Ethylene perception and NEP-like protein production by *Botrytis cinerea*

Yaite Cuesta Arenas

Thesis committee

Supervisor:	Prof. Dr. P.J.G.M. de Wit Professor of Phytopathology Wageningen University
Co-supervisor	Dr. J.A.L. van Kan Universitair Docent Phytopathology Wageningen University
Other members:	Prof. Dr. E.J. Woltering, Wageningen University Prof. Dr. B.J.C. Cornelissen, University of Amsterdam Dr. A.W. van Heusden, Wageningen UR Prof. Dr. R.P. Oliver, Murdoch University, Perth, Australia

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.

Ethylene perception and NEP-like protein production by *Botrytis cinerea*

Yaite Cuesta Arenas

Thesis
Submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Doctorate Board
to be defended in public
on Monday 17 May 2010
at 16.00 in the Aula.

Ethylene perception and NEP-like protein production by *Botrytis cinerea*.
Y. Cuesta Arenas

Thesis Wageningen University, the Netherlands, 2010
With references, with summaries in Dutch and English

ISBN: 978-90-8585-634-4

Table of contents

Chapter 1

General introduction

Chapter 1

General introduction

The necrