56	SAS405	Bc7	Bc12	Bc16	Bc18	Bc21	Bc25	Bc26	Bc29
SAS56	1									
SAS405	0.884	1								
Bc7	0.861	0.893	1							
Bc12	0.854	0.868	0.911	1						
Bc16	0.834	0.867	0.863	0.875	1					
Bc18	0.863	0.886	0.882	0.894	0.913	1				
Bc21	0.840	0.872	0.858	0.880	0.995	0.918	1			
Bc25	0.877	0.890	0.913	0.870	0.879	0.879	0.884	1		
Bc26	0.885	0.880	0.903	0.860	0.830	0.849	0.826	0.927	1	
Bc29	0.850	0.873	0.935	0.938	0.842	0.890	0.848	0.894	0.884	1

Computer analysis of these data resulted in a matrix with similarity coefficients for each pair of strains (Table 2). Numbers usually ranged from 0.83 to 0.94 indicating that every two strains are genetically highly similar but not identical. However, a similarity coefficient of 0.99 was found for the strains Bc16 and Bc21. Indeed, these two strains differed in only one RAPD marker out of 139 scored.

Sexual crosses. Reciprocal crosses, unspermatized and self-fertilized controls between eight Dutch strains and two Italian monoascospore strains resulted in 54 different crosses. Crosses forming mature apothecia are marked with a '+' in Table 3. Apothecia emerged from two up to five months after spermatization and they arose from any part of the sclerotial tissue, irrespective of the presence of conidiophores. The number of apothecia per sclerotium varied between one and eight, and of the 15 sclerotia used for each cross varying numbers (1 to 15) were successful in producing apothecia. Some malformed apothecia were observed as was also found by Faretra and Antonacci (1987).

Table 3: Sexual crosses of *B. cinerea*.

♀ \ ♂	SAS56	SAS405	Bc7	Bc12	Bc16	Bc18	Bc21	Bc25	Bc26	Bc29	USP
SAS56	–	+	+	+	+	–	–	–	+	+	–
SAS405	+	–	+	+	+	+	+	+	–	–	–
Bc7	nd	nd	nd								nd
Bc12	–	–		–							–
Bc16	+	+			+						–
Bc18	–	+				–					–
Bc21	–	+					–				–
Bc25	–	+						–			–
Bc26	+	–							–		–
Bc29	+	–								–	–

♀, female parent; ♂, male parent; usp, unspermatized control; +, production of apothecia; -, no production of apothecia; nd, prodcution of apothecia could not be tested.

Bc7, Bc12 and Bc16 successfully crossed as male with both SAS56 and SAS405 (Table 3), indicating that these Bc strains contain both mating type alleles. Bc16 also produced apothecia in the reciprocal crosses and in the self-fertilization control. This was not observed for Bc12 suggesting that sclerotia of Bc12 are possibly defective in formation of apothecia. As no sclerotial structures for Bc7 are known (Salinas and Schot, 1987) reciprocal crosses could not be tested for this strain. All other Bc strains successfully crossed either with SAS56 or SAS405 in both reciprocal crosses. The results show that Bc18, Bc21, Bc25 contain mating type *MAT1-1* and Bc26 and Bc29 contain mating type *MAT1-2*.

Phenotypical variation in progeny. Monoascospore cultures from a complete set of ascospores of the same ascus usually showed great uniformity of morphological characteristics. However, in a few sets (13 out of 146) a segregation of these characteristics (1:1 or 1:3 or 1:2:1) was observed giving rise to slower growing cultures or cultures with more aerial hyphae than usual (Fig. 2A and 2B). In several different crosses 4 of the 8 spores did not germinate at all or they died shortly after germination (Fig. 2C). Segregation of morphological characteristics observed in monoascospore cultures derived from one ascus was not always found in monoascospore cultures from the second ascus of the same apothecium.

A surprising phenomenon was the sometimes aberrant spore order found in the orderly collected ascospores from one ascus (Fig. 2B). In the model organism *Neurospora crassa* the meiosis is followed by a synchronic mitosis, causing every pair of ascospores to be identical (Raju, 1992). However, for *B. cinerea*, we observed in 5 out of 13 asci morphological characteristics which were not found in two consecutive monoascospore cultures, but in cultures of ascospores two positions apart. These results indicate that

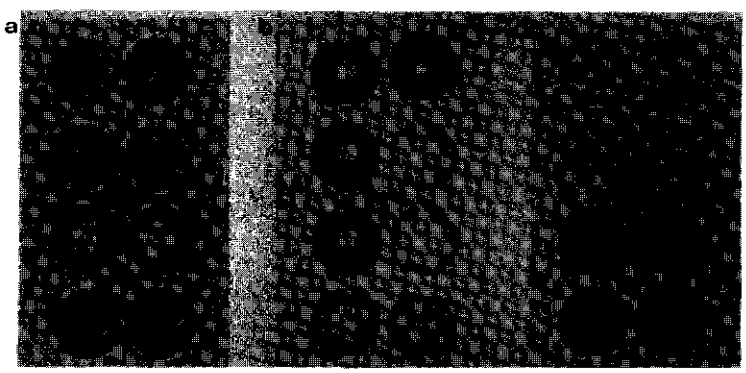

Figure 2: Segregation of morphological characteristics in cultures of 8 ascospores orderly collected from one ascus of cross SAS56 x Bc12 (A), cross SAS56 x Bc26 (B) and cross SAS405 x Bc18(C). Cultures at the right side are numbered 1, 3, 5, 7 and at the left side 2, 4, 6, 8.

ascospores of two different sets have changed places in the ascus during the last mitotic division.

Progeny of crosses in which sclerotium deficient strain Bc7 was involved gave varying results concerning sclerotium formation. Progeny of the cross SAS405 x Bc7 were all successful in producing sclerotia, whereas in cross SAS56 x Bc7, cultures of 1 to 3 ascospores per ascus gave rise to sclerotia. From this cross only 6 or 7 viable monoascospore cultures per ascus were recovered.

Since strain SAS405 contains resistance genes *Mbc1HR* and *Daf1LR* conferring resistance to benomyl and vinclozolin, respectively, segregation of these resistance genes was analyzed in the progeny of cross SAS405 x Bc7. Results showed an uncoupled 1:1 segregation of both genes, which is in agreement with the work of Faretra and Pollastro (1991). The aberrant ascospore order was again found in 6 out of the 8 ordered ascospore sets we tested.

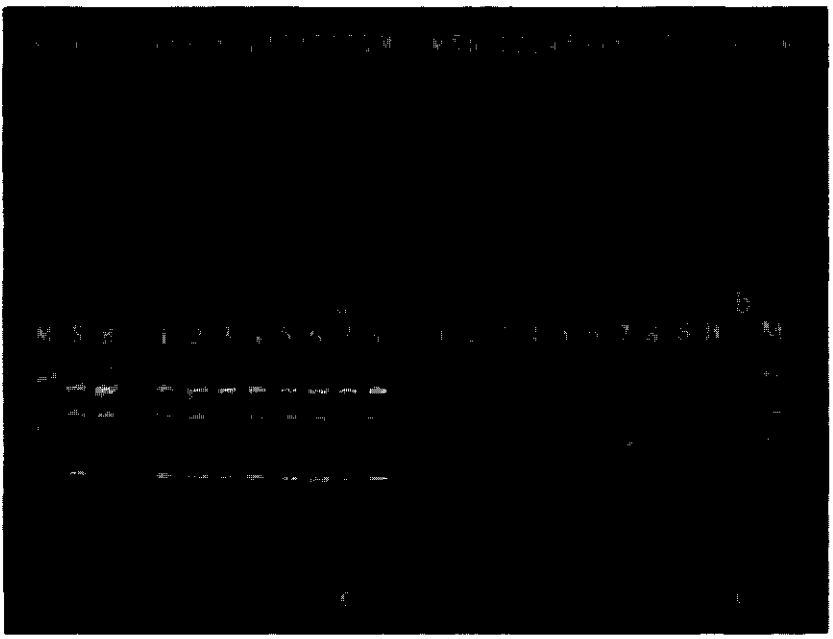

Figure 3: RAPD profiles of progeny of cross SAS405 x Bc7. **A** and **B**: 1:1 segregation of RAPD marker B18-1.4 in progeny obtained from asci A112 (Fig.A, 1-8 left), A211 (Fig.A 1-8 right), A311 (Fig.B 1-8 left), and A312 (Fig.B 1-8 right). RAPD marker B18-0.6 was absent in the progeny. **C**: 1:0 segregation of RAPD marker B13-0.8 shown for the progeny of ascus A112. **D**: 1:1 segregation of marker D02-1.1 and the appearance of two new markers (D02-1.4 and D02-1.2) in the progeny of ascus A312. Numbers of lane 1 to 8 represent the ascospore order as found in the ascus. B: parent Bc7, S: parent SAS405, M: marker with fragment sizes of 2605, 1605, 1198, 676, 517, 460, 396, 350 and 222 bp.

Table 4: Occurrence of RAPD-markers in parental strains SAS405 and Bc7, and the segregation in their progeny grown from orderly released ascospores numbered S1 to S8 of four asci. Progeny of each ascus (A112, A211, A311 and A312) is presented in sets of four genotypes and correction for aberrant spore order has been made. RAPD-markers were named after the primer with which they were amplified and this number is followed by the size (in Kb) of the fragment amplified.

	RAPD markers																
	B03	B12	B12	B13	B16	B18	B18	B19	B20	B20	B20	D02	D02	D02	D20	R07	R08
	2.2	1.8	0.8	0.8	1.5	1.4	0.6	1.7	1.7	1.0	0.9	1.4	1.2	1.1	1.4	0.7	0.9
SAS405	-	-	-	+	+	-	-	-	+	-	-	-	-	+	+	+	-
Bc7	+	+	-	-	-	+	+	+	-	+	+	-	-	-	-	-	+
A112 S1, S2	-	-	-	+	+	+	-	-	+	-	+	-	+	-	-	+	nd
A112 S3, S4	-	+	-	+	+	-	-	-	-	-	+	-	+	+	+	+	nd
A112 S5, S6	-	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	nd
A112 S7, S8	-	-	+	+	-	+	-	-	-	+	-	+	-	-	-	+	nd
A211 S1, S3	-	-	-	+	-	+	-	-	-	+	+	-	+	-	+	+	nd
A211 S2, S4	-	+	+	+	+	-	-	-	+	+	-	+	-	+	+	+	nd
A211 S5, S6	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	+	nd
A211 S7, S8	-	+	+	+	+	-	-	-	+	-	+	+	+	+	-	+	nd
A311 S1, S2	-	+	nd	+	-	+	-	-	+	-	+	+	-	-	nd	+	nd
A311 S3, S5	-	+	nd	+	-	-	-	-	-	-	+	+	-	+	nd	+	nd
A311 S4, S6	-	-	nd	+	+	-	-	-	-	+	-	-	+	+	nd	+	nd
A311 S7, S8	-	-	nd	+	+	+	-	-	+	+	-	-	+	-	nd	+	nd
A312 S1, S3	-	-	nd	+	+	-	-	-	+	-	+	+	+	-	nd	+	nd
A312 S2, S4	-	+	nd	+	+	+	-	-	-	-	+	-	+	+	nd	+	nd
A312 S5, S7	-	+	nd	+	-	-	-	-	-	+	-	+	-	+	nd	+	nd
A312 S6, S8	-	-	nd	+	-	+	-	-	+	+	-	-	-	-	nd	+	nd

Segregation of DNA polymorphisms in the progeny. To study segregation of DNA polymorphisms in the progeny of SAS405 and Bc7, a number of intense and reproducible RAPD markers was selected. These markers were amplification products of the primers B03, B12, B13, B16, B18, B19, B20, D02, D20, R07 and R08. New sets of ordered ascospores from the cross SAS405 x Bc7 were analyzed: two asci (A311 and A312) from one apothecium and two asci (A112 and A211) from two different apothecia. In the analysis of segregation of DNA polymorphisms, the conditions under which the polymorphisms were originally found were precisely followed, as the reproducibility is fully dependent on the reaction conditions. RAPD analysis also determined an aberrant spore order in three of the four asci (Fig. 3A and 3B). In ascus A211 spores 2 and 3 have changed places, in ascus A311 spores 4 and 5 (not detectable in Fig. 3B), and in ascus A312 spores 2,3 and 6,7 both have changed places. This observation was confirmed by

all other RAPD profiles in which neighbouring spore sets could be distinguished. In Table 4 the correct spore sets per ascus are presented, together with the occurrence of all RAPD markers tested in the four genotypes per ascus. Segregation of RAPD markers was mostly found in a 1:1 ratio (Fig. 3A, 3B and 3D), although a 1:0 ratio (Fig. 3C) was observed for two markers (B13-0.8 and R07-0.7), both originating from parent SAS405. Sometimes RAPD markers were lost in the progeny (B03-2.2, B18-0.6 (Fig. 3A, 3B) and B19-1.7). In contrast to this, new polymorphisms showed up in the progeny (RAPD marker B12-0.8, D02-1.4 and D02-1.2 (Fig. 3D)). No obvious linkage between RAPD markers could be detected.

Discussion

It has been shown by many workers that strains of *B. cinerea* can differ in growth rate, sclerotium production, pathogenicity or production of enzymes *in vitro* and *in vivo* (Grindle, 1979; Lorenz, 1983; Salinas and Schot, 1987; Leone, 1990; Movahedi and Heale, 1990b). However, the species is genetically poorly identified. Since the introduction of RAPD analysis in 1990 by Williams *et al.*, this technique has proven to be a very powerful tool in genetic analysis and has applicability to a wide range of organisms (Welsh *et al.*, 1991; Reiter *et al.*, 1992). RAPD markers are amplified from genomic DNA in a PCR assay using arbitrary oligonucleotide primers, and are based on natural DNA polymorphisms. In this study we have shown by using a set of 50 different primers, that all ten *B. cinerea* strains analyzed were genetically distinct. However, computer analysis of the RAPD data showed a similarity coefficient of 0.99 for Bc16 and Bc21. This was surprising since these strains were collected from different hosts in different years, and they contain different mating types. Similarity coefficients of all other pairs of strains (Table 2) allowed no grouping of strains according to their host, year of isolation, geographical origin or mating type. Taken together, these data show that strains of *B. cinerea* of the same host are not genetically more alike than strains of different hosts. Therefore, although these observations should be tested on larger scale, host specialization does not seem to occur in strains of *B. cinerea*.

Several primers (B1, D5, D6) resulted in identical RAPD profiles for all 10 strains suggesting that RAPD markers could be used as interspecific markers as well as intraspecific. Preliminary data indicate that indeed some RAPD markers are specific for *B. cinerea* (Van Kan, unpublished).

A mating experiment performed to study segregation of DNA polymorphisms revealed that both mating types *MAT1-1* and *MAT1-2* were present equally among the Dutch strains. The presence of both mating types, found in three of the eight strains, could be an indication of heterokaryosis according to Lorenz and Eichhorn (1983) and Faretra *et al.* (1988c). However, since the Dutch strains were obtained from naturally infected hosts and mass-transferred, presence of both mating types could also be due to mixed cultures of homokaryons.

The isolation of ordered octads, although laborious compared to the collection of random progeny, has enabled us to investigate several fundamental genetic aspects in *B. cinerea*. First, we were able to study single meiotic events and calculate segregation of RAPD markers in only a few asci. Second, we were able to identify changes of spore order during the final mitotic division. This occurred with a significant frequency: 5 out of 13 asci analyzed for morphological characteristics showed the aberrant spore order. This was further confirmed by analyzing the segregation of fungicide markers and RAPD markers. In 6 out of 8 and 3 out of 4 asci, respectively, markers were not present in two consecutive ascospores, but in ascospores two positions apart. This implies that one cannot simply collect ascospores 1, 3, 5 and 7 when it is necessary to collect the four distinct genotypes from one ascus.

In the collected progeny of several different crosses, the first observation was an occasional segregation of aberrant growth characteristics, either in 1:1, 1:2:1 or 1:3 ratio. Since this aberrant growth may be due to several unidentified loci or combinations of loci, it is hard to account for these segregation ratios. Segregation ratios of RAPD markers are simpler to understand as these are amplified from single loci.

In the segregation of DNA polymorphisms in the progeny, a ratio of 1:1 was usually observed, being a normal Mendelian segregation of a genetic marker in a haploid organism. However, also a 1:0 segregation has been found for several RAPD markers. Since the ploidy level of *B. cinerea* has never been determined unequivocally, the 1:0 segregation ratio might be explained if *B. cinerea* were diploid. In this way, a 1:0 segregation would result from a homozygous locus, and a 1:1 segregation from a heterozygous locus. This assumption could easily be tested by backcrossing the progeny with the parental strain lacking the RAPD marker. However, due to the time-consuming procedure for sexual crosses this has not been proven yet. Other experimental observations, encountered by several authors, can also be indicative of diploidy. For example, Grindle (1979) found that mutants were produced with very low frequency or not at all. Mutants obtained were not stable. Secondly, Lorenz (1983) studied the phenotypical stability of monoascospore isolates. Since these isolates originate from homokaryotic ascospores (Lorenz and Eichhorn, 1983), variation due to heterokaryosis could be excluded. However, after repeated passage of monoascospore cultures a variation in morphotypes was observed. The unknown ploidy level was proposed by Lorenz (1983) as one possible explanation for this observation. Thirdly, Faretra *et al.* (1988b) showed that about 5% of monoascospore cultures derived from 24 crosses were compatible with both reference isolates containing *MAT1-1* or *MAT1-2*. We suggest that, due to diploidy, this progeny is heterozygous for the *MAT1* locus. Recently, measurements of DNA contents in nuclei of SAS56 and benomyl treated subcultures of this homokaryotic isolate have given further evidence that *B.cinerea* is not haploid (Tudzynski, personal communication).

Furthermore, two unexpected observations were made during the segregation analysis. First, three markers (all from the putative heterokaryotic parent Bc7) completely

disappeared from the progeny, and second, two new markers appeared in the progeny in a 1:1 ratio, which were not detected in either of the parents. The appearance or disappearance of RAPD markers in the progeny could possibly be a limitation of the RAPD technique itself. In amplification of genomic fragments there is a strong competition among DNA sequences. After meiosis the genetic background has changed and this also changes the competition between DNA sequences willing to amplify (Debets, personal communication). However, we never observed the disappearance of RAPD markers encoded by parental strain SAS405 or SAS56 (Van der Vlugt-Bergmans *et al.*, unpublished) and this prompted us to suggest the following explanation. Parent Bc7 can be regarded as heterokaryotic based on the presence of both mating types which could be due to a mix of nuclei in either one thallus (heterokaryon) or in several thalli (mix of homokaryons). In a cross, mononucleate microconidia of this parent fuse with sclerotial cells of the homokaryotic parent SAS405, giving rise to dikaryons. The genetic constitution of the dikaryon depends on which nucleus of Bc7 has been involved in the cell fusion. So, if a nucleus of Bc7, which does not encode the particular RAPD marker screened for, is involved in dikaryon formation, this particular RAPD marker scored in the parental strain Bc7 would not be scored in the progeny. Similarly, if a RAPD marker is present in only one nucleus of low abundance in the population of nuclei of Bc7, this marker might not be detected in Bc7 due to titration effects. If, however, this particular nucleus is involved in dikaryon formation, the marker will become detectable in the progeny resulting from the cross.

The appearance or disappearance of RAPD markers in all progeny analyzed can hardly be explained by the partitioning of one class of nuclei from a heterokaryotic parent in three independent fertilization events. The above mentioned mechanism could be elucidated by analyzing the progeny of the cross SAS405 x SAS56 in which both parents are homokaryotic. Disappearance or appearance of RAPD markers in the progeny of this cross can not be explained by heterokaryosis. Furthermore, RAPD markers should be used to develop more specific markers like RFLPs.

Acknowledgments

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CHAPTER 3

Employing molecular strategies to clone a cutinase gene of *Botrytis cinerea*

SUMMARY. In penetration of host tissue by *Botrytis cinerea* an important role for cutinase was suggested. To investigate the relevance of cutinase in more detail, cloning of the encoding gene is an essential step to enable studies on gene expression and the effect of gene disruption. Different cloning strategies were employed. First, probes of cutinase genes of *Fusarium solani* f.sp. *pisi*, *Magnaporthe grisea* and *Ascochyta rabiei* were used to screen a genomic library of *B. cinerea*. Second, amino acid sequences conserved among cutinases of five different fungi were used for the design of degenerated primers for use in a PCR based gene cloning strategy. Third, a monoclonal antibody raised against cutinase of *B. cinerea* strain Bc7 was used for screening of a cDNA expression library of *B. cinerea*. All three approaches appeared to be unsuccessful. A future strategy is discussed.

Introduction

A possible role for cutinase in penetration of host tissue by *Botrytis cinerea* Pers.:Fr. was suggested by electron microscopy studies (McKeen, 1974; Rijkenberg *et al.*, 1980) and the inhibitory effect of monoclonal antibodies raised against cutinase of *B. cinerea* on the infection of gerbera flowers (Salinas, 1992) (see Chapter 1). Evidence for the importance of cutinase during the penetration process can be obtained by cloning the encoding gene. This will enable mutation of the gene by gene disruption and functional analysis during penetration of host tissue.

Production of cutinase by *B. cinerea* in liquid cultures was demonstrated by several authors (Shishiyama *et al.*, 1970; Baker and Bateman, 1978). A cutinase of *B. cinerea* was purified to homogenity and identified as an 18 kD protein (Salinas, 1992). It belongs to a group of serine esterases, which share several characteristics: a catalytic triad comprising the amino acids serine, histidine and aspartic acid, of which serine is present in the consensus motif glycine-X-serine-X-glycine (Martinez *et al.*, 1992). Moreover, serine esterases hydrolyse the substrate para-nitrophenyl butyrate (PNB). This substrate is widely used to monitor cutinase activity in fungal cultures (Purdy and Kolattukudy, 1975; Dickman *et al.*, 1982; Bonnen and Hammerschmidt, 1989; Trail and Köller, 1990; Salinas, 1992). Cutinase is produced in the presence of cutin, cutin hydrolysate or cutin monomers like 16-hydroxyhexadecanoic acid in the medium, but its production is repressed by glucose (Lin and Kolattukudy, 1978; Nasraoui, 1992; Salinas, 1992).

Different strategies to clone the gene encoding cutinase were feasible, since cutinase genes of five different fungi were already cloned. These genes were isolated from *Fusarium solani* f.sp. *pisi*, (Soliday *et al.*, 1989), *Colletotrichum gloeosporioides, C. capsici* (Ettinger *et al.*, 1987), *Magnaporthe grisea* (Sweigard *et al.*, 1992a) and *Ascochyta rabiei* (Tenhaken, unpublished) and could be used as a probe for heterologous screening of a *B. cinerea* library. Sequence identity at the amino acid level varied from 48 to 56%. In addition, an alignment of the five cutinases showed the presence of conserved amino acid sequence stretches which could be used to design degenerated primers for PCR based gene cloning. Besides the DNA and protein sequence information of other fungal cutinases, a monoclonal antibody raised against cutinase of *B. cinerea*

strain Bc7 was available, which could be used in the screening of a cDNA expression library to isolate a cutinase cDNA clone.

In this chapter the *in vitro* induction profile of cutinase in *B. cinerea* strain SAS56 is described. Different cloning strategies, as outlined above, are employed in an attempt to clone the cutinase gene.

Materials and Methods

Culturing of *B. cinerea*. *B. cinerea* strain SAS56 was grown on malt extract agar (Oxoid) or tPDA (potato dextrose agar containing 300 g homogenized tomato leaves per litre) at 18°C in the dark. After 3 days the cultures were exposed to near UV-light for 16 hours to induce sporulation. One week later conidia were collected from sporulating cultures and used for inoculation of liquid Gamborg's B5 medium (Duchefa) supplemented with 0.3% glucose. Cultures of 100 ml of B5 medium were inoculated with 10^7 conidia and incubated in a rotary shaker at 20°C, 150 rpm. For induction of cutinase, 0.05% (w/v) 16-hydroxyhexadecanoic acid (16-hha, Sigma) or 0.02% tomato cutin (purified according to Salinas *et al.*, 1986) was added after 4 days and the cultures were further incubated for 9 days at 20°C, 150 rpm. Every day samples of the culture filtrate were taken to measure PNB hydrolytic activity and to detect cutinase by western blotting.

Enzyme assay for PNB hydrolytic activity. Culture filtrate was diluted in 25 mM potassium phosphate buffer, pH 8.0, 0.05% (v/v) Triton X-100 in a final volume of 450 µl. To this, 50 µl para-nitrophenyl butyrate (PNB, Sigma) resuspended at a concentration of 10 mM in 0.25 mM potassium phosphate buffer, pH 7.0, 0.5% (v/v) Triton X-100 was added. After one hour incubation at 22°C PNB hydrolysis was measured spectrophotometrically at 405 nm. Background caused by yellowish culture filtrate and/or non-enzymatic substrate degradation was determined in a parallel assay containing culture filtrate which was heated for 5 minutes at 100°C.

Western blotting. Samples of 1 ml of culture filtrate were lyophilized and analyzed by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). Electrophoresis was performed according to Laemmli (1970) with a 2% (w/v) stacking gel and a 15% (w/v) running gel using the Mini-Protean II dual slab gel system (BioRad). Molecular mass markers ranging from 14.2 to 66 kD (Sigma) were co-electrophoresed to estimate molecular masses of the various proteins. Proteins were stained with Coomassie Brilliant Blue R250 or blotted onto a nitrocellulose membrane in 25 mM Tris, 0.2 M glycin and 20% methanol (v/v) during 3 hours at 200 mA. For immunological detection monoclonal antibody 21C5 against cutinase of *B. cinerea* strain Bc7 (Salinas, 1992) was used. As a second antibody goat anti-mouse IgG labelled with alkaline phosphatase (Pierce) was used with NBT (p-nitro blue tetrazolium chloride, Sigma) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, Sigma) as substrates.

Screening of a genomic library. A genomic library of *B. cinerea* strain SAS56 in lambda EMBL3 was kindly provided by Dr M Kusters-Van Someren (Section Molecular Genetics of Industrial Micro-organisms, Wageningen Agricultural University, The Netherlands). In total, $2x10^4$ recombinant phages were plated. Replica filters (Hybond-N^+, Amersham) were made and hybridized for 16 hours at 56° or 60°C in modified Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS according to Church and Gilbert, 1984) in the presence of a random-primed [α-^{32}P]dATP labelled probe. Filters were washed in 0.5xSSC, 0.5%SDS at 56° or 60°C and exposed to Kodak X-OMAT AR film. Positive plaques were purified by a second round of hybridization. DNA from selected phages was isolated using Qiagen columns according to the manufacturer's instructions and analyzed by digestion with *Sal*I. Hybridizing fragments detected by Southern blot analysis (Sambrook *et al.*, 1989) were cloned and used for further subcloning and sequence analysis. DNA sequence analysis was performed on double-stranded DNA using the CircumVent Thermal Cycle Sequencing Kit (Biolabs, New England). Analyses of the sequence data and alignment to known sequences in GenEMBL and SWISSPROT databases were performed using the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711. All DNA manipulations not described were performed according to Sambrook *et al.* (1989).

PCR assays. For RT-PCR, total RNA was isolated from mycelium of *B. cinerea* strain SAS56 harvested three and four days after induction with 16-hha, according to the Extract-A-plant RNA isolation kit protocol (Clontech Lab). Poly(A)$^+$ RNA was isolated using the Oligotex-dT mRNA kit (Qiagen) and used as template in first strand cDNA synthesis employing an oligo(dT) primer and Superscript Reverse Transcriptase (RT) (GibcoBRL/Life Technologies) according to the manufacturer's instructions. For PCR 1 μl RT mix or 100 ng genomic DNA of SAS56 (isolated according to Drenth *et al.*, 1993) was combined with 250 ng of the degenerated primers or 50 ng oligo(dT) primer, 5 mM dNTP and 1.2 units Ultma Polymerase (Perkin Elmer). Buffer and $MgCl_2$ were added according to the manufacturer's instructions. The amplification started with 5 min 95°C followed by 35 cycles of 1 min 95°C, 1 min 50 or 55°C, 1 min 72°C and a final 2 minutes at 72°C before cooling to 4°C. Amplified products were analyzed on a 2% agarose gel in TAE buffer and cloned in pCR-script SK$^+$ (Stratagene). DNA sequencing was performed on double stranded DNA using the CircumVent Thermal Cycle Sequencing Kit (Biolabs, New England).

Construction and immunoscreening of a cDNA library. Total RNA was isolated from mycelium of *B. cinerea* strain SAS56 harvested four days after induction with 16-hha, according to the Extract-A-plant RNA Isolation kit protocol (Clontech Lab). Poly(A)$^+$ RNA was isolated using the Oligotex-dT mRNA kit of Qiagen (Promega). cDNA syntesis and cloning in lambdaZAP II vector arms (Stratagene) was done according to the manufacturer's instructions.

For immunoscreening, five plates with $5x10^4$ phages each were used. Induction of protein expression and screening of the filters using the monoclonal antibody 21C5 against cutinase of *B. cinerea* strain Bc7 (Salinas, 1992) was done according to the manufacturer's instructions (Stratagene). As a second antibody goat anti-mouse IgG labelled with alkaline phosphatase was used with NBT and BCIP as substrates.

Results and Discussion

Cutinase production by *B. cinerea* strain SAS56. To analyse the cutinase production by strain SAS56 of *B. cinerea* in the presence of different inducers, the fungus was grown in three replicate cultures. After four days of growth, the cutin monomer 16-hha was added to culture A, tomato cutin to culture B, while culture C remained non-induced. During each of the following nine days culture filtrate was harvested and cutinase activity was measured (Fig. 1) in a spectrophotometric assay using PNB as substrate (Purdy and Kolattukudy, 1975; Salinas, 1992). Maximum PNB hydrolytic activity was measured in culture filtrate of SAS56 at 5 days post induction (d.p.i.) with 16-hha (culture A). Induction by cutin resulted only in a slow increase in PNB hydrolytic activity (culture B), which was reported to reach a maximum level at day 16-18 (Salinas *et al.*, 1986). Without inducer (culture C), PNB hydrolysis remained at a low, constant level.

Since the substrate PNB is not specific for cutinase and might be hydrolyzed by other serine esterases present in the culture filtrate, the presence of cutinase in culture filtrate A harvested at 0 to 5 d.p.i., was investigated by western blotting. Using the monoclonal antibody 21C5 raised against an 18 kD cutinase of *B. cinerea* strain Bc7 (Salinas, 1992), a single protein band of 18 kD was detected at 3, 4 and 5 d.p.i..

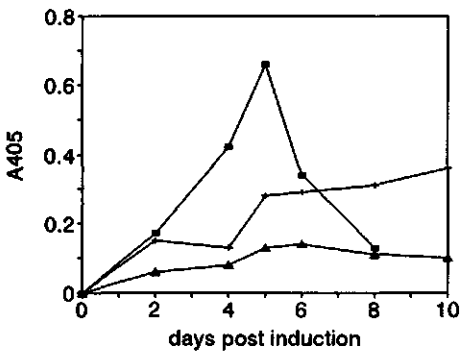

Figure 1: *In vitro* induction of cutinase by 16-hha (■) and tomato cutin (+) in *B. cinerea* strain SAS56. A third culture (▲) was non-induced. Cutinase activity was measured as a PNB hydrolytic activity in a spectrophotometric assay (Y-axis) during day 0 to 9 (X-axis) after induction.

Heterologous screening of a genomic library. A genomic library of *B. cinerea* strain SAS56 was screened with an 0.5 kb cutinase probe derived from the coding sequence of the cutinase gene of *F. solani* f.sp. *pisi* (corresponding to nt 161-559 as published in Soliday *et al.*, 1989). Hybridization under heterologous conditions (60°C) yielded six putative positive phages. In a second round of hybridization, however, all six phages failed to hybridize.

A new screening of the library was done with a labelled 1.0 kb fragment containing the coding sequence of the cutinase gene of *A. rabiei* and 150 bp and 200 bp of promoter and terminator sequence, respectively (Tenhaken, unpublished). This screening yielded 8 putative positive phages, which were further purified in a second screening. Southern blot analysis of these recombinant phages showed a hybridizing fragment of 3.0 kb in phage 8, which was cloned. Sequence analysis of several subclones covering different parts of the original hybridizing 3.0 kb fragment showed no homology to known cutinase sequences.

The Southern blot containing DNA of the 8 selected phages was also hybridized with a 2.1 kb probe containing the cutinase gene of *M. grisea* (*Xba*I/*Eco*RI fragment of plasmid pCB623, Sweigard *et al.*, 1992a). One 3.0 kb fragment of phage 6 showed a strong and unique hybridization signal. After subcloning and Southern blotting, the hybridization signal could be assigned to a 0.5 kb fragment. Sequence analysis of this fragment showed 45% identity to the probe. Unfortunately, this was present in 200 nt of the terminator sequence, immediately downstream of the coding region in the *M. grisea* gene. In the cloned *B. cinerea* fragment no cutinase-like sequence was found upstream of this homologous terminator sequence.

A final screening of the genomic library was performed using probes containing only coding sequences of cutinase genes of *A. rabiei* and *M. grisea*. Four replica filters were made from the library, two filters were hybridized with the *M. grisea* probe and two with the *A. rabiei* probe. In a control hybridization, the *M. grisea* probe clearly detected the cutinase sequence of *A. rabiei*, and vice versa. However, no recombinant phages of the *B. cinerea* library hybridized reproducibly with both heterologous probes.

Amplification of cutinase encoding sequences using degenerated primers. A multiple alignment of cloned cutinases of *F. solani* f.sp. *pisi* (Soliday, 1989), *C. gloeosporioides*, *C. capsici* (Ettinger *et al.*, 1987), *M. grisea* (Sweigard *et al.*, 1992a) and *A. rabiei* (Tenhaken, unpublished) is shown in Fig. 2. Conserved amino acid stretches (shaded boxes, Fig. 2) were selected for the design of degenerated primers. These primers were employed in a PCR assay on genomic DNA of SAS56, cDNA (RT-PCR) and a plasmid containing the cutinase gene of *A. rabiei* as a control. cDNA was synthesized from poly(A)$^+$ RNA isolated from *B. cinerea* grown for 3 days in the presence of 16-hha, 2 days before maximum PNB hydrolytic activity was measured in culture filtrate. Using all possible combinations of degenerated primers and an oligo(dT) primer in case RNA was used as template, different polymerases (Ultma polymerase and Amplitaq of Perkin Elmer)

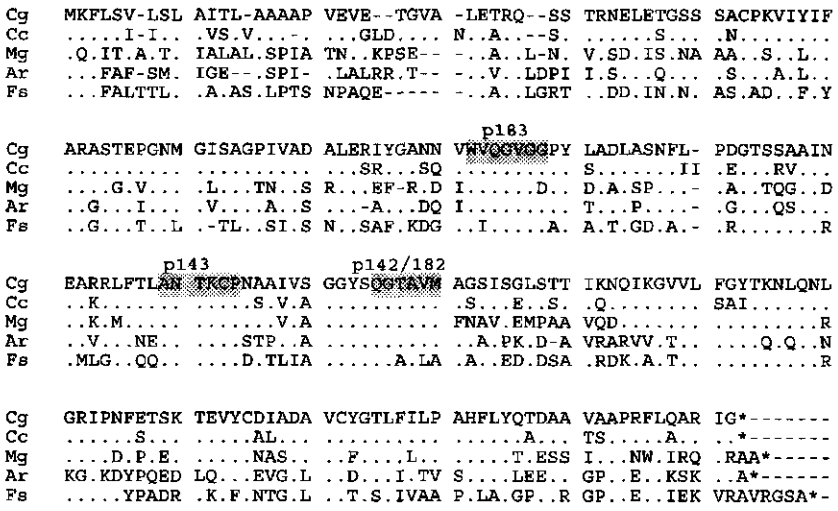

Figure 2: Computer alignment of cutinase sequences from *Colletotrichum gloeosporioides, C. capsici* (Ettinger *et al.*, 1987), *Magnaporthe grisea* (Sweigard *et al.*, 1992a) *Ascochyta rabiei* (Tenhaken, unpublished, accession number of GenEMBL database: X65628) and *Fusarium solani* f.sp. *pisi* (Soliday *et al.*, 1989). Amino acids identical to the first sequence are indicated by dots. Primers are based on amino acid sequences shown in shaded boxes. Primer 182 is in antisense orientation, primer 183, 143 and 142 in sense orientation. Primer sequences:
p142: 5'-CARGGNACNGCNGTNATG-3'; p143: 5'-GCNAAYACNAARTGYCC-3';
p182: 5'-CATNACNGCNGTNCCYTG-3'; p183: 5'-TGGGTNCARGGNGTNGGNG-3'.
N = A, C, G or T; Y = C or T; R = A or G.

and different annealing temperatures in the PCR assay, fragments were amplified. Only two fragments of 220 and 280 bp, respectively, amplified from genomic DNA using primers 182 and 183, were of a correct size as estimated from the coding sequence of known cutinase genes and considering the presence of introns. Both fragments were cloned and their sequence was determined. Primers used for amplification were present on each side, but the intervening sequence did not show any homology to known cutinase sequences.

In a final experiment all possible PCR assays were performed again with the only modification that RT-PCR was done on polyA$^+$RNA isolated from *B. cinerea* harvested at 4 d.p.i.. After gel electrophoresis, blotting and hybridization to a probe derived from the coding sequence of the *A. rabiei* cutinase, no amplified fragments hybridized, except for the 180 bp fragment amplified by primer 183 and 182 on the plasmid containing the *A. rabiei* cutinase.

Immunoscreening of a cDNA expression library. A cDNA library constructed from poly(A)$^+$RNA isolated from *B. cinerea* grown for 4 days in the presence of 16-hha, was screened with the monoclonal antibody 21C5. This antibody was raised against cutinase of *B. cinerea* strain Bc7, but also clearly detected cutinase in culture filtrate of *B. cinerea* strain SAS56. In total, $2.5x10^5$ recombinant phages were screened using this antibody. However, no phages expressing cutinase were detected.

The cDNA library was checked for size and orientation of inserts in recombinant phages by restriction and sequence analysis. In ten random phagemids insert sizes ranged from 0.5 to 2.0 kb, a size distribution which should contain a full length cutinase insert, of which the estimated size was about 0.8 kb. In addition, all ten inserts contained open reading frames in the correct orientation for expression of the coding region. Inserts of two phagemids showed homology to sequences coding for catalase (see Chapter 6) and ubiquitin, respectively.

The procedure of immunodetection was checked by including a western blot as mentioned before in the screening. Cutinase was clearly and specifically detected on this blot, but no positive phages were detected on the filters. Since the antibody detects denatured cutinase on a western blot while native protein is present on the filters, the sensitivity of the antibody for native and denatured cutinase was checked. Culture filtrate sampled 4 days after induction was spotted on a membrane before and after denaturation by boiling. In both fractions cutinase was detected with similar efficiency indicating that also native cutinase was detected by the monoclonal 21C5.

Conclusions and Future strategy

The *in vitro* induction of cutinase by 16-hha was demonstrated for *B. cinerea* strain SAS56. Maximal PNB hydrolytic activity at 5 d.p.i. was correlated with the presence of cutinase in the culture filtrate by western blotting. In subsequent experiments RNA was isolated at 3 and 4 d.p.i. preceding maximal PNB hydrolytic activity.

Attempts to clone the cutinase encoding gene of *B. cinerea* were based on DNA or protein homology. Unfortunately, all three molecular strategies failed. Control experiments revealed no indications for failure of the techniques used. Apparently, cutinase of *B. cinerea* is not sufficiently homologous to other cutinases to clone its gene by heterologous screening or to design degenerated primers for PCR based cloning. Moreover, the expression library of *B. cinerea* might have lacked full length cutinase clones in the correct frame for translation, which might explain the failure of isolating positive phages.

Molecular techniques which were considered feasible in a straight forward cloning of the cutinase gene appeared to be unsuccessful. A remaining possibility to obtain the gene encoding cutinase is by following a biochemical approach. Purification of cutinase from an *in vitro* induced culture of *B. cinerea* and partial sequence analysis of the protein enables the design of *B. cinerea* specific cutinase primers. These primers can be used to amplify the cutinase encoding sequence by PCR and subsequent sceening of the

genomic library of *B. cinerea* enables the isolation of the gene. This approach is described in the next chapter.

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CHAPTER 4

Purification, cloning and expression of cutinase A and a putative esterase of *Botrytis cinerea*

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Summary. Cutinase of *Botrytis cinerea* was suggested to play an important role in penetration of host tissue. An 18 kD protein purified from *in vitro* grown *B. cinerea* showed cutin hydrolyzing activity. Its sequence was partially determined to enable the design of specific primers for PCR based gene cloning. The corresponding gene *cutA* was cloned and showed significant homology to known cutinases. It was present in the *B. cinerea* genome as a single copy gene. During purification the substrate para-nitrophenyl butyrate was used to detect cutinase activity, but this substrate is also hydrolyzed by other esterases. A fraction with esterase activity was co-purified. The activity was attributed to an 11 kD protein of which the corresponding gene was cloned. Sequence analysis of this gene, named *ekdA*, revealed no homology to known proteins and the lack of a signal peptide predicts an intracellular origin. Expression of the *cutA* and *ekdA* genes of *B. cinerea* was studied by northern blot analysis. The *cutA* gene was induced *in vitro* by the cutin monomer 16-hydroxyhexadecanoic acid and repressed by glucose. Expression of the *ekdA* gene was not influenced by these compounds and was only detectable late during infection of tomato leaves. The expression of *cutA in planta* was low during the early phases of the infection, but high when the fungus had colonized the tomato leaf and started to sporulate. The presence of glucose in the inoculum buffer might have influenced the expression early during the infection.

Introduction

The cuticle of aerial plant parts is the first barrier a plant pathogenic fungus has to breach for direct penetration of the epidermis. The structural component of the cuticle is cutin, a polymer mainly composed of C16 and C18 hydroxy fatty acids, which are predominantly linked by ester bonds and some ether and peroxide bonds (Martin and Juniper, 1970). The enzyme cutinase is able to release fatty acids from cutin by hydrolysis of the ester bonds. Production of cutinase during growth on cutin in *in vitro* cultures has been reported for over 20 plant pathogenic fungi (Baker and Bateman, 1978; Ettinger *et al.*, 1987; Trail and Köller, 1990).

Botrytis cinerea Pers.:Fr., a ubiquitous plant pathogen with a very wide host range (Jarvis, 1977), is able to infect its host by direct penetration of the cuticle. Evidence for enzyme-mediated penetration came from electron microscopy studies (McKeen, 1974; Rijkenberg *et al.*, 1980). An important role for cutinase was proposed, because gerbera flowers sprayed with antibodies raised against cutinase of *B. cinerea* were protected against infection (Salinas, 1992).

Cutinase activity in *B. cinerea* was first demonstrated by Shishiyama *et al.* (1970) and Bateman and Baker (1978). The enzyme was purified and its production could be induced by cutin, cutin hydrolysate and cutin monomers such as 16-hydroxyhexadecanoic acid (16-hha). Like in *Fusarium solani* f.sp. *pisi* (Lin and Kolattukudy, 1978), cutinase expression was repressed by glucose (Salinas, 1992). Inhibition of enzyme activity by diiospropylfluorophosphate indicated the involvement of a serine residue in the active centre of the enzyme. Cutinases, including the one of *B. cinerea*, belong to the class of serine esterases that contain the catalytic triad (serine, histidine and aspartate), with the serine residue of the active centre in the consensus sequence glycine-X-serine-X-glycine

(Martinez *et al.*, 1992). This group of enzymes hydrolyses the model substrate para-nitrophenyl butyrate (PNB; Purdy and Kolattukudy, 1973) which is widely used to determine cutinase activity. However, to demonstrate specific cutin hydrolysis, the enzyme should be tested on cutin, for which mostly radioactively labelled cutin is used (Purdy and Kolattukudy, 1973; 1975).

The present work was undertaken to determine whether *B. cinerea* indeed requires cutinase for successful penetration. In this chapter the purification, cloning and expression analysis of a cutinase and a putative esterase that was co-purified are described.

Materials and Methods

Fungal growth. *B. cinerea* strain SAS56 (Van der Vlugt-Bergmans *et al.*, 1993) was grown on malt extract agar (Oxoid) or tPDA (potato dextrose agar containing 300 g homogenized tomato leaves per litre) at 18°C in the dark. After 3 days the cultures were exposed for 16 hours to near UV-light. One week later conidia were collected from sporulating cultures and inoculated with 10^8 conidia per litre in Gamborg's B5 medium (Duchefa) supplemented with 0.3% (w/v) glucose (start culture). After 4 days of growth in a rotary shaker at 160 rpm, 20°C, 16-hha (Sigma) was added as an inducer of cutinase expression, at a final concentration of 0.05% (w/v). Incubation was prolonged for another 6 days.

For analyses of gene expression *in vitro*, a two-day-old start culture was diluted five-fold in the following media: B5 medium + 0.3% glucose + 0.05% 16-hha (A), B5 medium + 0.05% 16-hha (B), B5 medium + 0.3% glucose (C). Remaining mycelium of the start culture was harvested. All three sub-cultures were incubated at 20°C, 160 rpm and mycelium and culture filtrate were sampled during each of the following eight days.

Enzyme assay for PNB hydrolytic activity. Culture filtrate or (partly) purified protein fractions were diluted in 25 mM potassium-phosphate buffer, pH 8.0, 0.05% (v/v) Triton X-100 in a final volume of 450 µl. To this, 50 µl para-nitrophenyl butyrate (PNB, Sigma) resuspended at a concentration of 10 mM in 0.25 mM phosphate buffer, pH 7.0, 0.5% (v/v) Triton X-100 was added. After one hour incubation at 22°C PNB hydrolysis was measured spectrophotometrically at 405 nm. Background caused by yellowish culture filtrate and/or non-enzymatic substrate degradation was determined in a parallel assay containing protein fraction which was heated for 5 minutes at 100°C.

Purification of PNB hydrolytic enzymes. A *B. cinerea* culture induced with 16-hha and showing highest PNB hydrolytic activities at 6 days after induction was filtered and polysaccharides were removed from the culture filtrate by 15-20% (v/v) acetone precipitations. The culture filtrate was subsequently concentrated to 30 ml by ultrafiltration using an Amicon PM-10 membrane (cut off 10 kD). The resulting 30 ml suspension was freeze-dried and resuspended in 2 ml of elution buffer (50 mM Tris-HCl, pH 7.5, 100 mM

NaCl). After centrifugation (5 minutes at 13,000*g*), the supernatant was applied to a Sephadex G-50 column (2.6 x 100 cm), equilibrated with elution buffer and the column was eluted at a flow rate of 10 ml·h^{-1}. Fractions of 5 ml were collected during 48 hours and the even fractions were monitored for PNB hydrolytic activity. Fractions of each peak were pooled, dialysed against 25 mM imidazole-HCl, pH 7.4 and applied to a Polybuffer Exchanger 94 column (Pharmacia, 1.2 x 35 cm) equilibrated with 25 mM imidazole-HCl, pH 7.4. Elution with 1:8 diluted Polybuffer 74-HCl, pH 4.0 at 30 ml h^{-1} during 12 hours resulted in a pH gradient from 7.0 to 4.5. Fractions of 5 ml were collected and in the even fractions pH and PNB hydrolytic activity were determined. Fractions containing high activity were dialysed (cut off 12-14 kD) against distilled water to remove the Polybuffer and analysed by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). Electrophoresis was performed according to Laemmli (1970) with a 2% (w/v) stacking gel and a 15% (w/v) running gel using the Mini-Protean II dual slab gel system (BioRad). Molecular mass markers ranging from 14.2 to 66 kD (Sigma) were co-electrophoresed to estimate molecular masses of the various proteins. Proteins were stained with Coomassie Brilliant Blue R250.

Enzyme assay for cutinase activity. Tomato cutin, kindly provided by Dr J. Salinas (Research Institute for Plant Protection, IPO-DLO, Wageningen, The Netherlands), was coupled with the chromogenic group Remazol Brilliant Blue R (Wolf and Wirth, 1990) by Dr G.A. Wolf (Institut für Pflanzenpathologie und Pflanzenschutz der Georg-August-Universität, Göttingen, Germany). After coupling, colourless cutin became dark blue.

Optimal pH, temperature and concentration of Triton X-100 for the enzyme assay were determined using partly purified cutinase fractions. Cutin-RBB hydrolysis was optimal in 100 mM sodium-phosphate buffer pH 5.6, 0.5% (v/v) Triton X-100 supplemented with 1 mg cutin-RBB. This suspension was incubated at 30°C for one hour. Background was measured in a parallel assay using protein fractions heated for 5 minutes at 100°C. Cutin-RBB hydrolysis was determined by pelleting solid cutin-RBB particles and measuring RBB-labelled cutin monomers in the supernatant spectrophotometrically at 590 nm.

Determination of amino acid sequences. Purified protein fractions were subjected to SDS-PAGE (Laemmli, 1970) and blotted onto PVDF-membrane in 50 mM Tris, 50 mM boric acid, 0.1% SDS and 20% methanol. Blotting was carried out for 8 hours at 35 V. After blotting the membrane was washed three times for 10 minutes in 10 mM boric acid pH 8, 25 mM NaCl. Protein bands were visualized by Coomassie Brilliant Blue staining and cut out. *In situ* membrane tryptic digestions, separations of peptides and amino acid sequence determination were performed as described by Bauw *et al.*, 1990.

RT-PCR. Total RNA was isolated from mycelium of *B. cinerea* strain SAS56 harvested three days post induction with 16-hha, according to the Extract-A-plant RNA isolation kit protocol (Clontech Lab). Poly(A)$^+$ RNA was isolated using the Oligotex-dT mRNA kit

(Qiagen) and used as template in first strand cDNA synthesis employing an oligo(dT) primer and Superscript Reverse Transcriptase (RT) (GibcoBRL/Life Technologies) according to the manufacturer's instructions. For PCR 1 μl RT mix was combined with 250 ng of the degenerated primers or 50 ng oligo(dT) primer, 5 mM dNTPs and 1.2 units Ultma Polymerase (Perkin Elmer). Buffer and $MgCl_2$ were added according to the manufacturer's instructions. The amplification started with 5 min 95°C followed by 35 cycles of 1 min 95°C, 1 min 55°C, 1 min 72°C and a final 2 minutes at 72°C before cooling to 4°C. Amplified products were analyzed on a 2% agarose gel in TAE buffer and cloned in pCR-script SK$^+$ (Stratagene). DNA sequencing was performed on double stranded DNA using CircumVent Thermal Cycle Sequencing Kit (Biolabs, New England).

Screening of genomic and cDNA libraries. A genomic library of *B. cinerea* strain SAS56 in lambda EMBL3 was kindly provided by Dr M. Kusters-Van Someren (Section Molecular Genetics of Industrial Micro-organisms, Wageningen Agricultural University, The Netherlands). A lambda ZAP II cDNA library was constructed from poly(A)$^+$-RNA isolated from mycelium grown in the presence of 16-hha during 4 days (see Chapter 3).

From both the genomic and cDNA library, 2.10^4 recombinant phages were plated. Replica filters (Hybond-N$^+$, Amersham) were made and hybridized for 16 hours at 65°C in modified Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS according to Church and Gilbert, 1984) in the presence of a random-primed [α-^{32}P]dATP labelled probe. Filters were washed in 0.5xSSC, 0.5%SDS at 65°C and exposed to Kodak X-OMAT AR film. Positive plaques were purified by a second round of hybridization. In case of the cDNA library, phagemids were excised from positive phages and used for sequence analysis. DNA from selected phages of the genomic library was isolated using Qiagen columns according to the manufacturer's instructions and analyzed by digestion with *Sal*I. Hybridizing fragments detected by Southern blot analysis were cloned and used for further subcloning and sequence analysis. DNA sequence analysis was performed on double-stranded DNA using the Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). Analyses of the sequence data and alignment to known sequences in GenEMBL and SWISS_PROT databases were performed using the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711. All DNA manipulations not described were performed according to Sambrook *et al.*, 1989).

Southern blot analysis. Genomic DNA of *B.cinerea* strain SAS56 was isolated according to Drenth *et al.* (1993), digested with restriction enzymes, size-separated on 0.7% agarose/TAE gel and blotted according to the manufacturer's instuctions onto Hybond-N$^+$ membranes (Amersham). The blot was hybridized in modified Church buffer, as described above, at 65°C for 18 hours in the presence of a random-primed [α-^{32}P]dATP labelled probe. Blots were washed in 0.2xSSC, 0.5% SDS at 65°C and exposed to Kodak X-OMAT AR film.

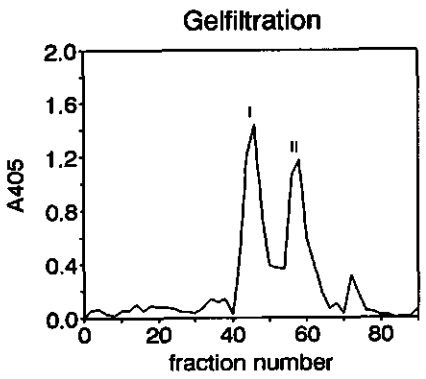
Analysis of gene expression *in vitro*. Total RNA was isolated from mycelial fractions according to the Extract-A-plant RNA isolation kit protocol (Clontech Lab). Samples of 10 μg total RNA were denatured with formamide and formaldehyde and subjected to electrophoresis on a 1.0% agarose gel containing formaldehyde (Sambrook *et al.*, 1989). After blotting to Hybond-N^+ membranes (Amersham), blots were hybridized as described for Southern blots.

Analysis of gene expression *in planta*. Conidia of sporulating cultures of *B. cinerea* strain SAS56 were harvested and resuspended in Gamborg's B5 medium (Duchefa) supplemented with 10 mM glucose and 8.5 mM potassium phosphate, pH 5.0, and sprayed at a density of 10^6 conidia per ml onto detached compound tomato leaves (*Lycopersicon esculentum*, cv. Moneymaker). Compound leaves were incubated with their stem inserted in wet florist's foam oases, in closed plastic boxes with a transparent lid to obtain a humidity of 100%. Before closing the boxes, the inoculum was air-dried. The boxes were placed at 18°C, with a light-dark cycle of 16 hours light and 8 hours darkness. Leaves were harvested at 0, 4, 8, 12, 16, 20, 24, 32, 48, 72, 120 hours after inoculation. Isolation of total RNA from the leaves, electrophoresis under denaturing conditions, blotting and hybridization were performed as mentioned above.

Results

Purification of PNB hydrolytic enzymes. In *B. cinerea* cutinase production is induced by cutin or monomers of cutin, such as 16-hha (Salinas, 1992). Enzyme activity can be determined using radioactively labelled cutin or PNB as a substrate (Purdy and Kolattukudy, 1973, 1975). In this study, PNB was used to quickly monitor cutinase activity in fractions obtained during the purification process. However, esterases other than cutinase can also hydrolyse this substrate and might be co-purified as well. Therefore, purified fractions are referred to as fractions containing PNB hydrolytic activities.

A liquid culture of *B. cinerea* grown in the presence of 16-hha was used to purify cutinase. Culture filtrate of *B. cinerea* showing maximal PNB hydrolytic activity 6 days post induction was concentrated and proteins were separated according to their size by gelfiltration. PNB hydrolytic activity was detected in fractions 42 to 48 (peak I) and 56 to 64 (peak II) of the eluent (Fig. 1). From each peak, pooled fractions were separately subjected to chromatofocusing and the resulting fractions were again monitored for PNB hydrolysis. PNB hydrolytic activity from peak I eluted at pH 7.0 (Fig. 1, fractions 20-22, peak IA) and the activity from peak II eluted at pH 5.8 (fractions 36-38, peak IIA). This suggests the presence of two different PNB hydrolytic enzymes. Protein composition of peak IA and IIA was analyzed on SDS-PAGE (Fig. 2). Peak IA contained two proteins of about 80 kD and 11 kD, while in peak IIA two proteins of 45 and 18 kD were present.



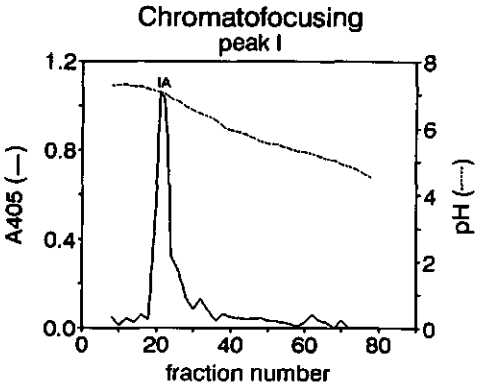
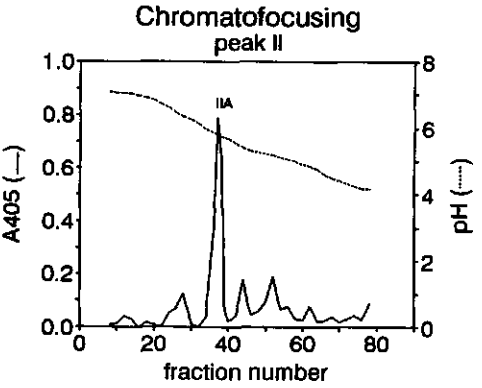


Figure 1: Profiles of PNB hydrolytic activity in fractions obtained after gelfiltration and chromatofocusing. PNB hydrolysis (Y-axis) was measured in even fractions (X-axis). Two peaks I (fractions 42 to 48) and II (fractions 56 to 64) obtained after gel-filtration, showed significant activity. Both peaks were subjected independently to chromatofocusing. In even fractions (X-axis) PNB hydrolytic activity (left Y-axis (—)) and pH (right Y-axis (···)) were measured. Peak IA (fractions 20 to 22) and peak IIA (fractions 36 to 38) contained highest PNB hydrolytic activity.



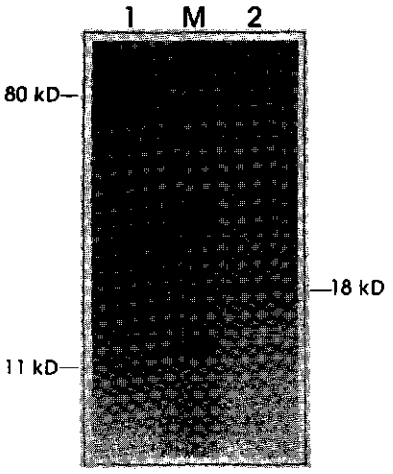


Figure 2: SDS-polyacrylamide gel electrophoresis of peak IA (lane 1) and peak IIA (lane 2). Lane M contains marker proteins with molecular weights of 66, 45, 36, 29, 24, 20, 14.2 kD. Proteins were stained with Coomassie Brilliant blue R250.

Characterization of purified proteins

Determination of cutinase activity. To demonstrate cutinase activity in peak IA and/or peak IIA, cutin labelled with the chromogenic group Remazol Brilliant Blue R (RBB, Wolf and Wirth, 1990) was used as a substrate. Cutin hydrolytic activity could be measured by an increase in the absorption at 590 nm caused by the release of RBB-labelled fatty acids into the solution from insoluble cutin-RBB. In peak IIA a ΔA590 of 0.566 was measured, whereas in peak IA a ΔA590 of only 0.057 was measured. These results clearly demonstrate cutinase activity in peak IIA. Since a cutinase purified from *B. cinerea* strain Bc7 was identified as a 18 kD protein by Salinas (1992), the 18 kD protein in peak IIA was assumed to be cutinase. This protein was subjected to amino acid sequence analysis for further identification and the design of gene specific primers for PCR based gene cloning.

In peak IA no significant cutin-RBB hydrolysis could be measured. PNB hydrolytic activity due to esterase activity could either reside in the 80 kD or the 11 kD protein and therefore both proteins were subjected to N-terminal sequence analysis.

Amino acid sequence analysis. The N-terminal and four internal sequences (p7, p8, p9 and p11) of the 18 kD protein were determined (Table 1). The N-terminal sequence showed significant identity (50-60% in 17 amino acids) to sequences present in the N-terminal region of cutinases of *Colletotrichum gloeosporioides, C. capsici, Alternaria brassicicola, Magnaporthe grisea, Ascochyta rabiei* and *F. solani* f.sp. *pisi* (see below). Peptides p7, p8 and p11 also aligned with these cutinases, whereas peptide p9 could not be aligned. The serine residue present in the motif G-X-S-X-G, characteristic for the active centre of all serine esterases, was identified in peptide p8. Based on the sequence homologies to fungal cutinases, the presence of the active site residue serine in the purified protein and its cutin hydrolyzing activity, the 18 kD protein was confirmed to be cutinase.

Of the 11 kD and 80 kD proteins present in peak IA, only N-terminal sequences were determined (Table 1). The N-terminal sequence of the 80 kD protein showed homology to the N-terminus of glucoamylase of *Neurospora crassa* (Stone *et al.*, 1993, 10 out of 16 amino acids were identical). The N-terminal sequence of the 11 kD protein showed no homology to sequences in the SWISS_PROT database. No PNB hydrolytic activity was detected in commercially available glucoamylase of *Aspergillus niger* and, therefore, it was assumed that PNB hydrolytic activity of peak IA resided in the 11 kD protein.

Isolation of *cutA* and *ekdA* encoding genes. Amino acid sequence information of the 18 kD cutinase and 11 kD protein was employed to design oligonucleotides for use as primers in RT-PCR to amplify encoding regions of the corresponding genes named *cutA* and *ekdA* (eleven kD protein), respectively.

Primer combinations N+11 and N+8 (Table 1) derived from the amino acid sequence of the N-terminus, p11 and p8 of cutinase, respectively, amplified fragments of 300 and 380 basepairs. After cloning and sequence analysis of the 380 bp fragment, an open

Table 1: Sequences of N-termini and peptides obtained after tryptic digests, of 18 kD, 11 kD and 80 kD proteins. Underlined amino acid sequences refer to degenerated primer sequences. Primer 8 and 11 of the 18 kD sequences are complementary to the coding sequences.
X = undetermined amino acid residue, N = A, C, G or T; Y = C or T; R = A or G; H = A, C or T; I = inosine. A slash means 'or'.

Peptide		amino acid sequence	primer	nucleotide sequence
18 kD -	N	AXSDVI/TVIFARGXSEXG	N	5'-GAYGTIAYIGTIATHTTYGC-3'
	p7	LLPAETTAK		
	p8	LVISGYSQGGQLVHNAAK	8	5'-CCNGTYRTICAIGTRTTRCG-3'
	p9	TYGMDTTAAAAFVK		
	p11	ISXAVIFGDPDNGDPV	11	5'-GGRTCICCRTTRTCIGGRTCNCC-3'
11 kD -	N	AXGITVTNKST	N1	5'-GGIATHACIGTNACNAAYAARTC-3'
			N2	AG-3'
80 kD -	N	XVDSFIAXEXPIAFRNL		

reading frame coding for 120 amino acids was deduced, comprising the peptide sequences of the N-terminus, p7, p8 and p11.

Two N-terminal primers N1 and N2 derived from the N-terminal sequence of the 11 kD protein and differing in only two nucleotides at the 3'-end due to degenerated codon choice, were used in combination with an oligo(dT) primer. Only primer N1 amplified a fragment of 370 bp, which was cloned and used for sequence analysis. The DNA sequence showed the presence of both primers used for PCR at the 5'- and 3'-end, and started at the 5'-end with an open reading frame coding for 78 amino acids.

Using both cloned PCR fragments as a probe, a genomic library of *B. cinerea* was screened to isolate the corresponding genes *cutA* and *ekdA*. After a second round of screening 5 and 3 recombinant phage clones, respectively, were isolated. Coding regions of the cutinase gene were mapped by Southern blot analysis on two *Sal*I fragments in each recombinant phage; one of 5.0 kb and a second fragment of variable size. This is in agreement with the presence of a *Sal*I site in the probe. A single 7.0 kb *Sal*I fragment was detected in each recombinant phage containing the *ekdA* gene.

A cDNA library was also screened with both probes. No positive clones were obtained using the *cutA* probe, whereas the *ekdA* probe yielded two positives, containing an insert of 0.6 kb.

Analysis of the *cutA* gene. Two 2.5 and 5.0 kb *Sal*I fragments from one positive recombinant phage were subcloned and a restriction map of the *cutA* locus was made (Fig. 3A and B). Both subclones were used for further subcloning and various overlapping clones were sequenced (Fig. 3B). The complete sequence represented in plasmid pCut1 (Fig. 3C) is shown in Figure 4. This sequence was aligned with the cDNA sequence of the PCR fragment (Fig. 3D) to identify the coding region and the position of introns. The coding sequence is interrupted by two introns of which the boundaries start and finish with

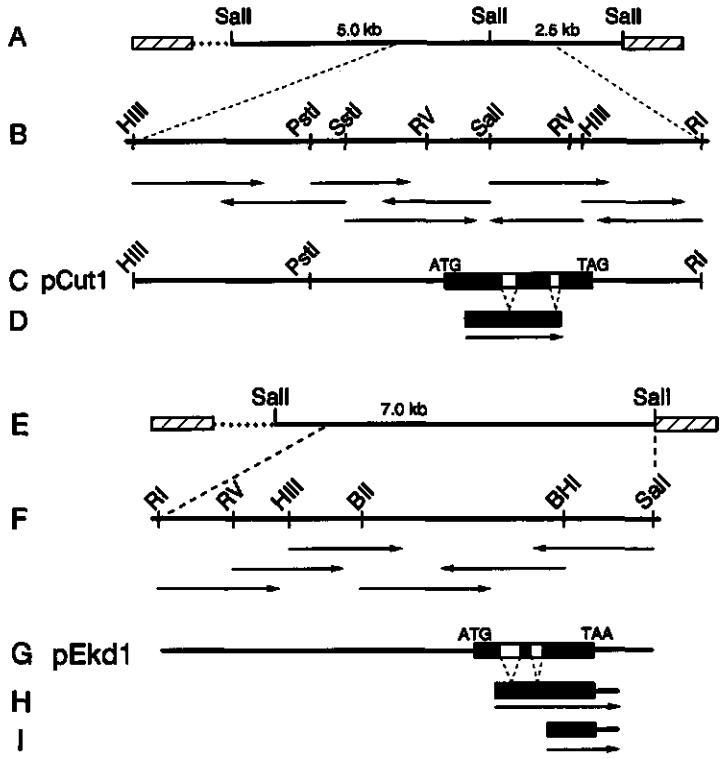


Figure 3: Analysis of the *B. cinerea cutA* and *ekdA* genes.
A: Map of recombinant phage containing 5.0 and 2.5 kb *SalI* fragments hybridizing to *cutA* probe. **B**: Partial restriction map and sequencing strategy of *cutA*. **C**: Organization of the *cutA* gene. The complete sequence cloned in plasmid pCut1 is presented in Fig. 4. **D**: Position of cDNA fragment amplified by primers N and 11. **E**: Map of recombinant phage containing 7.0 kb *SalI* fragment hybridizing to *ekdA* probe. **F**: Partial restriction map and sequencing strategy of *ekdA*. **G**: Organization of the *ekdA* gene. The complete sequence cloned in plasmid pEkd1 is shown in Fig. 7. **H**: Position of cDNA clone isolated from cDNA library. **I**: Position of cDNA fragment amplified by primers N1 and oligo(dT). Discontinuous lines (A and E) represent DNA region of unknown length. Hatched bars represent phage arms. Arrows indicate direction and extent of sequencing. Closed bars represent coding sequences, open bars represent introns. Start and stopcodons are shown. Abbreviations of restriction enzymes include: RI = *Eco*RI, RV = *Eco*RV, HIII = *Hin*dIII.

the consensus sequences GT and AG respectively. The introns are 78 and 66 nt in length.

The deduced amino acid sequence, starting at the only methionine present in front of the N-terminus, is 202 residues in length. The potential cleavage site of the signal sequence, predicted according to the (-3,-1) rule (Von Heijne, 1986), is between position 20 and 21 (see ↓ in Fig. 4), whereas the N-terminal sequence of the purified protein starts at position 30. This suggests that additional N-terminal processing steps occur

either during or after secretion in the culture medium. Other peptide sequences obtained from protein sequencing (shaded in Fig. 4) all match with the deduced sequence.

A computer alignment of all cutinases known so far, including the one of *B. cinerea*, is shown in Fig. 5. Peptide sequences determined from the purified protein are indicated. Homology between the cutinase of *B. cinerea* and other cloned cutinases, varies from 50 to 53%. The identity, as shown by dots in Fig. 5, is only 31 to 35%. These scores are rather low in comparison to a homology of 64 to 74% and identity of 48-64% among cutinases of *F. solani* f.sp. *pisi, A. rabiei, C. capsici* and *A. brassicicola*. Nevertheless, the functional serine residue is present in a highly conserved region (GYSGG, position 139-

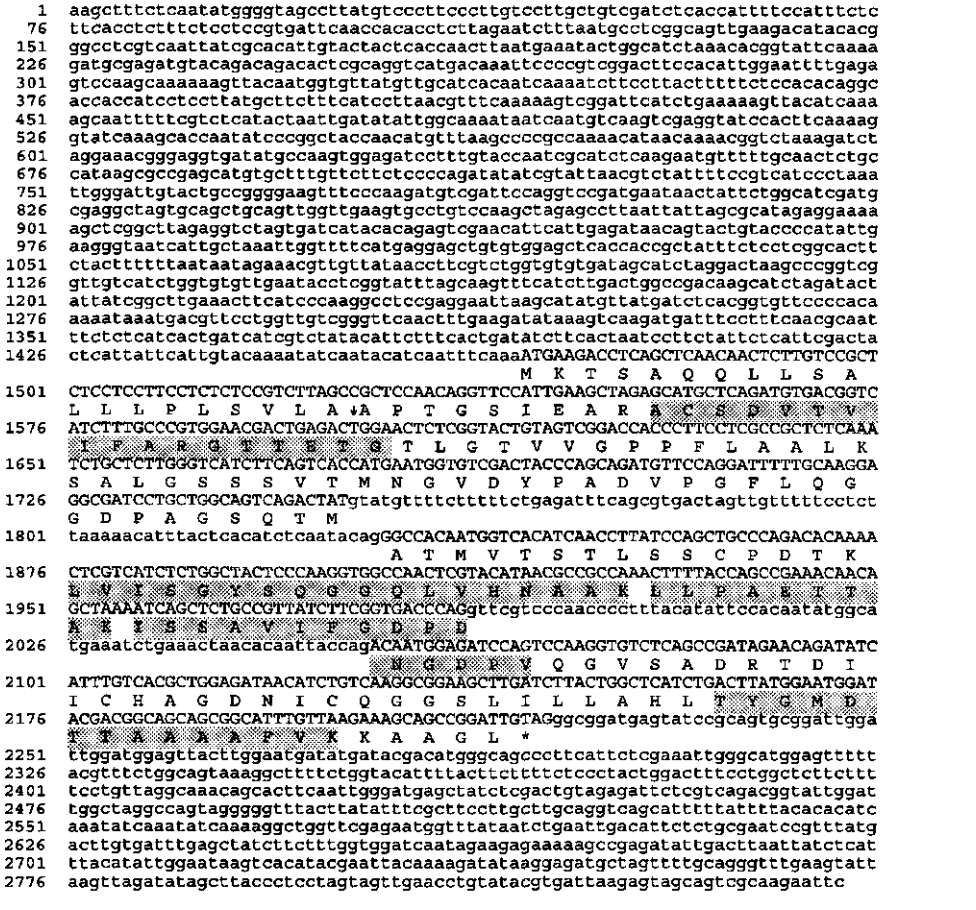


Figure 4: Nucleotide sequence of the *B. cinerea cutA* gene. The sequence starts at a *Hind*III site and extends up to an *Eco*RI site as shown in Fig. 3B. The deduced amino acid sequence is shown below the open reading frame. Sequences of the N-terminus and peptides p8, p7, p11 and p9, respectively, as determined by protein sequencing, are indicated by shaded boxes. The arrow indicates the potential cleavage site of the signal sequence. The nucleotide sequence data will appear in the EMBL and GenBank databases under accession number Z69264.

143) in all sequences. Aspartic acid (D) and histidine (H) at positions 196 and 209, respectively, participating with serine in the catalytic triad, are also conserved at similar positions in the cutinase of *B. cinerea*.

In front of the coding sequence 1.4 kb of the promoter region was sequenced (Fig. 4). No typical CAAT and TATA boxes for transcription initiation could be identified. At the 3'-end of the coding sequence 0.7 kb of the terminator was sequenced. A polyadenylation signal (AATAAA) is not found.

The copy number of the *cutA* gene was determined by Southern blot analysis of genomic DNA of *B.cinerea* digested with several enzymes (Fig. 6). A probe containing part of the cutinase encoding sequence hybridized to single fragments in the *Eco*RI, *Eco*RV and *Hin*dIII digestions. The *Sal*I digestion shows two hybridization signals due to the presence of a *Sal*I site in the coding region. A second, weakly hybridizing fragment in the *Pst*I digest was not predicted from the restriction map and was not reproducible. It is concluded that *cutA* occurs as a single copy gene in the genome of *B. cinerea*.

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                                                                    N
Bc   ---------- ---------M KTSAQQLLSA LLLPLSVLAA PTGSIEARAC SDVTVIPARG
Cg   MKFLSVLSLA ITLAA-AAPV EV--ETGVA- .ETRQ.STRN ELETGSSS.. PK.IY....A
Cc   MKFLSIISLA VSLVA-AAPV EVGLDTGVAN .EARQ.STRN ELE.GSSSN. PK.IY....A
Ab   ---------- ---------- -MMNLN..LS KPCQA.TTRN ELETGSSD.. PRTIF.....
Mg   MQFITVALTL IALAL-ASPI A.NVEKPSEL EARQ.NSVRS DLI.GN.A.. PS.IL....A
Ar   ---MKFFAFS MLIGE-ASPI VLALRRTTLE VRQLDPIIRS ELEQGSSSS. PKAIL.....
Fs   ---MKFFALT TLLAATASAL P..NPAQELE ARQLGRTTRD DLINGNSAS. R..IF.Y...

Bc   TTETGTLGTV VGPPFLAALK SALGSSSVTM NGVD--YPAD VPG---FLQG GDPAGSQTMA
Cg   S..P.NM.IS A..IVAD..E RIY.ANN.WV Q..GGP.L.. LASNF-LPD. TSS.AINEAR
Cc   S..P.NM.IS A..IVAD..E .RY.A.Q.WV Q..GGP.S.. LASNFIIPE. TSRVAINEAK
Ab   S..A.NM.AL ...FTAN..E ..Y.A.N.WV Q..GGP.T.G LVEN-ALPA. TSQ.AIREAQ
Mg   SG.V.NM.LS A.TNVASR.E REF-RNDIWV Q..GDP.D.A LSPNF-LPA. TTQGAIDEAK
Ar   S..I.NM.VS A..AVAS..E -.Y.ADQIWV Q..GGP.T.. L.SNF-LPG. TSQSAINEAV
Fs   S....N..-T L..SIASN.E ..F.KDG.WI Q..GGA.R.T LGDN-ALPR. TSS.AIRE.L

                          * P8          P7              P11
Bc   TMVTSTLSSC PDTKLVIEGY SQGGQLVHNA AKLLPAETTA KISSAVIFG- -DPDNGDPV
Cg   RLF.LANTK. .NAAI.SG.. ...TAVMAGS ISG.STTIKN Q.KGV.L..Y TKNLQ.LGRI
Cc   RLF.LANTK. .NSAV.AG.. ...TAVMASS ISE.SSTIQN Q.KGV.LSAI TKNLQ.LGRI
Ab   RLFNLAASK. .N.PITAG.. ...AAVMS.. IPG.S.AVQD Q.KGV.L..Y TKNLQ..GRI
Mg   R.F.LANTK. .NAAV.AG.. ...TAVMF.. VSEM..AVQD Q.KGV.L..Y TKNLQ.RGRI
Ar   RLFNEANTK. .S.PI.AG.. ...TAVMAG. IPK.-DAVR. RVVGT.L..Y TQNQQ.NKGI
Fs   GLFQQANTK. ..AT.IA... ...AA.AAAS IED.DSAIRD ..AGT.L..Y TKNLQ.RGRI

                     *             *        P9
Bc   QGVSADRTDI ICHAGDNICQ GGSLILLAHL TTGMDTTAAA A-FVKKAAGL *------ 202
Cg   PNFETSK.EV Y.DIA.AV.Y .TLF..P..F L.QT.AAV.. PR.LQARI.* ------ 224
Cc   PNF.TSK.EV Y.ALA.AV.Y .TLF..P..F L.QA.AATS. PR.LAARI.* ------ 228
Ab   PNFPTSK.T. Y.ET..LV.N .TLI.TP... L.SDEAAVQ. PT.LRAQIDS A*---- 209
Mg   PDFPTEK.EV Y.N.S.AV.F .TLFL.P..F L.TTESSI.. PNWLIRQIRA A*---- 228
Ar   KDYPQEDLQV Y.EV..LV.D .TLI.TVS.F L.LEEAAGP. PE.L.SKI.A *------ 223
Fs   PNYPADR.KV F.NT..LV.T .SLIVAAP.. A..P.ARGP. PE.LIEKVRA VRGSA* 230
```

Figure 5: Comparison of cutinase sequences from *B. cinerea*, *Colletotrichum gloeosporioides*, *C. capsici* (Ettinger *et al.*, 1987), *Alternaria brassicicola* (Yao and Köller, 1994), *Magnaporthe grisea* (Sweigard *et al.*, 1992a) *Ascochyta rabiei* (Tenhaken, unpublished, accession number of GenEMBL database: X65628) and *Fusarium solani* f.sp *pisi* (Soliday *et al.*, 1989). Amino acids identical to cutinase of *B. cinerea* are indicated by dots. Gaps introduced for optimal alignment are indicated by a dash. Asterisks above the alignment indicate amino acids participating in catalytic triad (Martinez, *et al*, 1992). Shaded boxes indicate the position of peptides determined by protein sequencing.

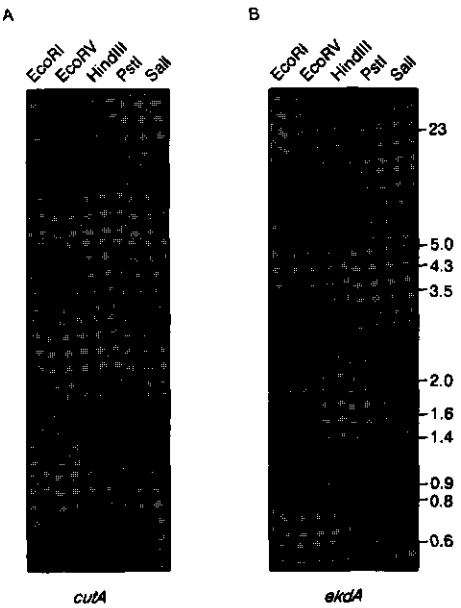

Figure 6: Autoradiographs of Southern blots containing genomic DNA of *B. cinerea* digested with different restriction enzymes as indicated. Molecular size-markers are indicated in kb. **A**: Blot hybridized with *cutA* probe. **B**: Blot hybridized with *ekdA* probe. Both probes were derived from the cloned PCR fragments.

Analysis of the *ekdA* gene. The 11kD encoding gene *ekdA* was located on a 7.0 kb *SalI* fragment (Fig. 3E) of a positive recombinant phage. Restriction analysis and Southern blotting identified the position of the coding region at the 3'-end of the fragment. Subclones were made for sequence analysis (Fig. 3F) and the complete sequence is shown in Fig. 7. Two cDNA clones (Fig. 3H) were isolated and sequenced to determine the complete primary structure of the *ekdA* gene. Both cDNA clones were not full-length and differed 6 codons in length. In the genomic sequence, the first methionine in frame is 25 amino acids upstream of the start of the longest cDNA (Fig. 7). This methionine is predicted to be the startcodon, since no other startcodons are present in frame. From these data an open reading frame encoding 144 amino acids, interrupted by two introns, was identified. The introns are 51 and 60 nt in length and both bounded by the consensus sequences GT and AG, respectively. The N-terminal sequence of the purified protein starts at position 65 (Fig. 7, shaded box). The length and amino acid composition of the deduced N-terminal sequence (residue 1 to 64), however, are not characteristic for a functional signal peptide (Von Heijne, 1986). Comparison of the deduced amino acid sequence to sequences in the SWISS_PROT database did not reveal any homology to known proteins. The amino acid sequence G-X-S-X-G, specific for serine esterases, or other motifs were not identified.

In front of the coding sequence 1.4 kb of the promoter region was sequenced (Fig. 7), in which no typical CAAT and TATA boxes for transcription initiation could be identified. At the 3'-end of the coding sequence a possible polyadenylation signal (AATAA,

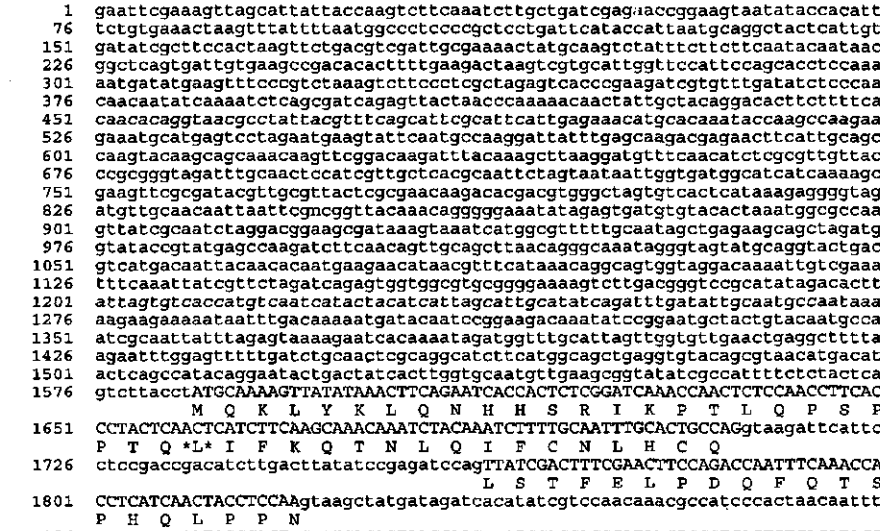

Figure 7: Nucleotide sequence of *B. cinerea ekdA* gene starting at the *Eco*RI site and extending to the *Sal*I site as shown in Fig. 3F. The deduced amino acid sequence is shown below the open reading frame. The N-terminal sequence as determined by protein sequencing is indicated by the shaded box. The amino acid in between asterisks is the first residue in the cDNA sequence. The underlined sequence indicates the putative polyadenylation signal and two bold, underlined nucleotides represent the start of the poly(A)-tail in the PCR fragment and cDNA clone, respectively. The nucleotide sequence data will appear in the EMBL and GenBank databases under accession number Z69265.

underlined in Fig. 7) is present, 117 nucleotides downstream of the stopcodon. The poly(A)-tail, as determined from the cDNA sequences, started 22-26 nt downstream of this putative polyadenylation signal.

Southern blot analysis of genomic DNA of *B. cinerea* digested with several enzymes showed single hybridizing fragments (Fig. 6), indicating that *ekdA* is a single copy gene.

***In vitro* expression of *cutA* and *ekdA*.** Gene expression of *cutA* and *ekdA* was analyzed in different cultures of *B. cinerea* and compared to measurements of PNB hydrolysis in the corresponding culture filtrates. To this end, a start culture of *B. cinerea* was diluted at day 0 in sub-cultures A (16-hha + glucose), B (16-hha) and C (glucose) and sampled

during each of the following 5 to 8 days. Determination of the glucose content in culture filtrates showed that glucose was already depleted from culture medium A and C at day 2. In the absence of glucose less fungal biomass had developed (Fig. 8, culture B).

Production of PNB hydrolytic activities in the start and sub-cultures sampled during 8 days post induction (d.p.i.) and northern blot analysis of RNA isolated from the start culture at day 0 and from cultures A, B and C during 1 to 5 d.p.i., is shown in Fig. 8. The blots were hybridized to coding regions of *cutA*, *ekdA*, and the constitutively expressed ß-tubulin gene of *B. cinerea* (*tubA*) to standardize mRNA levels. Single transcripts of approximately 900 and 600 nt in length were detected for *cutA* and *ekdA*, respectively, which is in agreement to the length calculated from the coding sequences.

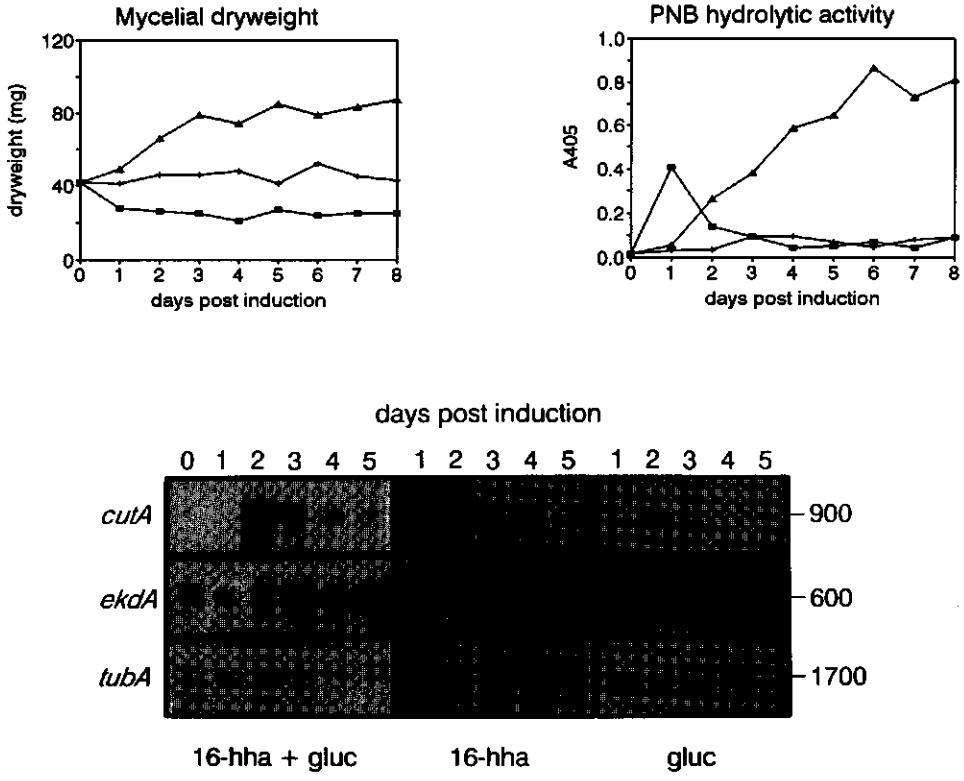

Figure 8: Mycelial dryweight, PNB hydrolytic activity in culture filtrate and northern blot analysis of *cutA* and *ekdA* expression in different cultures of *B. cinerea*. A start culture of *B. cinerea* was diluted at day 0 in sub-cultures A (16-hha + glucose, ▲), B (16-hha, ■) and C (glucose, +) and sampled during each of the following days. Mycelial dryweight was determined in samples of 50 ml culture medium. Northern blots containing total RNA isolated from *in vitro* grown *B. cinerea* were hybridized with probes derived from the *cutA*, *ekdA* and the ß-tubulin gene (*tubA*). Sizes of mRNAs (in nt) are shown in the right margin. All autoradiographs are overnight exposures.

In culture A, PNB hydrolytic activity was first detected at 2 d.p.i. reaching a maximum activity at 6 d.p.i. When glucose was depleted from the medium at 2 d.p.i., the *cutA* gene was immediately strongly expressed. From 3 d.p.i. onwards, the hybridization signal of *cutA* was fading, while the hybridization signal of the *ekdA* gene had increased. In contrast to *cutA*, expression of the *ekdA* gene was already detected in the start culture (day 0) and at a low level at 1 d.p.i..

In culture B, in the absence of glucose, the *cutA* gene was immediately expressed at a high level at 1 d.p.i., indicating that glucose repressed the expression of the *cutA* gene at day 1 in culture A. Both in cultures A and B the *cutA* expression follows the same pattern of a strong transient induction. Similarly, a transient peak activity in PNB hydrolysis was found at day 1 in culture B. The expression of *ekdA* displays a similar pattern in cultures B and C as in culture A: a relatively low expression at 1 and 2 d.p.i. and an increase in expression from 3 d.p.i. onwards, as demonstrated by a stronger hybridization signal (Fig. 8). There is no indication for specific induction of *ekdA* expression by 16-hha (culture B) or repression by glucose (culture A) as demonstrated for *cutA* expression. In the absence of the cutin monomer (culture C), no PNB hydrolytic activity was measured and only a very low hybridization signal of *cutA* mRNA was detected.

***In planta* expression of *cutA* and *ekdA*.** To analyse whether *cutA* and *ekdA* are expressed during infection of host tissue, tomato leaves were inoculated with conidia of *B. cinerea*. Standard inoculation for successful and synchronized infection was performed with conidia resuspended in a buffer containing glucose and phosphate (Van den Heuvel, 1981; Edlich *et al.*, 1989). The first visible symptoms on inoculated tomato leaves were small water-soaked lesions at 16 hours post inoculation (h.p.i.). These lesions became necrotic at 20 h.p.i. and their size remained unchanged until 72 h.p.i.. No new lesions

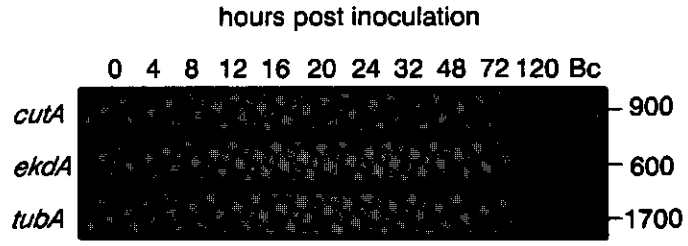

Figure 9: Autoradiograph of northern blot containing total RNA isolated from infected tomato leaves at different time points (numbers refer to hours post inoculation) and from one-day-old *B. cinerea* mycelium grown in standard medium (Bc). The blot was hybridized with probes derived from *cutA*, *ekdA* and *tubA* genes of *B. cinerea*. Sizes of the mRNA (in nt) are shown in the right margin.

appeared during that time, indicating that the primary infection occurred very synchronized. After 72 h.p.i., a subset of lesions developed into spreading lesions. At 120 h.p.i., the fungus had grown abundantly and started to sporulate from the expanded, necrotic lesions. Based on these symptom developments leaves were harvested at 4, 8, 12, 16 and 20 h.p.i., when penetration of host tissue occurs and the expression of *cutA* might be expected. Sampling continued until 120 h.p.i. to cover the whole infection process.

The *in planta* expression of *cutA* and *ekdA* was studied by northern blot analysis of total RNA isolated from infected tomato leaves (Fig. 9). The blot was also hybridized with the *tubA* gene of *B. cinerea* to demonstrate the ability to detect fungal mRNAs in the total population of plant and fungal RNAs. This probe hybridized specifically with the fungal ß-tubulin mRNA under the stringent conditions used. Under heterologous hybridization conditions a plant ß-tubulin mRNA was detected of a smaller size (results not shown).

Expression of the *cutA* gene was first detectable at 12 h.p.i., stayed at a similar level as the *tubA* signal and strongly increased at 120 h.p.i., when also the fungal biomass had increased. The *ekdA* gene is also highly expressed at this late time point, but no *ekdA* mRNA is detected earlier during the infection.

Discussion

A liquid culture of *B. cinerea* grown in the presence of the cutin monomer 16-hha, was used to purify cutinase. To quickly monitor cutinase activity during the purification process, the substrate PNB was used. Two fractions containing PNB hydrolytic activity resulted from the purification. PNB is however not specific for cutinase, as it is also hydrolyzed by other esterases. Therefore, to measure specifically cutin hydrolysis in these fractions, cutin labelled with the chromogenic group Remazol Brilliant Blue R (Wolf and Wirth, 1990) was used. Cutin-RBB hydrolysis was only measured in the fraction containing the 18 kD protein, later identified as cutinase, and not in the other PNB hydrolytic fraction containing 11 kD and 80 kD proteins. This is the first time that the use of cutin-RBB as a suitable substrate for cutinase is reported and it proved to be a good alternative for radioactively labelled cutin (Purdy and Kolattukudy, 1973, 1975).

The 18 kD protein was identified as cutinase, based on size, cutin hydrolytic activity and significant sequence homology to known fungal cutinases. The catalytic triad comprising the amino acids serine, aspartic acid and histidine was conserved. The deduced amino acid sequence of the *B. cinerea cutA* gene revealed an open reading frame of 202 amino acids, of which 29 N-terminal amino acids are removed during post translational processing to release a mature protein of 183 amino acids. Southern blot analysis showed cutinase to be encoded by a single copy gene.

The 80 kD and 11 kD proteins present in peak IA, were also characterized. The 80 kD protein showed, based on its N-terminus, homology to glucoamylase. A heterologous glucoamylase contained no esterase activity and, therefore, PNB hydrolytic activity was

assumed to reside in the 11 kD protein. The corresponding gene, named *ekdA*, was cloned and appeared to encode a protein of 144 amino acids. The N-terminal sequence determined from the purified protein started at position 65 of the deduced open reading frame. The length and amino acid composition of the 64 N-terminal amino acids do not correspond to a functional signal peptide (Von Heijne, 1986), which suggests an intracellular origin. Probably, the purified 11 kD protein is a relatively stable degradation product of an intracellular protein. A possible explanation for the purification of the 11 kD protein from a culture like culture A might be the release of some protein from desintegrating mycelium of vigorously grown *B. cinerea* (Fig. 8). The *ekdA* sequence has no homology to sequences present in databases, which could confirm its enzymatic activity or reveal its biological function.

Gene expression of *cutA* and *ekdA*, and PNB hydrolysis in culture filtrate were analyzed in different *B. cinerea* cultures. Expression of *cutA* gene is repressed by glucose (compare cultures A and B) and requires the presence of an inducer (compare cultures A and C). PNB hydrolytic activity in cultures B and C, correlated with the observed *cutA* expression. In culture A, however, PNB hydrolysis was increasing until 6 d.p.i., whereas *cutA* expression was only strong at 1 d.p.i.. A correlation between *cutA* expression and cutinase activity in cultures A, B and C should have been demonstrated with cutin-RBB. Unfortunately, in crude culture filtrate, containing cutinase, cutin-RBB hydrolytic activity could not be determined because of interference of other components in the culture filtrate.

No PNB hydrolysis was measured in the start culture and cultures B and C at 3-5 d.p.i., when the *ekdA* gene was clearly expressed. On the other hand, the increase in PNB hydrolytic activity in culture A might be attributed to the presumed esterase activity of the 11 kD protein, released from desintegrating mycelium in this culture.

Expression of *cutA* during infection of tomato leaves by *B. cinerea* was first observed at 12 h.p.i., just before lesions appear at 16 h.p.i. Cutinase expression was not strongly induced compared to the expression of the constitutively expressed *tubA* gene. The strong *cutA* expression observed at 120 h.p.i. might reflect cutin degradation during saprophytic growth of the fungus in final stages of plant colonization.

Analysis of a role for cutinase in penetration of host tissue by *B. cinerea* remains difficult when conidia are inoculated in the presence of glucose, which represses *cutA* expression. Inoculation of tomato leaves with dry conidia, however, results in inefficient and asynchronized infection, which makes it unsuitable to accurately determine gene expression in time by northern blot analysis. The next chapter reports on the use of the reporter gene GUS (Jefferson *et al*, 1987) to study cutinase expression at early time-points during infection in single germinating conidia. In addition, cutinase A-deficient mutants, obtained by gene disruption, were used to study the requirement of cutinase for successful penetration of host tissue by *B. cinerea*.

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CHAPTER 5

Cutinase A of *Botrytis cinerea* is expressed, but not essential during penetration of gerbera and tomato

This chapter has been submitted to *Molecular Plant-Microbe Interactions* by the authors C.J.B. van der Vlugt-Bergmans, J.W. van 't Klooster, C.A.M. Wagemakers, D.C.T. Dees, J.A.L. van Kan.

Summary. The plant pathogen *Botrytis cinerea* can infect undamaged plant tissue directly by penetration of the cuticle. This penetration was suggested to be enzyme-mediated and an important role for cutinase in the infection process was proposed.

In this study the expression of the cutinase encoding gene *cutA* of *B. cinerea* was analysed using a *cutA* promoter - GUS reporter gene fusion. Transformants containing this fusion construct were examined for GUS expression on gerbera flowers and tomato fruits. High GUS activity was found when conidia germinated and penetrated into epidermal cells, indicating that *cutA* is expressed during the early stages of infection.

To determine the biological relevance of cutinase A for successful penetration, cutinase A-deficient mutants were constructed by gene disruption. Pathogenicity of two transformants lacking a functional *cutA* gene was studied on gerbera flowers and tomato fruits. Their ability to infect and to develop disease was unaltered compared to the wild type strain. These results exclude an important role for the *cutA* gene during direct penetration of host tissue by *B. cinerea*.

Introduction

The ubiquitous plant pathogen *Botrytis cinerea* Pers.:Fr. infects fruits, flowers or green plant tissue of more than 200 plant species (Jarvis, 1977). Direct penetration of germ tubes into undamaged plant tissue occurs via natural openings, or through the cuticle (Verhoeff, 1980), the first plant layer the fungus encounters before entering the epidermis. To breach this cutin layer, mechanical or enzymatic mechanisms were suggested to be employed by *B. cinerea*. Clark and Lorbeer (1976) reported on the formation of appressoria-like structures by *B. cinerea* mediating penetration of onion leaves by mechanical force. McKeen (1974) and Rijkenberg *et al.* (1980) described enzyme-mediated penetration of the cuticle of bean leaves and tomato fruits. Using electron microscopy they observed a sharp pore through the cuticle without curled edges, and the infection peg did not cause an indentation of the cuticle or epidermal wall during penetration. Moreover, the absence of appressoria-like structures indicated that penetration is an enzymatic process. Esterase activity at the tip of germ tubes (McKeen, 1974) and the production of cutinase in culture media of *B. cinerea* supplied with cutin as carbon source (Shishiyama *et al.*, 1970; Baker and Bateman, 1978) provided support for a role for cutinase in penetration of the cuticle.

Further evidence supporting the role of cutinase in hydrolyzing the cutin layer and thus facilitating penetration, is the inhibition of infection by monoclonal antibodies raised against cutinase of *B. cinerea*. Lesion formation on gerbera flowers was reduced by 80% when flowers were sprayed with this antibody before inoculation with *B. cinerea* (Salinas, 1992).

The cloning of the cutinase encoding gene *cutA* of *B. cinerea* (Chapter 4) allows the use of a promoter - reporter gene fusion to detect *cutA* gene expression during penetration of host tissue. The *uidA* reporter gene encoding ß-glucuronidase (GUS, Jefferson *et al.*, 1987) has been successfully used in fungal pathogens to study expression of a particular gene during colonization of host tissue (Van den Ackerveken

et al., 1994; Wubben *et al.*, 1994). In this study, the GUS reporter system was employed to determine whether cutinase A is indeed produced by *B. cinerea* during penetration of host tissue. Expression from the *cutA* promoter is visualized as blue staining of fungal structures when GUS is expressed under control of the *cutA* promoter.

To unequivocally determine the biological relevance of cutinase A of *B. cinerea* during penetration of its host, cutinase A-deficient mutants are required. The establishment of a transformation system for *B. cinerea* (Hilber *et al.*, 1994; Hamada *et al.*, 1994) and the availability of haploid *B. cinerea* strains (Büttner *et al.*, 1994) allowed disruption of the single copy gene *cutA*, to obtain these mutants. Transformants lacking a functional cutinase A gene were assayed for their ability to infect gerbera and tomato tissue.

Materials and Methods

Fungal growth. Strain SAS56 of *B. cinerea* (Van der Vlugt-Bergmans *et al.*, 1993), haploid strain B05.10 (derived from SAS56, a gift from P. Büttner and Dr P. Tudzynski, Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), and resulting transformants were grown on malt extract agar (Oxoid) at 18°C in the dark. Prior to plant inoculations strains were grown on tPDA (potato dextrose agar containing 300 g homogenized tomato leaves per litre). After 3 days the cultures were exposed for 16 hours to near UV-light. One week later conidia were collected from sporulating cultures and inoculated (10^6 conidia per litre) in Gamborg's B5 medium (Duchefa) supplemented with 0.3% glucose. After two days of growth in a rotary shaker at 160 rpm, 20°C, mycelium was harvested for isolation of genomic DNA. For analysis of gene expression, two-day-old cultures (start cultures) were diluted ten-fold in the following media: B5 medium + 0.05% 16-hydroxyhexadecanoic acid (16-hha; A), B5 medium + 0.05% 16-hha + 0.3% glucose (B), B5 medium + 0.3% glucose (C). Remaining mycelium of the start cultures was harvested. All three sub-cultures were incubated at 20°C, 160 rpm and sampled during the following days.

Construction of transformation vectors. Vector pCutGUS containing a fusion between the *cutA* promoter of *B. cinerea* and the GUS reporter gene *uidA* was constructed in the following way. A *Nco*I site (CCATGG) was introduced at the ATG startcodon of the *cutA* gene by PCR using a primer complementary to a region upstream of the ATG (5'-CTCCATGGTGAAATTGATGTATTGATATTTTG-3') in combination with a M13 primer at the 5'-end of the promoter on a subclone containing part of the *cutA* gene as template. The resulting 1.5 kb PCR fragment was ligated in pBluescript SK$^-$ (Stratagene) digested with *Eco*RV. Plasmid pCF20 (Van den Ackerveken *et al.*, 1994) contained the *gpd* promoter of *Aspergillus nidulans*, the *Escherichia coli uidA* gene encoding ß-glucuronidase (GUS, Jefferson *et al.*, 1987) and the terminator of the *A.nidulans trp*C gene. The *gpd* promoter was excised from pCF20 by digestion with *Eco*RI and *Nco*I, and replaced by the *cutA* promoter present on a 1.5 kb *Eco*RI/*Nco*I fragment excised from the

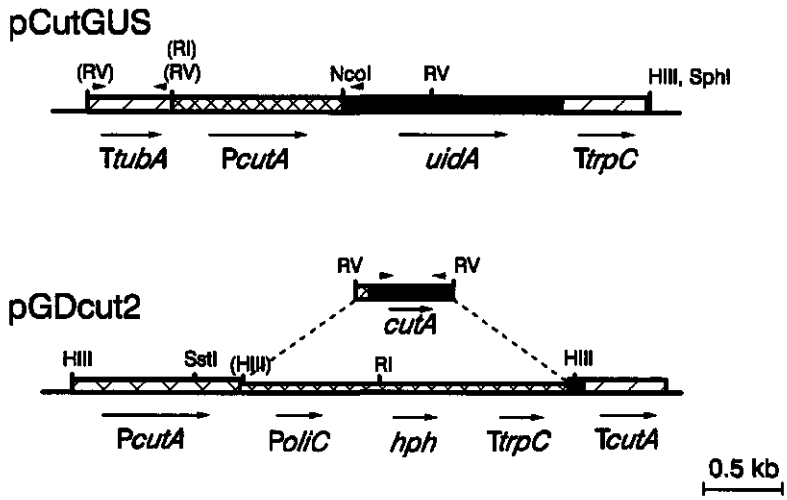

Figure 1: Schematic representation of plasmid pCutGUS and pGDcut2 used for transformation of *B. cinerea*. Sequences *tubA* and *cutA* are derived from *B. cinerea* genes, *uidA* and *hph* are *Escherichia coli* genes, *trpC* and *oliC* are from *Aspergillus nidulans*. Arrows indicate the orientation of the fragment. Small arrowheads above the boxes represent primers used for PCR. RI = *Eco*RI, RV = *Eco*RV, HIII = *Hin*dIII. Restriction sites between brackets are deleted during subcloning.

Bluescript construct. In the resulting construct, an additional 0.7 kb *Eco*RV fragment containing the terminator sequence of *tubA* gene of *B.cinerea* was cloned in the *Eco*RI site, upstream of the *cutA* promoter (Fig. 1A). This *Eco*RI site was first made blunt by incubation with Klenow large fragment DNA polymerase according to the prescribed reaction conditions. Correct orientation of the *tubA* terminator in vector pCutGUS was checked by PCR using one of the primers complementary to 5'- and 3'-sequences of the *tub*A terminator in combination with a primer in the *uid*A gene (Fig. 1A).

Vector pGDcut2 was derived from plasmid pCut1 (Chapter 4). The main part of the coding sequence of *cutA* was deleted by digestion with *Eco*RV and replaced by a selection marker digested from plasmid pOHT (Hilber *et al.*, 1994; provided by Dr M. Ward, Genencor International, San Francisco, USA). The selection marker was present on a 2.6 kb *Sma*I/*Hin*dIII fragment of pOHT and consisted of the bacterial hygromycin phosphotransferase gene (*hph*, conferring resistance to hygromycin B) fused to the *oli*C promoter and *trp*C terminator of *A. nidulans*. This fragment was incubated with Klenow large fragment DNA polymerase to fill in the sticky ends and used to replace the *cutA* *Eco*RV fragment from plasmid pCut1 (Fig. 1B). The orientation of the hygromycin B selection marker in the resulting pGDcut2 construct was determined by restriction analysis.

Transformation of *B. cinerea*. Conidia from sporulating cultures of *B. cinerea* strain SAS56 or B05.10 were collected and inoculated in bacto malt extract (Difco, 10^5conidia per ml). Cultures were incubated in a rotary shaker at 150 rpm, at 22°C for 2 days. The following transformation protocol was adapted from Hamada *et al.*, 1994. Mycelium was harvested by filtration and washed twice in sterile water and once in 0.6 M KCl, 50 mM $CaCl_2$ (KC-buffer). Protoplasts were generated by adding 10 ml of a filter-sterilized solution containing 5 mg Novozyme 234 (Novolabs) in KC-buffer per gram of fresh weight mycelium. This suspension was incubated for 2 hours at 23°C with shaking at 80 rpm. The digested mycelium was filtered using filters with 25 µm and 10 µm mesh size, respectively. Protoplasts were pelleted by centrifugation at 2,000 g for 10 minutes, washed once and resuspended in KC-buffer to a final concentration of 10^8 protoplasts per ml. Portions of 100 µl were maintained on ice for 5 min and, subsequently, plasmid DNA in 40 µl water, 5 µl of 50 mM spermidine and 100 µl of 25% PEG 3350 (Sigma) in 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5 were, one by one, gently added to the protoplasts. The mixture was kept on ice for 20 min. Five-hundred microlitres of PEG solution were added, gently mixed and incubated for 10 min at room temperature. Finally, the volume was adjusted to 1 ml with KC-buffer. For selection, 100 µl of protoplasts were spread on 10 ml SH-agar (0.6 M sucrose, 5 mM HEPES, pH 5.3, 1 mM $(NH_4)_2HPO_4$, 1.2% agar) and incubated for 3 hours at room temperature. Protoplasts were then overlayed with 3 ml of SH-topagar (0.6% agar) containing 100 µg/ml of hygromycin B (Duchefa Biochemie BV). After 3 to 7 days of incubation at 22°C, hygromycin B-resistant colonies of *B. cinerea* were individually transferred to malt extract agar and grown for 2 days at 22°C. Agar plugs with mycelium from the edge of each colony were subsequently transferred to malt extract agar amended with 100 µg/ml hygromycin B.

Prior to transformation of *B. cinerea* strain SAS56, vector pCutGUS was linearized with *Sph*I (Fig. 1A) and pOHT with *Hin*dIII. Ten microgram of linearized vector and 1 µg of linearized pOHT as selection marker were used for cotransformation. The haploid strain B05.10 of *B. cinerea* was transformed using 2 µg of circular pGDcut2.

Southern blot analysis. Genomic DNA was isolated according to Drenth *et al.* (1993), digested with restriction enzymes, size-separated on a 0.7% agarose gel and blotted onto Hybond-N^+ (Amersham) according to Sambrook *et al.* (1989). The blot was hybridized in modified Church buffer (0.5 M sodium phosphate buffer, pH 7.2, 7% SDS according to Church and Gilbert, 1984) at 65°C for 18 hours in the presence of a random-primed [α-^{32}P]dATP labelled probe. Blots were washed in 0.2xSSC, 0.5% SDS at 65°C and exposed to Kodak X-OMAT AR film.

Northern blot analysis. Total RNA was isolated from mycelial samples using the method of Extract-A-plant RNA kit (Clontech Lab). Samples of 10 µg total RNA were denatured with formamide and formaldehyde and subjected to electrophoresis on a 1.0 % agarose gel containing formaldehyde (Sambrook *et al.*, 1989). After blotting to Hybond-N^+

membranes, blots were hybridized in modified Church buffer (as described above) in the presence of a random-primed [α-^{32}P]dATP labelled probe and washed in 0.2xSSC, 0.5% SDS at 65°C before exposure to Kodak X-OMAT AR film.

Fluorometric GUS assay. One hundred milligram fresh mycelium was resuspended in GUS Extraction Buffer (GEB: 50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1% N-laurylsarcosine, 0.1% Triton X-100, 10 mM ß-mercaptoethanol, Jefferson *et al.*, 1987), ground with a pestle, vortexed and after centrifugation the supernatant was stored at 4°C. Protein concentration of the samples was determined using the BioRad protein assay (Bradford *et al.*, 1976) and bovine IgG as a standard. Ten microgram of protein of each sample was diluted to 100 μl in GEB and added to 500 μl 1 mM MUG (4-methyl umbelliferyl glucuronide, Research Organics) dissolved in GEB. After mixing, enzyme assays were incubated at 37°C in the dark. After 1 and 2 hours of incubation 100 μl of each enzyme assay was mixed with 900 μl stopbuffer (0.2 M Na_2CO_3). Fluorescence was measured in a spectrofluorimeter (excitation 365 nm, emission 450 nm), calibrated with MU (4-methyl umbelliferone, Sigma) in stopbuffer.

Bioassays. Gerbera flowers cv. Sirtaki and Romana and tomato fruits cv. Moneymaker (green fruits of 3-4 cm in diameter) were dry-inoculated with transformants or wildtype strains of *B. cinerea*. Conidia were transferred from sporulating cultures by a brush, or they were dusted on the host tissue by tapping the bottom of an inverted petri dish with a sporulating culture.

Flowers and fruits were incubated in closed plastic boxes with a transparent lid to obtain a humidity of 100%, at 20°C with a dark-light cycle of 16 hours light and 8 hours dark. Development of disease symptoms was followed by eye and fungal penetration was examined microscopically in whole-mount preparations of inoculated petals or in epidermal strips of tomato fruits. Flower petals harvested at different time-points post inoculation, were fixed in absolute alcohol-glacial acetic acid (1:1 v/v), cleared in 75% lactic acid for 48 h, 37°C and mounted on a microscope slide in lactophenol-cotton blue. Epidermal strips of tomato fruits were treated similarily with omission of the clearing step.

For histochemical localization of GUS activity, gerbera petals or epidermal strips of tomato fruits were incubated with 0.5 mg/ml X-Gluc (5-bromo-4-chloro-3-inolyl ß-D glucuronide, Biosynth AG) in 50 mM phosphate buffer, pH 7.0, 1 mM KFeCN and 0.05% (v/v) Triton-X100, overnight at 37°C. The tissue was mounted on microscope slides in 50% (v/v) glycerol.

The samples were examined with a Zeiss Axioscope microscope and photographs were taken using a Zeiss MC-100 camera unit. Bioassays were performed two times.

Reisolation of mycelium from infected tomato fruit was performed by incubating a piece of infected fruit tissue on wateragar at 18°C. After 3 days of incubation mycelium bearing agar plugs were excised from the edge of the developing colony and incubated on malt extract agar as described above.

Results

Characterization of transformants containing the pCutGUS construct. To study the expression of the cutinase gene (*cutA*) during penetration of host tissue by *B. cinerea*, a fusion was made between the *cutA* promoter and the *uidA* reporter gene encoding ß-glucuronidase (GUS, Jefferson *et al.*, 1987). To prevent disturbance by other regulatory sequences near the site of integration, the terminator sequence of the *tubA* gene of *B. cinerea* was cloned in front of the *cutA* promoter (Fig. 1A). This construct called pCutGUS, was introduced into *B. cinerea* strain SAS56 by cotransformation with the vector pOHT. Twelve hygromycin B resistant transformants were assessed for the presence of pCutGUS insertions by PCR using primers complementary to the *tubA* terminator sequence and the *uidA* coding sequence (Fig. 1A). In ten of the twelve transformants a fragment of 2.0 kb was amplified, indicating that the *cutA* promoter - *uidA* reporter gene fusion was correctly cotransformed into the genome of *B. cinerea*. The number of pCutGUS insertions was determined by Southern blot analysis (Fig. 2). A blot containing genomic DNA of five transformants T1, T2, T3, T5, T7 and wildtype strain SAS56 digested with *Eco*RV was probed with a *Eco*RV/*Hin*dIII fragment of pCutGUS (Fig. 1A), containing parts of the *uidA* coding region and *trpC* terminator. Results show a single integration in T2 and T5 represented by a strongly hybridizing fragment and integration of many copies in T1, T3 and T7.

To assess whether these transformants exhibit GUS activity upon induction with the cutin monomer 16-hha (see Chapter 3 and 4), transformants and the wild type strain SAS56 were grown in standard medium. After 2 days of growth 16-hha was added (day 0) and incubation followed for another 3 days. In two independent series of cultured transformants intracellular GUS activity was measured quantitatively using a fluorometric assay with MUG as substrate. Only in transformant T2 and T7 induction of GUS activity upon incubation with 16-hha was reproducibly detected (Table 1). At day 0 no GUS activity was measured, but in the presence of 16-hha high

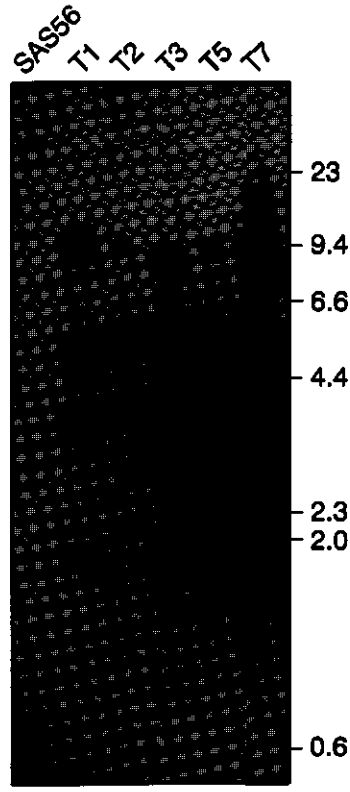

Figure 2: Autoradiograph of a Southern blot containing genomic DNA of *B. cinerea* wildtype strain SAS56 and the transformants T1, T2, T3, T5 and T7, digested with *Eco*RV. The blot was hybridized with the *Eco*RV/*Hin*dIII fragment of pCutGUS (Fig. 1A), containing parts of the *uidA* coding region and *trpC* terminator. Molecular size markers are indicated in kb at the right margin.

Table 1: GUS activity measured in mycelial samples of transformants T2 and T7 of *B. cinerea* containing a *cutA* promoter - GUS fusion, and the wildtype strain SAS56. At day 0 the cutin monomer 16-hha was added to induce expression from the *cutA* promoter. A fluorometric assay using MUG as substrate was used to monitor GUS activity quantitatively. GUS activity is measured in nmol MU / hour / 10 μg protein. Data of one experiment are shown and were reproducible in a second independent experiment.

	T2	T7	SAS56
day 0	0.95	0.39	0.04
day 1	11.89	5.45	0.07
day 2	12.85	6.38	0.06
day 3	9.60	1.56	0.02

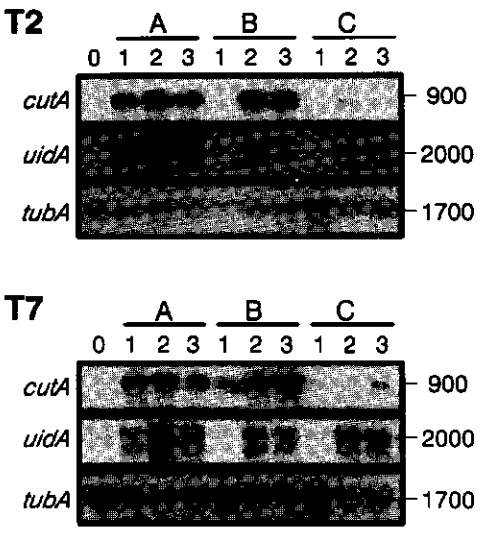

Figure 3: Autoradiographs of a northern blot containing total RNA of transformants T2 and T7 of *B. cinerea*, isolated from mycelium grown under different inducing conditions. The blot was hybridized with probes containing part of the coding regions of the *cutA*, *uidA* and *tubA* genes. Sizes of the transcripts are indicated in nt at the right margin.

activity was measured at day 1 and day 2 and at a lower level at day 3. In the wild type strain SAS56 lacking the *cutA* promoter - GUS fusion, no GUS activity was detected (Table 1).

To investigate whether GUS and cutinase A are expressed similarly from the *cutA* promoter under different cutinase inducing and repressing conditions, expression of the genes was studied at the RNA level by northern blot analysis. Transformants T2 and T7 were grown in a start culture for 2 days and subsequently diluted in three different sub-cultures (day 0): culture A contained 16-hha, culture B contained 16-hha and glucose and in culture C only glucose was present. Mycelium from the start culture and cultures A to C was sampled at day 0 and day 1 to 3, respectively, for isolation of total RNA. Results of northern blot analysis are shown in Fig. 3. Both *cutA* and *uidA* transcripts of 900 and 2000 nt, respectively, were detected from day 1 onwards when mycelium was grown in the presence of 16-hha (culture A). In culture B of transformant T2, *cutA* and *uidA* transcripts were detected when glucose was depleted from the medium at day 2 and 3, indicating that glucose repression of the *cutA* promoter affects the expression of the *uidA* gene as well. In the absence of the inducer (T2, culture C), only weak hybridization signals of *cutA* and *uidA* were present. Hybridization of the blot with a probe derived from the constitutively expressed ß-tubulin gene of *B. cinerea* (*tubA*) showed

similar amounts of fungal RNA in each lane on the blot. Co-expression of *cutA* and *uidA* was also observed in transformant T7, except for the samples harvested at day 1 (culture B) and day 2 and 3 (culture C). Moreover, hybridization of the blot containing T7 RNA with the *uidA* probe shows a second larger transcript, which is coregulated with the 2000 nt *uidA* transcript. Since the construct contained a terminator sequence in front of the *cutA* promoter, we think that this transcript results from termination downstream of the *trpC* terminator.

The northern blot analysis suggests that transcripts of *uidA* are more rapidly turned over than those of *cutA*. This was concluded from smears below the hybridizing *uidA* transcript and the decrease in the hybridization signal of the *uidA* transcript compared to that of *cutA* at day 3 (cultures A and B). The wild type *cutA* gene is shown to be still functional in transformants T2 and T7.

Histochemical localization of GUS activity. Expression from the *cutA* promoter during penetration of gerbera flowers and tomato fruits was examined by monitoring GUS activity in transformants T2 and T7. After inoculation, GUS activity was visualized microscopically in a histochemical assay using X-Gluc as substrate.

On gerbera flowers cv. Sirtaki, dry-inoculated with conidia of *B. cinerea*, necrotic lesions were clearly visible 24 hours post inoculation (h.p.i.). Penetration of the cutin layer had occurred before that time-point and, therefore, flower petals were harvested and examined for GUS activity at 6, 16 and 24 h.p.i.. At 6 h.p.i., several conidia showed strong GUS activity visualized by their dark blue colour, whereas other conidia were pale blue or colourless (Fig. 4A). At 16 h.p.i., nearly all conidia were stained blue and had germinated. Infection pegs were also stained dark blue (Fig. 4B). Penetration of host cells was observed and at 24 h.p.i. also cell death was visible by yellow-brownish cell contents of penetrated epidermal cells (Fig. 4C). In wildtype strain SAS56 similar observations on germination and penetration were made. Pathogenicity of T2 and T7 was unaltered compared with the wildtype strain SAS56.

On tomato fruits *B. cinerea* developed slower. Two days post inoculation (d.p.i.) 60% of the conidia had germinated and 40% of the total number of conidia had penetrated into epidermal cells. All fungal structures, except for some ungerminated conidia, showed GUS activity and were stained dark blue and penetration of host tissue was visible (Fig. 4D). At 3 d.p.i., up to 90% of the conidia had germinated and 50% had penetrated. Death of single epidermal cells was visible in the microscope, although no necrotic lesions were seen by eye.

Characterization of cutinase A-deficient transformants. To determine whether cutinase A is essential for successful penetration of host tissue by *B. cinerea*, the single copy gene *cutA* was mutated by gene disruption. Transformation vector pGDcut2 containing a hygromycin B selection marker flanked by 1.4 kb *cutA* promoter sequence at the 5'-end and 0.1 kb coding sequence followed by 0.6 kb terminator of *cutA* at the 3'-end (Fig. 1B),

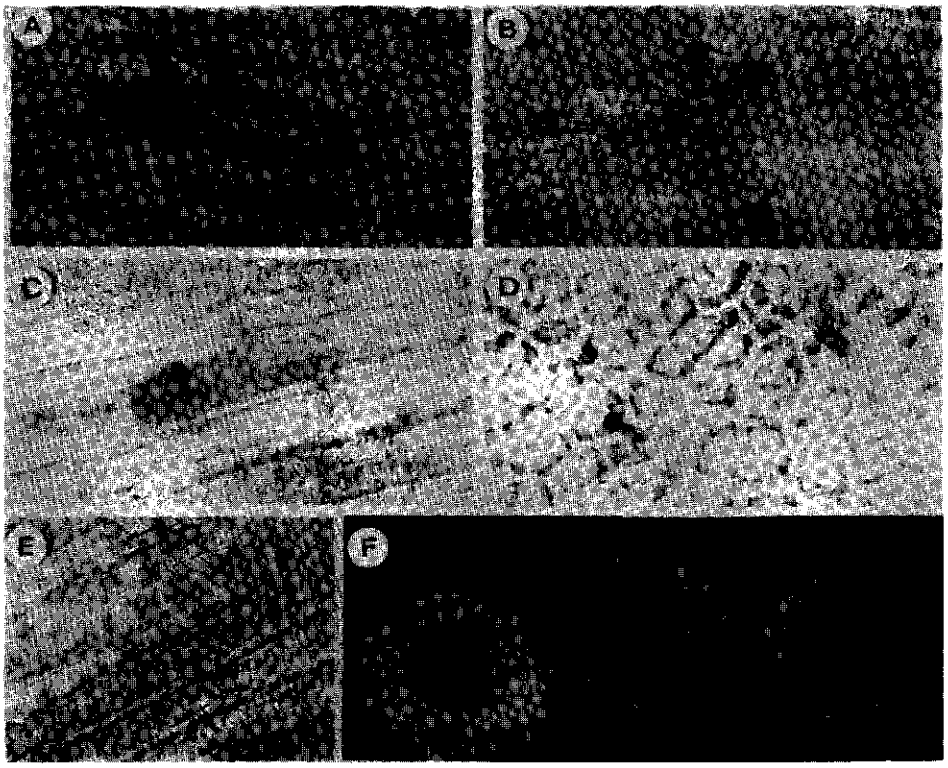

Figure 4: Histochemical localization of GUS activity on gerbera petals (A-C) and tomato fruit (D) inoculated with transformant T2 of *B. cinerea* containing the *cutA* promoter - GUS fusion. **A.** Conidia on a gerbera petal show different levels of GUS activity at 6 h.p.i.. **B.** At 16 h.p.i. most conidia have germinated and show high GUS activity in conidia and infection pegs. **C.** At 24 h.p.i. entry of an infection peg into an epidermal cell has led to cell death visualized by a darker cell content. **D.** Three d.p.i. on tomato fruit, conidia show different levels of GUS activity and one germinated conidium has penetrated an epidermal cell. **E.** Conidia of transformant T130 lacking a functional cutinase A gene, germinate and penetrate epidermal cells of gerbera petals equally well. Fungal structures are stained with lactophenol-cotton blue dye. Note the darker cell content of the penetrated epidermal cell. **F.** Gerbera flowers non-inoculated (left) and inoculated with B05.10 (middle) and T130 (right).

was used to transform the haploid *B. cinerea* strain B05.10. Pathogenicity of this strain was indistinguishable from strain SAS56 from which it was derived (Büttner and Tudzinsky, unpublished). Homologous recombination between the *cutA* locus and *cutA* sequences present in pGDcut2 allows replacement of the wildtype gene by the hygromycin cassette.

Eighty hygromycin B resistant colonies obtained after transformation of strain B05.10 with pGDcut2, were screened by PCR using primers complementary to *cutA* coding sequences which were deleted in pGDcut2 (Fig. 1B). A 400 bp PCR-fragment was

expected from the wildtype *cutA* locus, whereas no amplification product was expected in transformants in which gene disruption had occurred. In 23 of the 80 transformants the 400 bp fragment was absent or very weakly amplified compared to the intensity of the amplification product in the other transformants. Six of the 23 transformants were selected for further analysis by Southern blotting (Fig. 5A). Genomic DNA digested with *Hin*dIII was predicted to release a 2.1 kb fragment from the wildtype *cutA* gene or a 4.0 kb fragment from the mutated gene (Fig. 1B). Using a *Hin*dIII/*Sst*I fragment of the *cutA* promoter (see Fig. 1B) as probe, the presence of a 4.0 kb fragment was demonstrated in all six transformants indicating that homologous recombination had occurred at the *cutA* locus. However, in three transformants T124, T125 and T137 also the wildtype 2.1 kb fragment weakly hybridized, indicating that the wildtype *cutA* gene is still present in these transformants. Other, non-predicted hybridization signals in T137 (Fig. 5A) demonstrate that ectopic vector integration had also occurred. Hybridization of the same blot with a *Hin*dIII/*Eco*RI fragment of the hygromycin cassette (Fig. 1B) showed only hybridization of the 4.0 kb fragment in T124, T125, T130, T132, and T136, and the same additional

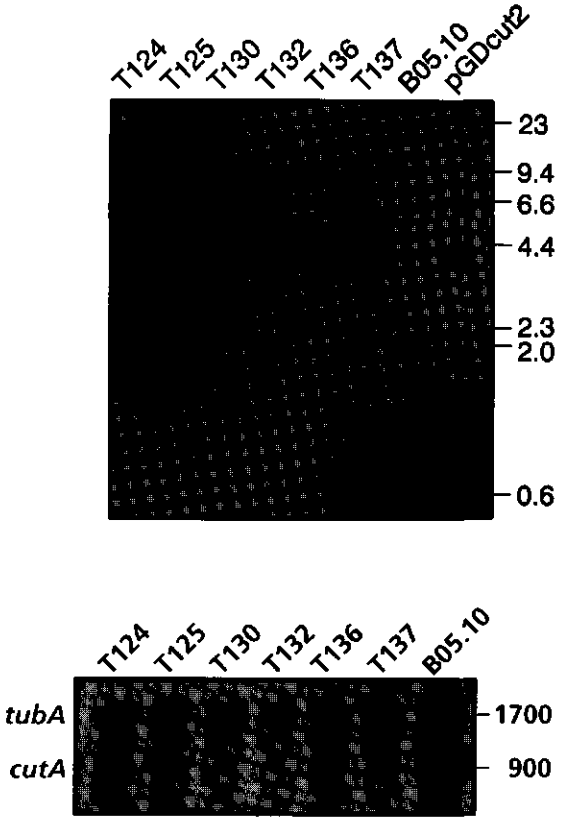

Figure 5: Autoradiograph of a Southern blot (upper panel) containing genomic DNA of *B. cinerea* wildtype strain B05.10 and the transformants T124, T125, T130, T132, T136, T137, and the gene disruption plasmid pGDcut2 digested with *Hin*dIII. The blot was hybridized with the *Hin*dIII/*Sst*I fragment of pGDcut2 (Figure 1A), containing a part of the *cutA* promoter sequence. Molecular-size markers are indicated in kb at the right margin.
Autoradiograph of a northern blot (lower panel) containing total RNA of *B. cinerea* wildtype strain B05.10 and the transformants T124, T125, T130, T132, T136, T137, isolated from mycelium induced for *cutA* expression. As probe parts of the *cutA* and *tubA* encoding regions of these *B. cinerea* genes have been used. Sizes of the transcripts are shown in nt at the right margin.

fragments of T137 as detected in Fig. 5A (results not shown). Since no ectopic integrations of the selection marker were detected in T124 and T125, untransformed nuclei harbouring the wildtype *cutA* gene might still be present in these tranformants.

To monitor *cutA* transcript levels in the wildtype strain and transformants, northern blot analysis was performed on total RNA isolated from transformants grown in cutinase inducing medium (Fig. 5B). A single band of 900 nt was detected in the wildtype and, to a lesser extent, in transformants T124, T125 and T137 in which the wildtype *cutA* gene was also detected by Southern blotting. No *cutA* mRNA was however detected in transformants T130, T132 and T136. Cohybridization with a probe derived from the constitutively expressed ß-tubulin gene (*tubA*) of *B. cinerea* demonstrated the presence of approximately equal amounts of RNA on the blot.

Southern and northern blot data demonstrate that the *cutA* gene is not functional in transformants T130, T132 and T136. Viability of transformants T130, T132 and the wildtype strain B05.10 was tested by plating conidia on solid medium. After two days of incubation 90% of T130 or T132 conidia had germinated compared to 100% germination in B05.10. Germinated conidia developed into normal colonies.

Pathogenicity assay of cutinase A-deficient mutants. Conidia of wildtype strain B05.10 and transformants T130 and T132 lacking a functional *cutA* gene, were dry inoculated on gerbera flowers cv. Sirtaki and tomato fruits. Symptoms on gerbera flowers were first visible 24 h.p.i. as necrotic lesions occurring everywhere on the petals. At 48 h.p.i. flowers were completely rotten (Fig. 4F).

On tomato fruits no necrotic lesions were visible. At 3 d.p.i. the interior of the fruit was colonized resulting in complete maceration of the tissue 5 d.p.i.. In two independent tests on two gerbera flowers and tomato fruits, no significant differences in lesion size, lesion number or rate of disease development were observed between the wildtype B05.10 and the cutinase A-deficient transformants T130 and T132.

Penetration of epidermal host cells was also examined microscopically: no differences in fungal penetration structures between B05.10, T130 or T132 were observed. Penetration occurred via epidermal cells and death of host cells was indicated by yellow-brownish stained cell content (Fig. 4E).

Structure and thickness of the cutin layer might vary with plant age or cultivar (Martin and Juniper, 1970). Therefore, gerbera flowers of different age and another cultivar Romana were tested. Again, no differences in pathogenicity of T130 and T132 as compared to B05.10 were visible on these flowers.

B. cinerea was reisolated from tomato fruits inoculated with B05.10, T130 and T132 and analyzed by PCR using primers complementary to *cutA* encoding sequences (Fig. 1B). From the reisolated strain B05.10 a 0.4 kb fragment was amplified, whereas this fragment was absent in PCR assays using reisolated strains T130 and T132. The isolates obtained from the infected tomato fruit were demonstrated to be identical, with respect to the *cutA* deletion, to the strains used for the inoculation.

Discussion

Cutinase was suggested to play an important role in penetrating host tissue by *B. cinerea*. This was based on use of monoclonal antibodies raised against cutinase which prevented infection of gerbera flowers (Salinas, 1992), and on electron microscopy studies (McKeen, 1974; Rijkenberg *et al.*, 1980). However, proof for production of cutinase at the site of penetration was lacking and, therefore, its role remained speculative. In this study the expression of cutinase A during penetration of host tissue by *B. cinerea* was analysed by using transformants with the GUS reporter gene *uidA* under control of the *cutA* promoter. The requirement for cutinase A in the penetration process was investigated by using cutinase A-deficient mutants obtained by gene disruption in pathogenicity assays.

Transformants of *B. cinerea* containing the coding region of the GUS reporter gene (*uidA*) fused to the promoter sequence of *cutA* randomly integrated in the genome of *B. cinerea*, were first tested *in vitro* to demonstrate that GUS and cutinase A mRNAs are similarly expressed from the *cutA* promoter. Two transformants T2 and, to a lesser extent, T7 showed co-expression of both *cutA* and *uidA* genes as demonstrated by northern blot analysis. Histochemical localization of GUS activity during penetration of host tissue was visualized by blue staining of GUS containing fungal structures. On gerbera petals and epidermal strips of tomato fruits inoculated with T2 and T7, blue staining was clearly observed in germinating conidia and in penetration pegs. The GUS activity, expressed from the *cutA* promoter reflects cutinase A expression during the penetration event.

These histochemical studies demonstrate that cutinase A is expressed during germination and penetration of host tissue. However, proof for a pivotal role for cutinase in cutin hydrolysis and an enzyme-mediated penetration process can only come from cutinase A-deficient mutants. These were obtained by replacement of the *cutA* coding sequence by a hygromycin selection marker (gene disruption). Southern blot analysis of six transformants showed 3 transformants lacking the wildtype *cutA* gene and 3 transformants containing both the disrupted and wildtype *cutA* gene. The latter gene was present in a much lower abundance as shown by weaker hybridization signals. Since the selection marker was not ectopically integrated in transformants T124 and T125 (data not shown), the wildtype *cutA* gene is present in nuclei which are hygromycin B sensitive and thus untransformed. Due to the multinucleate, heterokaryotic nature of *B. cinerea*, transformed and untransformed nuclei can coexist in one thallus. Apparently, untransformed nuclei can survive hygromycin B selection due to the presence of resistant nuclei in transformants T124 and T125. The origin of these mixed tranformants remains speculative. Protoplasts might have contained two or more nuclei, which have not all been transformed. The presence of both types of nuclei hampered the initial detection of gene disruption by PCR, since the PCR assay was based on absence or presence of an amplification product of, respectively, the mutated or wildtype *cutA* gene. In 23 out of 80 transformants the PCR fragment was weakly amplified or completely absent. Southern blot analysis of 6 out of these 23 showed gene disruption in all six transformants. This suggests that gene disruption had occurred with a frequency of about 25% (23:80).

However, the actual frequency of cutinase A-deficient mutants will be lower due to the presence of untransformed nuclei.

Transformants T130, T132 and T136 lacked a functional *cutA* gene, since no *cutA* mRNA was detected by northern blot analysis. Pathogenicity tests were conducted with the cutinase A-deficient transformants T130 and T132 and the wildtype strain B05.10 by inoculation on gerbera flowers and tomato fruits. Both T130 and T132 showed normal development of disease symptoms indistinguishable from B05.10 which carries a functional *cutA* gene. Using flowers of different age or another gerbera cultivar, of which the cutin layer might be of a different structure or thickness (Martin and Juniper, 1970), did not show altered symptoms. Microscopical examination at early stages of the infection showed that penetration of T130 into epidermal cells was similar to the wildtype strain. Cutinase A-deficient mutants of *B. cinerea* show no altered penetration ability and cutinase A seems not to be required for successful penetration of undamaged host tissue.

In contrast to the observation that gerbera flowers sprayed with monoclonal antibodies raised against cutinase of *B. cinerea* strain Bc7 were protected against infection due to inhibition of cutinase activity (Salinas, 1992), we find that cutinase is not essential for infection of gerbera flowers by *B. cinerea*. To exclude the possibility that different cutinases are involved, these monoclonals were tested on B05.10 and the cutinase A-deficient transformants. After growth in cutinase inducing medium, cutinase was clearly detected in culture filtrate of B05.10 by western blotting using the monoclonal antibody, but undetectable in culture filtrate of T130 and T132 (results not shown). This confirms that the gene which has been disrupted in this study encodes the same cutinase against which antibodies were raised by Salinas (1992).

This is the first report showing the feasibility of gene disruption in *B. cinerea* as a means of specifically mutating a single defined locus. The biological relevance of cutinase A during the infection by *B. cinerea* was assessed and our experiments exclude an essential role for this cutinase in pathogenicity of *B. cinerea*. Cutinase gene disruption in *Alternaria brassicicola* (Yao and Köller, 1995) did also not alter the penetration ability of the fungus. However, this cutinase gene was not expressed *in planta*, but cutinases, other than those expressed in *in vitro* cultures, were produced during the penetration of host tissue by this fungus. To our knowledge this is the first report of a cutinase gene in a plant pathogenic fungus which is expressed during cuticle penetration, but its expression is evidently not required for successful invasion. Possibly additional cutinases are present in *B. cinerea*.

Acknowledgments

We thank P. Büttner and Dr P. Tudzinsky (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany) for providing the haploid strain B05.10 of *B. cinerea*, Dr M. Ward (Genencor International, San Francisco, USA) for plasmid pOHT and Dr J. Salinas (Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands) for providing antiserum 21C5 against cutinase of *B. cinerea*.

CHAPTER 6

Cloning, characterization and expression analysis of catalase A of *Botrytis cinerea*

This chapter has been submitted as part of a paper to *Physiological and Molecular Plant Pathology* by the authors C.J.B. van der Vlugt-Bergmans, C.A.M. Wagemakers, D.C.T. Dees, J.A.L. van Kan.

Summary. Catalase mediates the enzymatic breakdown of hydrogen peroxide to water and molecular oxygen. During the infection of broad bean (*Vicia faba*) by *Botrytis cinerea* the release of hydrogen peroxide by fungal glucose oxidase activity is thought to facilitate the penetration by the pathogen. Catalase activity might play a role in protecting the fungus against the damaging effects of hydrogen peroxide.

A cDNA clone encoding catalase was isolated from a library of *B. cinerea*. Southern blot analysis of genomic DNA indicated the presence of a single copy gene, denoted as *catA*. The cDNA clone encoded a protein, CAT-A, of 480 amino acids showing 56 to 65% similarity to fungal catalases. Detailed analysis of sequence homologies between other fungal catalases enabled grouping of catalases according to their cellular location. CAT-A of *B. cinerea* resembled most to the peroxisomal catalases. Northern blot analysis showed a significant expression of the gene *in vitro*. *In planta*, however, no *catA* expression could be detected by northern blot analysis, whereas a constitutively expressed ß-tubulin gene was detectable and symptom development on the inoculated leaves was very clear. The possible occurrence of additional catalase encoding genes in *B. cinerea* is discussed.

Introduction

One of the most rapid plant responses to pathogens is the oxidative burst, the release of active oxygen species like superoxide anion (O_2^-), hydroxyl radical ($OH^\cdot$) and the relatively stable and diffusable hydrogen peroxide (H_2O_2) (Mehdy, 1994). The function of the rapid generation of H_2O_2 after pathogen attack is still unclear, but several explanations have been postulated (Apostol *et al.*, 1989). Firstly, H_2O_2 can be an important factor in triggering cell wall reinforcement (Bradley *et al.*, 1992; Brisson *et al.*, 1994), thus influencing the ability for the pathogen to penetrate the plant cell. Secondly, H_2O_2 may act as second messenger in the activation of the hypersensitive response (HR) in the plant and/or the induction of defence genes (Levine *et al.*, 1994). Thirdly, elevated levels of H_2O_2 might also be actively involved in killing pathogen and/or host cells during the HR. H_2O_2 is relatively unreactive but it can form $OH^\cdot$ radicals which cause DNA mutations, protein denaturation and lipid peroxidation. By breaking down H_2O_2 the plant and/or pathogen protect themselves from these toxic effects. Catalase mediates the enzymatic breakdown of H_2O_2 by converting it to molecular oxygen and water. The balance between generation and breakdown of H_2O_2 might play a critical role in determining processes in both the host and the pathogen and, therefore, in the outcome of the interaction.

In the infection of broad bean (*Vicia faba*) by *Botrytis cinerea* Pers.:Fr. a positive correlation was found between pathogenicity and glucose or xylose oxidase activity in some isolates (Edlich *et al.*, 1987, 1989). The authors proposed that the oxidase activity of the fungus releases H_2O_2 by oxidizing glucose or xylose. This exogenously released H_2O_2 can be converted to O_2^- and $OH^\cdot$ radicals, which are capable of destroying relatively inert material, such as cutin and membrane lipids. It is also able to diffuse rapidly across the cell membrane, possibly having toxic effects on the plant cell. Weakening of plant tissue is thought to facilitate penetration by the fungus. However, diffusion of H_2O_2 to the interior of fungal cells is also possible and could be damaging to the fungus itself.

In this paper the cloning and characterization of a catalase-encoding cDNA of *B.*

cinerea is described. In view of the suggested role of H_2O_2 during the infection of broad bean by *B. cinerea* and the possibly damaging effect of H_2O_2 to the fungus, it was thought that *B. cinerea* might produce elevated levels of catalase to break down H_2O_2. Therefore, the expression of the catalase gene was studied during the infection of tomato leaves by *B. cinerea*.

Materials and Methods

Culturing of *B. cinerea*. *B. cinerea* strain SAS56 was grown on malt extract agar (Oxoid) at 18°C in the dark. After 3 days the cultures were exposed overnight to near UV-light. One week later conidia were collected from sporulating cultures and used for inoculation of liquid Gamborg's B5 medium (Duchefa) supplemented with 0.3% glucose. One litre of medium was inoculated with 10^8 conidia and incubated in a rotary shaker at 20°C, 150 rpm. After 4 days 0.05% (w/v) 16-hydroxyhexadecanoic acid was added and the culture was incubated at 20°C, 150 rpm for another 4 days.

Construction of the cDNA library. Total RNA was isolated from mycelium harvested four days after induction with 16-hydrohexadecanoic acid, according to the Extract-A-plant RNA Isolation kit protocol (Clontech Lab). Poly(A)$^+$ RNA was isolated using the Oligotex-dT mRNA kit of Qiagen. cDNA syntesis and cloning in lambdaZAP II vector arms (Stratagene) was done according to the manufacturer's instructions. The quality of the cDNA library was checked by determining the average insert size and the orientation of the inserts of 10 random picked and excised phagemids. The insert size was determined by restriction analysis with *Eco*RI and *Xho*I and subsequent electrophoresis on a 0.7% agarose gel in TAE. The orientation of the coding sequence was determined by sequence analysis. Sequencing of DNA inserts was done on double-stranded plasmid DNA using the Dyedeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Analyses of the sequence data and alignment to sequences in GenEMBL and SWISS_PROT databases were performed using the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711.

Screening of the cDNA library. In total, $5x10^4$ phages were plated. Replica filters were hybridized for 16 hours at 65°C in modified Church buffer (0.5 M sodium phosphate, pH 7.2, 7% SDS according to Church and Gilbert, 1984). As a probe the 0.7 kb *Eco*RI/*Xho*I insert of phagemid CT1 was used. This probe was labelled with [α-^{32}P]dATP using a random primers DNA labelling system of Gibco-BRL/LifeTechnologies. Filters were washed in 0.5xSSC, 0.5% SDS at 65°C and exposed to Kodak AR film. Positive plaques were purified by a second round of hybridization. Phagemids were excised from positive phages. The insert size and nucleotide sequence was determined as mentioned above. Subcloning of large inserts was performed according to standard procedures (Sambrook *et al.*, 1989).

Genomic Southern blot analysis. Genomic DNA of *B. cinerea* strain SAS56 was isolated (according to Drenth *et al.*, 1993) from mycelium grown in a liquid culture for 2 days. DNA was digested with the enzymes *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Sal*I and size-separated on 0.7% agarose/TAE gel. Following electrophoresis, DNA was blotted to Hybond-N^+ membranes (Amersham) by capillary transfer (Sambrook *et al.*,1989) and hybridized in modified Church buffer, as described above, at 65°C for 18 hours. The *Eco*RV fragment of subclone CT3.2 was used as probe and labelled as mentioned before. Blots were washed in 0.2xSSC, 0.5% SDS at 65°C and exposed to Kodak X-OMAT AR film.

Analysis of gene expression *in vitro*. One litre of Gamborg's B5 medium (Duchefa) supplemented with 0.3% glucose, was inoculated with 10^9 conidia of strain SAS56 and shaken at 20°C, 150 rpm for four days. Each day, samples of 100 ml were taken to harvest mycelium. Total RNA was isolated from the mycelium as mentioned before. Samples of 10 μg total RNA were denaturated with formamide and formaldehyde and subjected to electrophoresis on a 1.0% agarose gel containing formaldehyde (Sambrook, *et al.*, 1989). RNA was blotted onto Hybond-N^+ membranes (Amersham) by capillary transfer (Sambrook *et al.*, 1989). The blots were hybridized in modified Church buffer at 65°C as described above using the random primer-labelled 0.7 kb *Eco*RI/*Xho*I insert of phagemid CT1 as a probe. The blot was washed in 0.5xSSC, 0.5% SDS at 65°C and exposed to Kodak X-OMAT AR film. After de-probing, the blot was rehybridized with a 0.5 kb *Bgl*II/*Kpn*I fragment containing part of the constitutively expressed ß-tubulin gene of *B. cinerea*.

Analysis of gene expression *in planta*. Conidia of sporulating cultures were harvested and resuspended in Gamborg's B5 medium (Duchefa) supplemented with 10 mM glucose and 8.5 mM potassium phosphate, pH 5.0, and sprayed at a density of 10^6 conidia per ml onto detached, compound tomato leaves (*Lycopersicon esculentum*, cv. Moneymaker). Compound leaves were incubated with their stem inserted in wet florist's foam oasis, in closed plastic boxes with a transparent lid to obtain a humidity of about 100%. Before closing the boxes, the inoculum was air-dried. The boxes were placed at 18°C, with a dark-light cycle of 16 hours light and 8 hours darkness. Leaves were harvested at 0, 4, 8, 12, 16, 20, 24, 32, 48, 72, 120 hours post inoculation. Isolation of total RNA from the leaves, electrophoresis under denaturing conditions, blotting and hybridization were performed as mentioned before.

Results

Isolation and characterization of catalase encoding cDNAs. An expression library aimed at the cloning of cutinase encoding cDNAs was constructed from mRNA isolated from a *B. cinerea* culture induced with 16-hydroxyhexadecanoic acid (see Chapter 3). This

fatty acid is a monomer of cutin and known to induce cutinase expression in fungi (Kolattukudy, 1985).

To check the quality of this cDNA library size and orientation of inserts in 10 random phagemids were checked by restriction and sequence analysis. Insert sizes ranged from 0.5 to 2.0 kb reflecting the size distribution of most of the full-length fungal mRNAs. All inserts contained open reading frames in the correct orientation for expression of the coding region and sequences were compared to EMBL and SWISS_PROT databases. One clone, later named CT1, showed 60% homology to amino acids 451-556 of the catalase protein sequence of *Hansenula polymorpha* (Didion and Roggenkamp, 1992). This clone was subjected to further study for reasons as mentioned in the introduction.

To obtain full length cDNA clones the library was screened under high stringency using the 0.7 kb insert of clone CT1 as a probe. Initially twelve positives were selected of which ten were still positive in a second round of hybridization. Inserts were checked by restriction analysis and Southern blotting. Four phagemids (CT2, CT3, CT4, CT6) contained inserts of about 1.8 kb, four phagemids (CT5, CT8, CT9, CT13) contained inserts ranging from 1.2 to 1.4 kb and two phagemids (CT11, CT12) contained inserts of 0.6 and 0.7 kb respectively (Fig. 1).

Phagemids CT1, CT4, CT6, CT8, CT9 were sequenced from the 5'-end and CT3, CT8, CT9 were sequenced from the 3'-end. Overlapping sequences determined from different phagemids were identical, indicating that they represented mRNAs transcribed from the same gene. A partial restriction map was constructed (Fig. 1) and subclones of CT3 and CT8 were used for further sequence analysis.

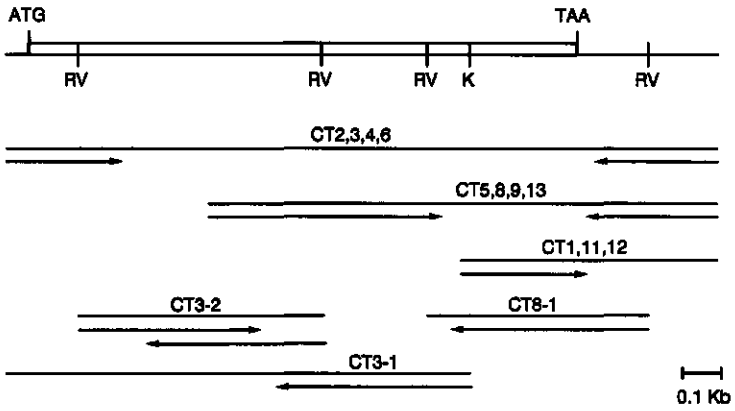

Figure 1: Partial restriction map of the *catA* cDNA of *B. cinerea* and the position of phagemids and subclones. Size of the longest phagemid in each class is shown. The open box indicates the coding sequence. Arrows indicate direction and extent of sequencing. RV=*Eco*RV, K=*Kpn*I.

In Fig. 2 the full length sequence of the catalase encoding cDNA of *B. cinerea*, denoted *catA*, and the deduced amino acid sequence are shown. The open reading frame encodes a protein, denoted CAT-A, of 480 amino acids. The sequence contains an in frame ATG codon at position 58. The sequence around this codon is in close agreement with the optimal context for the start of translation (CCACCATGGC, Kozak, 1986). A second in frame ATG codon was found at position 103 lacking this optimal translation context. We therefore assume the first ATG at position 58 to be the start of translation.

In the 3'-untranslated region of the cDNA sequence, a possible polyadenylation signal (AATAAA) is present at position 1751-1756 (underlined in Fig. 2), 253 nt downstream of the TAA stopcodon. The cDNA sequence stops with a polyA$^+$-tail starting at position 1853 (underlined in Fig. 2).

```
   1  cccaattgacaactaccattcatcacacataaatctgctcgagtaaattcaaacaacATGGCTCAAACCAATGGC
                                                          M  A  Q  T  N  G
  76  GTATTGCAAGAACCGGCCATCACAACGATGAACGGTGCTCCCGTTCTAAAGCCAGCATCTACCCAAAGAATTGGC
      V  L  Q  E  P  A  I  T  T  M  N  G  A  P  V  L  K  P  A  S  T  Q  R  I  G
 151  AATCAGCTCAGAGCCACTCTTCTACTTCAAGATATCAATCTCTTGGAATTGATCCAACACATCACCCATGAGCGA
      N  Q  L  R  A  T  L  L  L  Q  D  I  N  L  L  E  L  I  Q  H  I  T  H  E  R
 226  ATTCCTGAGCGTGTCGTCCATGCCAGAGGTACTAGCGCTCACGGATACTTTGAGGTCACCGATGACATATCAGAT
      I  P  E  R  V  V  H  A  R  G  T  S  A  H  G  Y  F  E  V  T  D  D  I  S  D
 301  GTCACATCTGCGGCTTTTCTAAACCGGGTCGGAAAACAAACCGATATATTTTGTCGGTTCTCCACCGTAGCTGGT
      V  T  S  A  A  F  L  N  R  V  G  K  Q  T  D  I  F  C  R  F  S  T  V  A  G
 376  CGAGCAGAATCTGCCGAAACGGTTCGGGATACTCGTGGTTTGCTTTTAAAATGTTTACCGAGGAAGGTAACTTA
      R  A  E  S  A  E  T  V  R  D  T  R  G  F  A  F  K  M  F  T  E  E  G  N  L
 451  GATTGGTTGTTCCTTAGCACTCCTGTCTTCCCAATTCGAGATGGAGCTAAATTCCCATCTTTCACTCATGCTACT
      D  W  L  F  L  S  T  P  V  F  P  I  R  D  G  A  K  F  P  S  F  T  H  A  T
 526  AAGAAGAATCCACGAAGTGGCTTACCTGATCACAAGGCATTCTGGGACTACTTCACTCACAACCAAGAGGGGATT
      K  K  N  P  R  S  G  L  P  D  H  K  A  F  W  D  Y  F  T  H  N  Q  E  G  I
 601  CACTTCCTGATGTTCCTCTTCAGTGATCGAGCTACACCAGTCGATTTCCAACATGCCGATATTTTCAGTATCAAC
      H  F  L  M  F  L  F  S  D  R  A  T  P  V  D  F  Q  H  A  D  I  F  S  I  N
 676  ACCTACAAGTTCACCAAATCAGACGGTTCATTCACATATGTGAAGATCCATCTCAAGACCAACCAAGGAGTGAAG
      T  Y  K  F  T  K  S  D  G  S  F  T  Y  V  K  I  H  L  K  T  N  Q  G  V  K
 751  AACTTCACACAAGATGAGGCTAATCAAAAGGCTGGTGTTGATCCAGACTTCCAAACCCGTAGTCTTTACGAGGAT
      N  F  T  Q  D  E  A  N  Q  K  A  G  V  D  P  D  F  Q  T  R  S  L  Y  E  D
 826  ATCGAAAATCAAAAGTACCCAACGTGGGACGTTTTTGCACAGATCATTGACCCTGTCAAGGCCGAGAATTATCAC
      I  E  N  Q  K  Y  P  T  W  D  V  F  A  Q  I  I  D  P  V  K  A  E  N  Y  H
 901  ATCAATATCTTCGACGCAACCAAGACATTCCCATTTTCCGAGTTCCCTCTTCGCAAGTTCGGTAAAATTACACTC
      I  N  I  F  D  A  T  K  T  F  P  F  S  E  F  P  L  R  K  F  G  K  I  T  L
 976  AACAGGAATGTGGATAATTTCTTCGCTGAGCAAGAGCAAAGTGCTTTTAGTCCAACAAATCTCGTTCCTGGTTGG
      N  R  N  V  D  N  F  F  A  E  Q  E  Q  S  A  F  S  P  T  N  L  V  P  G  W
1051  GCTCTAACTCCAGATCCTATCATTCAAACTCGTGCTCTTGCCTACGCAGATACTCAAAGATATCGTCTCGGAGCC
      A  L  T  P  D  P  I  I  Q  T  R  A  L  A  Y  A  D  T  Q  R  Y  R  L  G  A
1126  AACTTCGTCCAATTACCTGTCAATGCTCCATACAAAAAGCCCTTTACTCCCCTCATTAGGGATGGAGCTGCAACT
      N  F  V  Q  L  P  V  N  A  P  Y  K  K  P  F  T  P  L  I  R  D  G  A  A  T
1201  GTCAACGGCAACTTAGGTGGTACCCCAAATTACTTCCCATCATCTTTTTACAATGTTGGAGCAGCGACACAATAT
      V  N  G  N  L  G  G  T  P  N  Y  F  P  S  S  F  Y  N  V  G  A  A  T  Q  Y
1276  GCACAACCTGACGAAGAACAATTCCAAGGAACAGTTGTCAACTTTGAGAGTGAAGTCGTCGATGCAGACTACGTG
      A  Q  P  D  E  E  Q  F  Q  G  T  V  V  N  F  E  S  E  V  V  D  A  D  Y  V
1351  CAGCCAAGAATCTTCTGGGAAAAGACACTAGCTGAAGAGCCAGGTCAACAGGATAATCTTATCAGCAATGTTGCG
      Q  P  R  I  F  W  E  K  T  L  A  E  E  P  G  Q  Q  D  N  L  I  S  N  V  A
1426  GGTCATCTAAGCGCAGTCACTGGAGATAAAGGACTTGGAAGTTCGACAAGCGGTCTATGCAATGTTCGGTAAagt
      G  H  L  S  A  V  T  G  D  K  G  L  G  S  S  T  S  G  L  C  N  V  R  *
1501  taacgccgatcttggcaagcgtatcgagacttcgacagaaactttgatcaagcaaaacgagacaggaacgttggc
1576  caaaaatctgaacaacatcaagatcagcaaacaaaatggagtgaagaacgggatactaaatggcaacagccataa
1651  cagtggtatgttcgctcacaagaattggaactggagatatctgttttgttgtatttgtaatgtattgcgggaagt
1726  tggaatgagtcaagtccgttttctgaataaaacggtgtatgtagtgataagtgcggggatgtattttcctgaatc
1801  gtgtgaaagagaagagatagaagaagacgcggataattttaatttattttggaaaaaaaaaaaaaaaaa
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Figure 2: Nucleotide sequence of the *B. cinerea catA* cDNA and the deduced amino acid sequence of CAT-A. A possible polyadenylation recognition site and the polyA$^+$-tail are underlined. The nucleotide sequence data will appear in the EMBL and Genbank databases under the accession number Z54346.

Sequence homologies of CAT-A to other fungal catalases. The deduced CAT-A protein sequence was compared to catalases of *Hansenula polymorpha* (Hp-cat), *Candida tropicalis* (Ct-cat), *Saccharomyces cerevisiae* (Sc-cta1 and Sc-ctt1) and *Aspergillus niger* (An-catR). Fig. 3 shows the computer alignment of all fungal catalase sequences known to date.

Three amino acids suggested to participate directly in catalysis (Fita and Rossmann, 1985) and conserved in all known mammalian, plant and fungal catalases, are histidine (H), serine (S) and asparagine (N) (shaded in Fig. 3). This catalytic triad is represented in the motif H-X_{38}-S-X_{33-34}-N, which is also found in CAT-A of *B. cinerea*. The distance between S and N is, however, only 27 amino acids.

A second set of seven conserved amino acids (marked by asterisks in Fig. 3) are involved in the interaction with the prosthetic haem group (Murthy *et al.*, 1981). These amino acids are all, with the exception of valine at position 104 in the Sc-cta1 sequence (Fig. 3), conserved in fungal catalases, including CAT-A of *B. cinerea*.

```
          1                                                          60
Bc-catA   ---------- ----MAQTNG VLQEPAITTM NGAPVL-KPA STQRIGNQLR ----------
 Hp-cat   ---------- ---------- MSNP.VF.TS Q.C..S-D.F T....PLDST GYKY------
 Ct-cat   ---------- ---------- --MA.TF.NS ..Q.IP-E.F A...V.QH-- ----------
Sc-cta1   -----MSKLG QEKNEVNYSD VREDRVV.NS T.N.IN-E.F V.....EH-- ----------
Sc-ctt1   ------------MNVFGKKE EK..KVYSLQ ..F.YSHH.Y AS.------- ---Y------
An-catR   MRHFWLLPAV AGIAGAQCPY LSG.MSF.QE QDNAGDTIEV TE.P.D.T.Y VNDTGSYMTT

          61                                                  *      120
Bc-catA   ---------- -------ATL LLQDINLLEL IQHITHERIP ERVVHARGTS AHGYFEVTDD
 Hp-cat   ---------- ---APPIGP. ....FK.IDT LS.FDR.... ....H.K.AG .Y.V......
 Ct-cat   ---------- -------GP. ....F..IDS LA.FDR.... ....H.K.SG .Y.V......
Sc-cta1   ---------- -------GP. ....Y..IDS LA.FNR.N.. Q.NPH.H.SG .F........
Sc-ctt1   ---------- ---SRPDGPI ....FH...N .ASFDR..V. ....H.K.GG CRLE..L..S
An-catR   DFGTPISDQT SLKAGPRGPT ..E.FIFRQK L.RFD...V. ....H...AG .Y.T.KSYA.

          121                  *                                   180
Bc-catA   ISDVTSAAFL NRVGKQTDIF CRFSTVAGRA ESAETVRDTR GFAFKMFTEE GNLDWLFLST
 Hp-cat   .T..C..K.. DT...K.R.. T..S..G.EK G..D.A..P. ...T.FY..D ....LVYNN.
 Ct-cat   .T..CA.K.. DT...K.R.. T..S..G.EL G..D.A..P. ...T.FY... ....LVYNN.
Sc-cta1   .T.ICGS.MF SKI..R.KCL T..S..G.DK G..D....P. ...T.FY... .....VYNN.
Sc-ctt1   L..I.Y..PY QN..YKCPGL V..S..G.ES GTPD.A..P. .VS..FY..W ..H..V.NN.
An-catR   W.N..A.D.. SAND.E.PM. ...S..V.FR G.VD.A..VH .H.CRFY.D. ..Y.IVGINF

            *        *                                              240
Bc-catA   PVFPIRDGAK FPSFTHATKK NPRSGL---P DHKAF-WDYF T--HNQEGIH FLMFLFSDRA
 Hp-cat   .I.F...PI. ..H.I.TQ.R ..ATN.---K .PNM.-...L .A--.D.SL. QV.Y...N.G
 Ct-cat   ...F...PS. ..H.I.TQ.R ..ETH.---K .ANM.-...L .S--.E.SV. QV.V.....G
Sc-cta1   ...F...PS. ..H.I.TQ.R ..QTN.---R .ADM.-..FL .TPE..VA.. QV.I.....G
Sc-ctt1   ...FL..AI. ..V.I.SQ.R D.Q.H.NQFQ .TTIY-...L .L--.P.S.. QITYM.G..G
An-catR   AP.F.Q.AIQ ..DLV..I.P M.NNEIPQAA TAHTSA..FF S--QQSTAL. SALW.M.GNG

          241                                                        300
Bc-catA   TPVDFQHADI FSINTYKFTK SDGSFTYVKI HLKTNQGVKN FTQDEANQKA GVDPDFQTRS
 Hp-cat   ..ASYRTMNG Y.GH...WYN SK.EWV..QV .FIA....H. LLDE..GRL. .E...HS..D
 Ct-cat   ..ASYREMNG Y.GH...WSN NK.EWF..QV .FISD..I.T L.NE..GSL. .SN..YAQED
Sc-cta1   ..ANYRSMHG Y.GH...WSN KN.DWH..QV .I..D..I.. L.IE..TKI. .SN..YCQQD
Sc-ctt1   ..ASWASMNA Y.GHSFIMVN KE.KD...QF .VLSDT.FET L.G.K.AELS .SH..YNQAK
An-catR   I.RS.R.MNG YGVHSFR.VA AN.TSKV.RT PW.SQ...AS LVW.E.QAA. .KNS.YHRQD

          301                                                        360
Bc-catA   LYEDIENQKY PTWDVFAQII DPVKAENYHI NIFDATKTFP FSEFPLRKFG KITLNRNVDN
 Hp-cat   .W.A..KGD. .S.ECYI.TM TLEQSKKLPF SV..L..VW. HKD....H.. RF...E.PK.
 Ct-cat   .FKN.AAGN. .S.TCYI.TM TEAQ.KEAEF SV..L..VW. HGKY.M.R.. .F...E.PK.
Sc-cta1   .F.A.Q.GN. .S.T.YI.TM TERD.KKLPF SV..L..VW. QGQ....RV. ..V..E.PL.
Sc-ctt1   .FTQLQ.GEK .KFNCYV.TM T.EQ.TKFRY SVN.L..IW. HK..... T...TE....
An-catR   ..NAMP.GH. .KYELQ...M .EADMLRFGF DLL.P..LV. EEVV.YTPL. MME..A.PT.
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```
          361          *                          *   *                          420
Bc-catA   FFAEQEQSAF SPTNL-VPGW ALTPDPIIQT RALAYADTQR YRL-GANFVQ LPVN------
 Hp-cat   YY..T..I.. ..SHT-...M EPSN..VL.S .LFS.P..H. H..-.P.YH. I..-------
 Ct-cat   Y...V..A.. ..AHT-..HM EPSA..VL.S .LFS....H. H..-.T.YT. I.-------
Sc-cta1   ...QV..A.. A.STT-..YQ EASA..VL.A .LFS...AH. ...-.P..H. I.-------
Sc-ctt1   Y.Q.I..V.. ....TCI..I KPSN.SVL.A .LFS.P.... H..-...YQ. ....RPRNLG
An-catR   Y...V..AG. Q.GHV-...I DF.D..LL.G .LFS.L...L T.HG.P..E. I...RPRKPV

          421                                                          480
Bc-catA   ---------- ----APYK-K PFTPLIRDGA ATVNGNLGGT PNYF---PSS ----------
 Hp-cat   ---------- ----C.L.SG S.N.IN...P MC.D..... ...ANAYNCP ----------
 Ct-cat   ---------- ----C.VTGA V.N.HM.... MN......NH ...L-ASDKP ----------
Sc-cta1   ---------- ----C..AS. F.N.A....P MN....F.SE .T.L-ANDK. ----------
Sc-ctt1   CPYSKGDSQY TAEQC.F.AV N.Q---...P MSYY-.F.PE ...ISSL.NQ TLKFKNEVND
An-catR   HNNNRDG--F GQQQI.TNNW AY..NSMSNG YPMQA.QTQG HGF.TA---- ----------

          481                                                          540
Bc-catA   ---------- FYNVGAATQY AQPDEEQFQG TVV-----NF ES--EVVDAD YVQPRIFWEK
 Hp-cat   ---------- IQYAVSPKAS GNKPDEKYT. E..-----PY HW--.HT.Y. .F..KM..-.
 Ct-cat   ---------- VEF---KQFS L.E.Q.VWH. AAT-----P. HW--KATP.. FK.ATEL.-.
Sc-cta1   ---------- YTY-IQQDRP I.QHQ.VWN. PAI-----PY HWATSPG.V. F..A.NLY-.
Sc-ctt1   EVSDKFKGIV LDE.TEVSVR K.EQDQIRNE HI.DAKINQY YYVYGISPL. FE...ALY..
An-catR   ---------- -PYRY.SGHL VRQTSPT.ND ---------- ---------H WS..AM..NS

          541                                                          600
Bc-catA   TLAEEPGQQD NLISNVAGHL SAV-TGDKG- -----LGSST SGLCNVR*-- ----------
 Hp-cat   V.GRT..E.E S.VK...N.V ..A-DEFIQD RVYEYFSKAE PIIGDLIRKK VQELKRKASS
 Ct-cat   V.KKY.N..E H.AH...V.A ..A-DAPIQD RVIAYFTKVH PD.GDLIKKE ILELSPRK*-
Sc-cta1   V.GKQ....K ..AY.IGI.V EGA-CPQIQQ RVYDMFARVD K..SEAIKK- VAEAKHASEL
Sc-ctt1   VYND--E.KK LFVH..VC.A CKIKDPKVKK RVTQYFGLLN ED.GK.IAEG LGVPWEPVDL
An-catR   LI---.AE.Q MVVNAIVFEN .K.NSPHVRK NVVNQLNMVN NN.AVRVARG LGLDEPSPNP

          601                                                          660
 Hp-cat   PSKI*----- ---------- ---------- ---------- ---------- ----------
Sc-cta1   SSNSKF*--- ---------- ---------- ---------- ---------- ----------
Sc-ctt1   EGYAKTWSIA SAN*------ ---------- ---------- ---------- ----------
An-catR   TYYTSNKTSN VGTFGKPLLS IEGLQVGFLA SNSHPESIKQ GQAMAAQFSA AGVDLNIVTE

          661
An-catR   AYADGVNTTY ALSDAIDFDA LIIADGVQSL FASPALANQM NSTATSTLYP PARPFQILVD
          SFRYGKPVAA VGSGSVALKN AGIDSSRSGV YTGSSETTEK IAKEVLEGLY TFRFVDRFAL
          DE* 783
```

Figure 3: Multiple alignment of catalase sequences of *B. cinerea* (Bc-catA), *Hansenula polymorpha* (Hp-cat; Didion and Roggenkamp, 1992), *Candida tropicalis* (Ct-cat; Murray and Rachubinski, 1987) *Saccharomyces cerevisiae* (Sc-cta1; Cohen *et al.*, 1988 and Sc-ctt1; Hartig and Ruis, 1986) and *Aspergillus niger* (An-catR; Fowler *et al.*, 1993). Amino acids identical to the CAT-A sequence of *B. cinerea* are indicated by dots. Gaps introduced for optimal alignment are indicated by a dash (-). Shaded amino acids are part of the catalatic triad and asterisks above the sequences indicate conserved amino acid residues involved in haem binding.

The overall amino acid sequence of *B. cinerea* CAT-A is similar to other fungal catalase sequences. It is also obvious that N- and C-terminal regions are very heterogeneous in sequence as well as in length, a feature also found in alignments of mammalian or plant catalases (Okada *et al.*, 1987; Suzuki *et al.*, 1994). Homologies between the different fungal catalases were calculated and are presented as percentages similarity and identity in Table 1. Highest similarity and identity scores are found between the sequences Hp-cat, Ct-cat and Sc-cta1, which are identified as peroxisomal catalases (for references, see legend Fig. 3). Scores between Hp-cat, Ct-cat or Sc-cta1 and the cytosolic catalase Sc-ctt1 (Table 1) are lower, suggesting that peroxisomal catalases are more homologous to

Table 1: Similarity and identity scores between catalases of *B. cinerea* (Bc-catA), *Hansenula polymorpha* (Hp-cat; Didion and Roggenkamp, 1992), *Candida tropicalis* (Ct-cat; Murray and Rachubinski, 1987) *Saccharomyces cerevisiae* (Sc-cta1; Cohen *et al.*, 1988 and Sc-ctt1; Hartig and Ruis, 1986) and *Aspergillus niger* (An-catR; Fowler *et al.*, 1993). Numbers refer to percentages similarity (at the right site of the diagonal) or identity (at the left site of the diagonal) between sequences.

%	Bc-catA	Hp-cat	Ct-cat	Sc-cta1	Sc-ctt1	An-catR
Bc-catA	-	65.3	63.6	64.8	59.0	56.2
Hp-cat	47.0	-	78.4	75.1	63.3	56.6
Ct-cat	45.8	69.5	-	78.8	66.5	56.0
Sc-cta1	44.4	59.1	65.8	-	63.2	53.9
Sc-ctt1	39.5	45.7	49.6	42.8	-	51.4
An-catR	34.2	38.4	36.7	34.4	31.4	-

each other than to cytosolic catalase. Striking structural differences between peroxisomal and cytosolic catalases are observed in the alignment as well: the cytosolic Sc-ctt1 sequence contains three extra blocks of amino acids at sites where gaps (415-434, 470-490 and 514-518, Fig. 3) were introduced for optimal alignment in amino acid sequences Hp-cat, Ct-cat and Sc-cta1. Regarding the CAT-A sequence of *B. cinerea*, higher percentages of similarity and identity are observed with the peroxisomal catalases Hp-cat, Ct-cat and Sc-cta1 than with the cytosolic catalase Sc-ctt1. In addition, the sequence of CAT-A appears to be colinear to the peroxisomal catalases and lacks the three blocks present in the cytosolic catalase.

Since the An-catR sequence is about 170-250 amino acids longer, similarity/identity scores were lower when this sequence was used to calculate scores. In addition, the An-catR sequence was not colinear with any of the other sequences. Grouping of this protein with peroxisomal or cytosolic catalases is, therefore, less clear. Unfortunately, the cellular localization of CAT-R has not yet been described.

***catA* is encoded by a single-copy gene.** A Southern blot containing *B. cinerea* DNA digested with *Eco*RI, *Eco*RV, *Hin*dIII, *Pst*I and *Sal*I was hybridized with a probe derived from the coding region of *catA*. In all DNA digests only one DNA fragment showed strong hybridization (Fig. 4) indicating the presence of a single-copy gene. In the *Hin*dIII and, to

lesser extent, the *Eco*RI digest, however, a weak signal of a second hybridizing fragment was present. This weak hybridization signal was not reproducible.

***In vitro* expression of *catA*.** To determine whether the *catA* gene is transcriptionally activated during standard *in vitro* growth conditions, expression of the gene was analyzed by northern blot hybridization. Total RNA was isolated from mycelium sampled one to four days after inoculation in standard liquid medium. After hybridization with a probe derived from the *catA* cDNA clone, a single transcript of approximately 1900 nt was detected (Fig. 5), which is in close agreement with the length calculated from the cDNA sequence. Hybridization of the same blot with a probe derived from the constitutively expressed ß-tubulin gene (*tubA*) of *B. cinerea* enabled standardization of *catA* mRNA levels to a constant amount of fungal RNA. Based on the signal intensities of the *catA* and *tubA* hybridization (Fig. 5), the expression of the *catA* gene is high in young, fastly growing mycelium (day 1) and decreases in older mycelium (day4). Since the *in vitro* culture was not specifically induced for catalase production, the observed *catA* expression might reflect a basic expression level in growing mycelium.

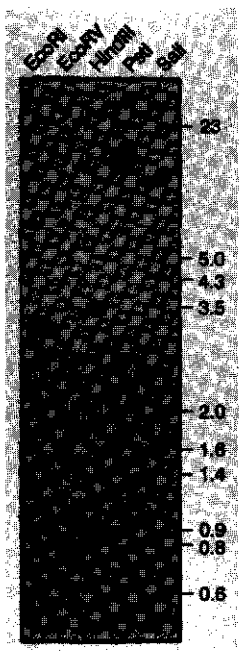

Figure 4: Autoradiograph of a Southern blot containing genomic DNA of *B. cinerea* digested with different restriction enzymes as indicated. The blot was hybridized with the *Eco*RV fragment of subclone CT3.2. Molecular-size markers are indicated in kb.

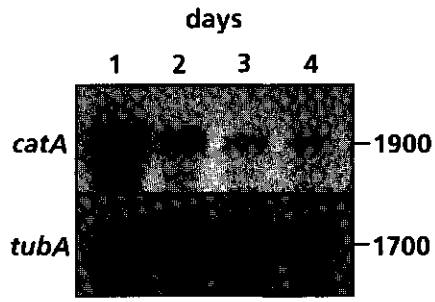

Figure 5: Autoradiograph of northern blot containing total RNA isolated from one to four-days-old *B. cinerea* mycelium grown in standard medium. The blot was hybridized with probes derived from the *B. cinerea* catalase encoding cDNA (*catA*) and the ß-tubulin gene (*tubA*). Sizes of the mRNAs (in nt) are shown in the right margin.

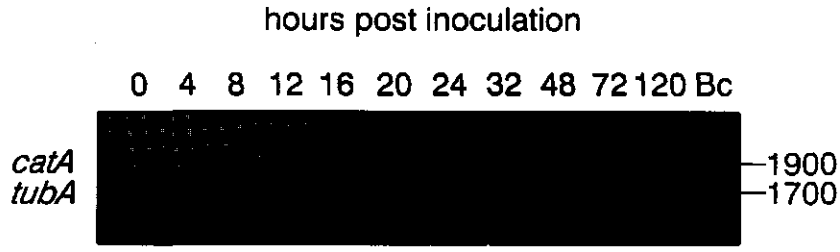

Figure 6: Autoradiograph of northern blot containing total RNA isolated from infected tomato leaves at different timepoints post inoculation (numbers refer to hours post inoculation) and from one-day-old *B. cinerea* mycelium grown in standard medium (Bc). The blot was co-hybridized with probes derived from the *B. cinerea* catalase encoding cDNA (*catA*) and the ß-tubulin gene (*tubA*). Sizes of the mRNAs (in nt) are shown in the right margin.

***In planta* expression of *catA*.** To analyse whether *catA* is expressed during infection of host tissue, tomato leaves were inoculated with conidia of *B. cinerea* resuspended in a buffer containing glucose as substrate for H_2O_2 generating glucose oxidase (Edlich *et al.*, 1987).

After inoculation of tomato leaves, the first visible symptoms were small, water-soaked lesions at 16 hours post inoculation (h.p.i.). These lesions became necrotic at 20 h.p.i. and their size remained unchanged until 72 h.p.i.. No new lesions appeared during that time, indicating that the infection occurred in a synchronized manner. After 72 h.p.i., a subset of lesions developed into spreading lesions. At 120 h.p.i., the fungus started to sporulate from these expanded, necrotic lesions. Based on these symptom developments leaves were harvested at 4, 8, 12, 16 and 20 h.p.i., when penetration of host tissue occurs and the production of H_2O_2 and catalase is expected. Sampling continued until 120 h.p.i. to cover the whole infection process.

The *in planta* expression of *catA* was studied by northern blot analysis of total RNA isolated from infected tomato leaves (Fig. 6). The blot was co-hybridized with a probe of the constitutively expressed ß-tubulin (*tubA*) gene of *B. cinerea* to demonstrate the ability to detect fungal mRNAs in the total population of plant and fungal RNAs, and the increase in fungal biomass during the infection. This probe hybridized specifically with the fungal ß-tubulin mRNA under the stringent conditions used. Under less stringent conditions a plant ß-tubulin mRNA was detected, however, its size is smaller (results not shown).

Before the occurrence of spreading lesions the *tubA* signal is weak (Fig. 6), corresponding with the low amount of fungal biomass at these stages of the infection. However, when lesions increased in size (beyond 72 h.p.i.) a stronger hybridization signal of *tubA* is visible at 120 h.p.i. correlating well with the expanded fungal biomass.

Detection of the *tubA* messenger *in planta* demonstrated that the low amount of fungal biomass does not hamper detection of a constitutively expressed gene like *tubA*. However, no transcript of the *catA* gene was detected in this time course (Fig. 6). Even after prolonged exposure of the blot for 6 days, no *catA* signal was visible.

Since expression of both *catA* and *tubA* in *in vitro* grown mycelium are more or less comparable (lane Bc, Fig. 6), the absence of a *catA* signal in the *in planta* time course indicates that its expression during infection is significantly lower than the expression of the constitutively expressed gene *tubA*.

Discussion

From a cDNA library of *B. cinerea* a catalase encoding clone, denoted *catA*, was isolated. Identification was based on overall homology to fungal catalases and the presence of conserved amino acids involved in the catalytic centre and haem binding. The open reading frame encoded a protein, CAT-A, of 480 amino acids in length representing the smallest fungal catalase to date. Southern blot analysis showed that the *B. cinerea* genome contains a single copy of the *catA* gene. During growth of *B. cinerea* in a standard liquid culture, expression of *catA* was high in young, rapidly growing mycelium and decreased in older mycelium. This *in vitro* expression could reflect a basic expression level in growing mycelium, since the culture was not specifically induced for catalase expression.

Edlich *et al.* (1987, 1989) demonstrated a correlation between pathogenicity of some isolates of *B. cinerea* on broad bean and production of H_2O_2 suggested to be released by fungal glucose or xylose oxidase activity. H_2O_2 can form other oxygen radicals ($O_2^{\cdot}$, $OH^{\cdot}$) which are also toxic and weaken plant tissue, thereby possibly facilitating the infection by *B. cinerea*. However, the released H_2O_2 could also be toxic to the fungus itself and it was hypothesized by the authors that the fungus might protect itself by producing elevated levels of catalase to detoxify H_2O_2.

The expression of *catA* was studied during infection of tomato leaves inoculated with conidia of *B. cinerea* resuspended in a buffer supplemented with glucose. Using this inoculation method Edlich *et al.* (1987, 1989) had demonstrated the production of H_2O_2 during infection and its effect on pathogenicity. Northern blot analysis of a time course containing RNA from *B. cinerea*-infected tomato leaves, showed no detectable *catA* expression, whereas symptom development on the tomato leaves demonstrated a successful infection. In addition, hybridization signals of the constitutively expressed ß-tubulin gene (*tubA*) showed that it was possible to detect fungal mRNAs in the total population of plant and fungal RNAs. Expression levels of *catA* and *tubA* in *in vitro* grown, non-induced mycelium were comparable (lane Bc, Fig. 6). However, during infection of host tissue *catA* expression is not detected and appears to be at a much lower level than *tubA* expression. Apparently the release of H_2O_2 by fungal glucose oxidase activity during the infection as demonstrated by Edlich *et al.* (1987, 1989), is not an inducing factor for

catA expression. It is unknown whether the exogenous H_2O_2 concentration is too low to induce *catA* expression, or whether H_2O_2 is detoxified by other catalase activities. For example, plant catalases might be induced earlier to break down exogenous H_2O_2, or catalases other than CAT-A are produced in *B. cinerea*. Presence of several, differentially regulated catalase encoding genes has been demonstrated in *S. cerevisiae* and *Neurospora crassa*. In *S. cerevisiae*, the *cta1* gene is known to be induced during growth on fatty acids and its product is mainly localized in peroxisomes where fatty acid oxidation occurs (Skoneczny *et al.*, 1988). The *ctt1* gene, however, is a soluble cytoplasmic catalase which is synthesized in response to a variety of metabolic and environmental stresses (Traczyk *et al.*, 1985). In *Neurospora crassa* three catalase loci have been identified. Activities of the three encoded enzymes varied in response to superoxide-mediated stress, heat shock and development (Chary and Natvig, 1989). However, no sequence data of these catalases are available.

Homology scores between fungal catalase sequences (Table 1) and the introduction of gaps in the sequence alignment (Fig. 3), allowed grouping of fungal catalases with respect to their cellular location. Peroxisomal catalases of *Hansenula polymorpha*, *Candida tropicalis* and CTA1 of *Saccharomyces cerevisiae* could be grouped and distinguished from the cytosolic CTT1 catalase. The CAT-A protein sequence of *B. cinerea* showed most structural homology with the peroxisomal catalases, including the gaps introduced for optimal sequence alignment suggesting that CAT-A is a peroxisomal catalase. A second, possibly cytosolic catalase might be expressed during infection of host tissue by *B. cinerea*. Low sequence identity, as observed between CTA1 and CTT1 of *S. cerevisiae* (42.8%, Table 1) makes cross-hybridization unlikely and, therefore, another catalase encoding gene in *B. cinerea* is not reproducibly detected in the hybridization experiments.

Future experiments are focussed on the induction of CAT-A of *B. cinerea* by H_2O_2 and the possible expression of tomato catalases during infection.

Acknowledgments

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CHAPTER 7

General discussion

Botrytis cinerea Pers.:Fr is a pathogen which exhibits great morphological, physiological and genetic variability. Its ability to employ various infection mechanisms may contribute to the success of this fungus as a pathogen. Due to a widespread distribution and destructive effects on economically important crops *B. cinerea* has been subject to many investigations in different disciplines during the last decades (Chapter 1). Nevertheless, molecular techniques were not yet applied to study this fungus at the onset of this PhD research.

Genetic variability

Genetic variation among different strains of a given fungus can be studied directly at the DNA level using RAPD analysis. This technique is a powerful method to detect differences among strains of which no genomic sequence information is available. The use of a series of different primers of random sequence enables the amplification of DNA fragments dispersed throughout the genome, including regions of non-coding and repeated sequences.

Chapter 2 describes the application of RAPD analysis to study DNA polymorphisms among 10 strains of *B. cinerea*, i.e. 2 homokaryotic strains of known mating type and 8 strains collected from different hosts (gerbera, tomato or rose) in The Netherlands. All ten *B. cinerea* strains analyzed were genetically more or less distinct. From this analysis it was concluded that strains isolated from the same host are genetically not more alike than those originating from different hosts. Analysis of more strains from different crops (Rudakoff and Van der Vlugt-Bergmans, unpublished) confirmed that host specialization does not seem to occur in *B. cinerea*.

Two other studies provided evidence for variation at the DNA level among strains of *B. cinerea*. Firstly, pulsed field gel electrophoresis revealed distinct chromosomal banding patterns for five strains of which three were isolated from the same host species and in the same year (Van Kan *et al.*, 1993). Secondly, a retrotransposon-like element was cloned from *B. cinerea* (Diolez *et al.*, 1995) which was present in varying copy numbers in different isolates of *B. cinerea*. It remains to be studied whether this element is able to transpose actively, thereby contributing to genetic variability.

Chapter 2 also reports the use of RAPD analysis to study the segregation of polymorphic markers in the progeny collected from ordered ascospore sets. Analyzing ordered octads rather than randomly collected progeny of squashed apothecia facilitates detailed analysis of single meiotic events and segregation ratios in only a limited number of progeny. The inheritance of RAPD markers was studied in the progeny of cross SAS405 x Bc7. A segregation ratio of 1:1 was usually observed for DNA polymorphisms; a normal Mendelian segregation of a genetic marker in a haploid organism. However, a 1:0 segregation of RAPD markers, the disappearance of existing and appearance of new RAPD markers in the progeny was also observed, which could be attributed to heterokaryosis, presumed polyploidy of the fungus or limitations of the RAPD technique itself.

Recent work of Büttner *et al.* (1994) provided evidence that oligoploidy or aneuploidy might be a common feature of *B. cinerea* strains. The commonly used Italian strains SAS56 and SAS405 were assessed to be triploid and diploid, respectively. In retrospect, the segregation ratio of 1:0 of a RAPD marker inherited from the diploid SAS405 parent could be explained by parental homozygosity for this marker. The ploidy level of parent Bc7 was not determined. The question how strains of presumably different ploidy level (e.g. SAS56 x SAS405) are capable of mating remains unresolved.

Heterokaryosis as a cause for the high variability in this fungus, was first demonstrated by the work of Akutsu *et al.* (1988). Nuclei carrying a fungicide resistance marker were shown to be transmitted from one thallus to the second by anastomosis. Unfortunately, Akutsu *et al.* did not continue their studies on the segregation of the fungicide markers by monoconidial transfers. It is yet unknown whether multinucleate macroconidia originating from heterokaryotic mycelium can maintenain the heterokaryotic status. Macroconidia may either contain a subset of different parental nuclei, or originate from a conidium containing one parental nucleus which divides mitotically. The availability of nuclear markers (fungicide resistance markers, RAPD markers or markers in transgenic strains) allows to examine this process in detail which is essential to understand the actual contribution of heterokaryosis to natural variability.

Artefacts of the RAPD technique itself were also proposed as an explanation for the unexpected segregation of RAPD markers in the progeny of cross SAS405 x Bc7. RAPD analysis in itself is useful to gain insight in the genetic background of the organism. In additional studies, however, it should be combined with well characterized markers. As such, RAPD markers can serve as basis to design more specific RFLP (restriction fragment length polymorphism) or STS (sequence tagged site) markers. In addition, further studies on the segregation of markers and design of a genetic map for *B. cinerea* should be based on progeny of crosses between homokaryotic, haploid strains.

The work described above demonstrates the complex genetic background of *B. cinerea*. Much more research is needed to understand the mechanisms contributing to genetic variability and to study the relevance of various mechanisms in genetic variation at the population level.

Cloning of the *B. cinerea* cutinase gene

Many genes, from a wide range of organisms, have been cloned on the basis of DNA or protein homology. Since several fungal cutinases had been cloned already and showed considerable sequence homology, it was tempting to exploit this strategy to clone the *B. cinerea* cutinase gene as well. In Chapter 3 the application of heterologous hybridization screening of a genomic library and immunoscreening of a cDNA library of *B. cinerea* is described, as well as a PCR based cloning strategy using degenerated primers based on conserved amino acid sequences within the cutinases. All three approaches proved unsuccessful. The reason became obvious once the gene was finally cloned and its

structure and expression were analyzed (Chapter 4). The sequence identity at the protein level among cloned cutinases was 48-64%, whereas the identity of these sequences with that of *B. cinerea* appeared to be only 31-35%. The absence of specific, reproducible hybridization signals during heterologous screening of the genomic library of *B. cinerea* was apparently due to this low sequence homology. In addition, the identical amino acid regions in the five other cutinases were not, or only partially, present in the *B. cinerea* cutinase sequence. Therefore, the primers we designed did not match the cutinase sequence and gene specific amplification was impossible. The time point for isolation of mRNA from a cutinase induced *B. cinerea* culture and construction of the cDNA library was based on maximal enzyme activity in culture filtrate. Since a correlation between PNB hydrolysis and cutin hydrolysis was previously demonstrated in culture medium of *B. cinerea* (Salinas, 1992), mRNA was isolated at day 3-4, preceding the peak of PNB hydrolytic activity at day 5-6. When the library was hybridized with the *B. cinerea* cutinase probe (Chapter 4), no cutinase cDNA clones were detected. Cutinase mRNA expression in cultures induced with the cutin monomer appeared to occur transiently with a sharp peak on the first day post induction. The time point chosen for isolation of mRNA for the library construction was, in retrospect, incorrect.

Eventually, the biochemical approach (Chapter 4), i.e. purification of cutinase and subsequent PCR based gene cloning using primers based on determined amino acid sequences, was successful. The purified 18 kD enzyme hydrolyzed cutin labelled with the chromogenic group Remazol Brilliant Blue (RBB) and the encoding gene (*cutA*) showed clear sequence homology to other cutinases. During the purification of cutinase a second fraction resulted, which contained two proteins of 80 and 11 kD and showed PNB hydrolytic activity. In view of the possibility that fungi produce a second, unrelated cutinase (Sweigard *et al.*, 1992b; Yao and Köller, 1995) this fraction was further analyzed. Although no cutin-RBB hydrolyzing activity was detected, it was taken in consideration that an endo-cutinase activity would hydrolyse internal ester bonds, without releasing soluble RBB-coupled monomers from insoluble cutin-RBB in the supernatant. Based on the N-terminal amino acid sequence, the 80 kD protein was identified as a glucoamylase. By deduction, the 11 kD protein was assumed to be the putative esterase. Sequence analysis of the 11 kD encoding gene (*ekdA*), however, revealed no homology to serine esterases or other proteins. Its expression *in vitro* was not induced by the cutin monomer 16-hha or repressed by glucose. Besides, it was expressed late (120 h.p.i.) during the infection of tomato leaves. The lack of a signal peptide in the *ekdA* gene predicted an intracellular location for this protein, making it unlikely that it plays a direct role at the plant - fungus interface. Therefore, the subsequent work was focussed on the role of the 18 kD cutinase in the infection process by *B. cinerea*.

Enzymatic penetration of undamaged host tissue

Cutinase-mediated penetration. The mechanism by which *B. cinerea* breaches the cuticle has been debated for a long time. During the last twenty-five years, evidence pointed to an enzyme-mediated penetration in which cutinase was involved (discussed in Chapter 1). Using the GUS reporter gene under control of the *cutA* promoter, we demonstrated that the cutinase gene is expressed early during infection of host tissue (Chapter 5). Gerbera flowers and tomato fruits were used for inoculation, since much research on the role of cutinase in the penetration process by *B. cinerea* was carried out with these hosts. The way to investigate a requirement for cutinase in this process was to analyse the infection ability of cutinase A-deficient mutants. These mutants were obtained by means of cutinase gene disruption in a haploid *B. cinerea* strain (Chapter 5). Pathogenicity assays, however, demonstrated that cutinase A-deficient mutants were still able to penetrate and infected host tissue equally well as the wild type haploid strain. Microscopically, penetration of host tissue by the mutant was also indistinguishable from penetration by the wild type.

If not by the action of the 18 kD cutinase, how is *B. cinerea* able to penetrate undamaged host tissue? Enzymatic penetration could be mediated by another cutinase-like enzyme, since the production of additional cutinase activities was reported for *Magnaporthe grisea* (Sweigard *et al.* 1992b) and *Alternaria brassicicola* (Yao and Köller, 1995). Otherwise, mechanical penetration by formation of appressoria and/or another enzymatic process, involving the generation of active oxygen species, may aid the penetration.

Generation of active oxygen species. Edlich *et al.* (1989) demonstrated a correlation between the release of H_2O_2 by fungal glucose oxidase and pathogenicity of several *B. cinerea* isolates. H_2O_2 and other active oxygen species (AOS) are in principle capable of destroying cutin and membrane lipids, which would facilitate penetration by the fungus. H_2O_2 is able to diffuse across the cell membrane and exert toxic effects on the interior of the cell. Production of AOS at the host-pathogen interface might also be toxic to the fungus itself. Therefore, *B. cinerea* has to protect itself against autotoxic effects, possibly by enhanced synthesis of enzymes degrading AOS such as catalase. A catalase cDNA clone of *B. cinerea* (*catA*) was characterized and its expression analyzed during penetration and subsequent colonization of tomato leaves (Chapter 6). No *catA* messenger was detected during the infection by northern blot analysis. Detailed comparison of the *B. cinerea* catalase sequence with other fungal catalases revealed homology to peroxisomal catalases. The presumed peroxisomal location of the *catA* gene product, the absence of a hybridization signal at any time in *B. cinerea*-infected tomato leaves and the possibility that other, cytosolic, catalases may be involved, make an important role for this catalase in detoxification of H_2O_2 during the infection unlikely.

To gain insight in the function of AOS for the infection ability of *B. cinerea*, enzymes directly involved in the generation of AOS rather than enzymes involved in breakdown

should be studied. Currently, attempts to clone the glucose oxidase gene of *B. cinerea* are undertaken in the laboratory of Dr P. Tudzynski (Münster, Germany). It will be interesting to study its expression during the infection process.

Alternative roles for cutinase

Investigations on the function of *B. cinerea* cutinase were primarily focussed on its presumed role during penetration of undamaged host tissue (Chapter 4 and 5). Since no indications for a prominent role in the penetration process were obtained, it is tempting to speculate about other possible functions.

During standard inoculations of tomato leaves and subsequent analysis of *cutA* expression by northern blot analysis, a strong signal was detected late during infection (120 h.p.i., Chapter 4). Although GUS reporter gene expression under control of the *cutA* promoter has shown early gene expression, it should be noted that this was observed during dry inoculation of gerbera flowers and tomato fruits (Chapter 5). On tomato leaves glucose was present in the inoculum buffer and could have repressed cutinase expression. The late expression as shown by northern blot analysis could indicate a role for cutinase during saprophytic growth of the fungus on dead host tissue (Köller *et al.*, 1995). The fact that sporulation already occurs at this late time point could indicate that conidia contain cutinase activity, possibly in the extracellular matrix. However, in washing fluid of ungerminated spores the 18 kD cutinase was never detected (Salinas, 1992).

From *Aspergillus flavus*, a 22 kD cutinase has been purified and preliminary experiments have suggested a role for cutinase in aflatoxin production and pathogenicity of *A. flavus* on maize kernels (Guo *et al.*, 1995). Recently, a cutinase of the non-pathogenic fungus *Aspergillus oryzae* has been cloned and characterized (Ohnishi *et al.*, 1995). Initially, the enzyme was purified as a lipase produced during fermentation. However, the encoding gene appeared to be homologous to cutinases of phytopathogenic fungi. Both studies indicate that cutinase might have additional functions besides hydrolyzing cutin.

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Samenvatting

De schimmel *Botrytis cinerea* veroorzaakt ziekten in meer dan 200 plantesoorten waaronder veel economisch belangrijke gewassen zoals druif, aardbei, tomaat, gerbera en roos. Het ziektebeeld wordt vaak pas duidelijk na de oogst van het produkt. Bloemen blijken dan necrotische vlekken te vertonen die kunnen uitbreiden en leiden tot totale verrotting. Vruchten worden volledig gekoloniseerd door de schimmel, waarna uitbundige sporulatie volgt. Deze grijze sporenmassa geeft *B. cinerea* de algemene naam 'grauwe schimmel'. Bestrijding van de schimmelziekte is moeilijk omdat de schimmel uitgebreid voorkomt, zich makkelijk verspreidt via sporen die door de wind meegevoerd worden, en door de snelle resistentie-ontwikkeling tegen diverse fungiciden. Vanwege het economisch belang van deze schimmelziekte, wordt er al vanaf het begin van deze eeuw onderzoek gedaan aan *B. cinerea*, voornamelijk op het gebied van pathogenese, epidemiologie, fysiologie en biochemie. Het moleculair-biologisch onderzoek is pas de laatste vijf jaar in diverse onderzoeksgroepen op gang gekomen. Door toepassing van moleculair-biologische technieken tijdens het hier beschreven promotie-onderzoek, is inzicht verkregen in de variatie binnen de soort *B. cinerea* op DNA-niveau. Daarnaast is de interactie met de waardplant, op het moment dat de schimmel de plant binnendringt, nader onderzocht.

Verschillende isolaten van *B. cinerea* vertonen onderling vaak aanzienlijke variatie in groeikarakteristieken, pathogenese, productie van conidia, sclerotia of enzymen. Ook mono-spore cultures van een isolaat laten vaak een uitsplitsing van uiterlijke kenmerken zien. Om deze fenotypische variatie op DNA-niveau te onderzoeken is de RAPD (randomly amplified polymorphic DNA)-analyse uitermate geschikt, met name omdat er geen gedetailleerde kennis van het DNA vooraf beschikbaar hoeft te zijn. Hoofdstuk 2 beschrijft het gebruik van deze techniek om de variatie binnen een tiental verschillende isolaten en hun nakomelingen, verkregen uit kruisingen, te analyseren.

RAPD-analyse toonde aan dat de *B. cinerea* isolaten, die afkomstig waren van tomaat, gerbera of roos, onderling allemaal verschillend waren. Het was niet zo dat isolaten van roos onderling meer op elkaar leken dan isolaten afkomstig van gerbera. Deze resultaten suggereren dat er geen sprake is van aanpassing van isolaten van *B. cinerea* aan hun gastheer. RAPD-analyse van nakomelingen verkregen uit kruisingen liet zien dat niet alle RAPD-markers volgens de verwachte 1:1 ratio segregeerden, maar dat ook een 1:0 uitsplitsing voorkwam, dat RAPD-markers afwezig waren in nakomelingen of dat juist nieuwe RAPD-markers gedetecteerd werden, die vervolgens 1:1 uitsplitsten. Deze fenomenen zouden verklaard kunnen worden door het voorkomen van heterokaryose en/of polyploïdie in *B. cinerea*. Voor bewijs van deze verklaringen is echter verder onderzoek noodzakelijk. Een mogelijkheid hiertoe is de analyse van nakomelingen verkregen door kruising van homokaryotische isolaten, omdat hierbij slechts twee typen

ouderkernen betrokken zijn in de kruising. Bovendien zouden beter gedefinieerde markers zoals RFLP (restriction fragment length polymorphism)- of STS (sequence tagged sites)-markers gebruikt kunnen worden in plaats van, of in aanvulling op, RAPD-markers. Uit recent onderzoek van een andere onderzoeksgroep is gebleken dat poly- en aneuploïdie inderdaad voorkomt in *B. cinerea*. Dit betekent dat gebruik van goed gedefinieerde haploïde of diploïde isolaten vereist is voor genetische analyses.

B. cinerea is voornamelijk aanwezig op verzwakt, beschadigd of dood plantaardig materiaal, maar is ook in staat om intact planteweefsel te infecteren. Penetratie van onbeschadigd, gezond weefsel wordt verondersteld plaats te vinden met behulp van enzymen of door de vorming van appressoria, dan wel door een combinatie van beiden. Cutinase is een enzym dat al vaak onderwerp van studie is geweest en waarvoor in de literatuur duidelijk aanwijzingen bestaan dat het een belangrijke rol speelt bij de penetratie van het planteweefsel. De eerste barrière die de schimmel tegenkomt tijdens het binnendringen van planteweefsel is immers cutine. Cutinase zou in staat zijn dit cutine af te breken om zo de toegang tot het onderliggende planteweefsel te vergemakkelijken. Een ander enzymatisch proces dat het binnendringen van planteweefsel mogelijk zou maken, is de vorming van zuurstofradikalen door enzymen van de schimmel. Zuurstofradikalen zouden het planteweefsel verzwakken waardoor de schimmel makkelijker kan binnendringen. Uiteraard moet de schimmel zich wel zelf beschermen tegen deze ook voor hem toxische zuurstofradikalen.

Deze twee enzymatische processen zijn in het hier beschreven promotie-onderzoek nader onderzocht. De nadruk lag hierbij op het analyseren van de rol van cutinase tijdens de penetratie.

Om te kunnen onderzoeken hoe belangrijk cutinase is voor *B. cinerea* tijdens het binnendringen van intact planteweefsel, is het nodig om het gen dat codeert voor dit enzym te isoleren. In hoofdstuk 3 is beschreven hoe met behulp van moleculair-biologische technieken getracht is het cutinasegen van *B. cinerea* te isoleren. Omdat er cutinasegenen beschikbaar zijn van andere schimmels zijn deze genen als probe gebruikt om een genomische bank van *B. cinerea* te screenen. Dit lukte echter niet doordat, zoals later bleek, er te grote verschillen bestaan tussen deze cutinase sequenties en die van *B. cinerea*. Door deze verschillen bleken ook primers, ontworpen op basis van geconserveerde cutinase sequenties, niet geschikt om de *B. cinerea* cutinase sequentie via PCR te amplificeren. Behalve andere cutinasegenen, was er ook een monoklonaal antilichaam tegen het cutinase van *B. cinerea* beschikbaar. Dit antilichaam is gebruikt om een expressiebank van *B. cinerea* te screenen. Ook deze methode leidde niet tot het isoleren van het gen. Zoals later uit northern blot analyses bleek, kwam het cutinase gen veel eerder tot expressie dan verwacht werd op basis van de gemeten enzymactiviteit in het cultuurfiltraat. Hierdoor bleek achteraf het tijdstip van RNA-isolatie voor het maken van de expressiebank niet goed gekozen te zijn.

Een biochemische aanpak (hoofdstuk 4) leidde uiteindelijk tot het kloneren van het cutinase gen. Cutinase werd gezuiverd uit cultuurfiltraat van een *in vitro* cultuur van *B. cinerea*. De gezuiverde fractie bevatte duidelijk cutine hydrolyserende activiteit die gemeten werd met behulp van cutine gelabeld met een kleur-groep (Remazol Brilliant Blue R), in plaats van het veel gebruikte radio-aktief gelabelde cutine. De aminozuur sequentie van het enzym, dat een molekuulgewicht van 18 kD had, werd gedeeltelijk bepaald en met behulp van deze sequenties konden primers gesynthetiseerd worden om uiteindelijk de cutinase coderende sequentie via PCR te amplificeren. Screening van een genomische bank leidde tot de isolatie van het *B. cinerea* cutinase gen (*cutA*). Sequentiehomologie met andere cutinases was significant, hoewel veel lager dan de homologie die de andere cutinase sequenties onderling vertoonden. Geconserveerde aminozuren die het actieve centrum van het enzym vormen, werden ook in de *B. cinerea* cutinasesequentie geïdentificeerd.

Tijdens de zuivering van cutinase uit cultuurfiltraat bleek er een tweede enzym aanwezig te zijn dat, net als cutinase, een esterase-activiteit bezat. Hoewel de gezuiverde fractie met esterase-activiteit geen cutine hydrolyserende activiteit bezat, werd er rekening gehouden met een endo-cutinase activiteit, die door de onoplosbaarheid van cutine brokstukken wellicht slecht meetbaar was. De fractie bevatte twee eiwitten, een van 80 kD en een van 11 kD. Bepaling van de N-terminale aminozuursequentie identificeerde het 80 kD eiwit als glucoamylase. Dit enzym mist esterase-activiteit en derhalve werd aan het 11 kD eiwit de esterase-activiteit toegeschreven. Het coderende gen (*ekdA*) werd op dezelfde wijze als beschreven voor *cutA*, geïsoleerd. Sequentieanalyse en vergelijking met sequenties in databanken kon echter de biologische functie van het eiwit niet achterhalen. Bovendien deed de afwezigheid van een signaalpeptide vermoeden dat het 11 kD eiwit een intracellulaire oorsprong heeft. Dit maakt het onwaarschijnlijk dat dit enzym een belangrijke rol speelt op het raakvlak tussen de plant en de schimmel.

Onderzoek naar de genexpressie en het belang van cutinase tijdens de penetratie van planteweefsel door *B. cinerea* wordt beschreven in Hoofdstuk 5. Expressie van het cutinasegen werd onderzocht door gebruik te maken van een reportergen dat achter de regulerende sequenties van de cutinasepromoter werd gezet. Wanneer deze promoter geactiveerd wordt, komt het reportergen, dat codeert voor het enzym glucuronidase, tot expressie. Glucuronidase zet een substraat om waardoor de schimmelstructuur waarin het enzym voorkomt, blauw kleurt. Aan de kleur van de schimmel, die microscopisch bekeken werd, kon dus afgeleid worden of het cutinasegen actief was. Transformanten van *B. cinerea* die dit promoter-reporter construct bezaten, bleken *in vitro* het glucuronidase en cutinase onder dezelfde omstandigheden tot expressie te brengen. Geïnoculeerd op gerberabloemen en tomaten bleken kiemende sporen en kiembuizen die het planteweefsel penetreerden sterk blauw te kleuren. Dit toont aan dat het cutinasegen tot expressie komt wanneer *B. cinerea* het planteweefsel binnendringt.

Of cutinase ook werkelijk nodig is voor succesvolle penetratie, werd onderzocht met transformanten waarin het cutinasegen uitgeschakeld was. Deze uitschakeling werd bewerkstelligd door 'gendisruptie', een techniek die niet eerder voor *B. cinerea* beschreven was. Omdat cutinase in een enkel kopie in het genoom van *B. cinerea* voorkomt en omdat er inmiddels haploïde isolaten beschikbaar waren, leek gendisruptie haalbaar. Analyse van 80 transformanten leverde drie transformanten op waarin het cutinase gen volledig uitgeschakeld was. Na inoculatie van sporen van deze transformanten op gerberabloemen en tomaten, bleek dat deze cutinase A deficiënte transformanten even goed in staat waren het planteweefsel te penetreren als het wildtype isolaat met een intact cutinasegen. Ook op microscopisch niveau kon geen onderscheid gemaakt worden in het penetratieproces van de transformanten en van het wildtype. Hieruit werd geconcludeerd dat dit cutinase niet essentieel is voor een succesvolle penetratie van het planteweefsel. Het is echter niet uitgesloten dat een ander cutinase geproduceerd wordt, of dat penetratie volgens een ander enzymatisch en/of mechanisch proces verloopt.

Een dergelijk ander enzymatisch proces is de mogelijke productie van H_2O_2 door glucose oxidase van de schimmel. In de literatuur was beschreven dat infectie van boon door *B. cinerea* gecorreleerd is met glucose oxidase-activiteit. Verondersteld werd dat het gevormde H_2O_2, en daarvan afgeleide zuurstofradikalen, het planteweefsel verzwakken waardoor de schimmel makkelijker toegang krijgt. Echter, H_2O_2 is niet alleen toxisch voor de plant maar ook voor de schimmel. Door het kloneren van een *B. cinerea* gen coderend voor catalase A (*catA*) dat H_2O_2 omzet in H_2O and O_2, kon onderzocht worden of *catA* tijdens de penetratie tot expressie komt om de schimmel bescherming te bieden tegen mogelijke toxische effecten van H_2O_2 (Hoofdstuk 6). Het catalase A-gen bleek *in vitro* duidelijk tot expressie te komen, maar werd tijdens de infectie van tomatebladeren niet geïnduceerd. Sequentievergelijking met andere schimmel catalases liet zien dat catalase A van *B. cinerea* meer verwant is met peroxisomale catalases dan met in het cytoplasma vorkomende catalases. Het is dus mogelijk dat een ander catalase tot expressie komt tijdens de infectie. Nader onderzoek, met name ook naar de H_2O_2 genererende enzymen zoals glucose oxidase, is hiervoor echter nodig.

Curriculum vitae

Cécile Johanna Beatrix Bergmans werd op 6 juli 1966 te Venlo-Blerick geboren. Na het behalen van het Gymnasium-ß diploma aan het Collegium Marianum te Venlo, begon zij in 1984 aan de studie Planteziektenkunde aan de Landbouwuniversiteit te Wageningen. De studie werd afgerond met afstudeeropdrachten bij de vakgroepen Erfelijkheidsleer, Virologie en Fytopathologie en een stage aan het Plant Research Institute, Burnley, Melbourne te Australië. In juni 1990 behaalde ze het ingenieursdiploma met lof. Van augustus 1990 tot augustus 1991 was ze als Onderzoeker in Opleiding werkzaam bij de vakgroep Moleculaire Celbiologie van de Rijksuniversiteit te Utrecht. Onder leiding van dr. J.C.M. Smeekens en prof. dr. P.J. Weisbeek verrichtte zij onderzoek aan een geconditioneerd genexpressie systeem in planten. Van augustus 1991 tot augustus 1995 werkte ze als Assistent in Opleiding, onder leiding van dr. J.A.L. van Kan en prof. dr. ir. P.J.G.M. de Wit, bij de vakgroep Fytopathologie van de Landbouwuniversiteit Wageningen. Het onderzoek uitgevoerd tijdens deze periode staat beschreven in dit proefschrift. Per 1 februari is ze als Post-doc werkzaam bij de vakgroep Levensmiddelen-technologie, sectie Industriële Microbiologie aan het onderwerp expressieklonering in *Kluyveromyces lactis*.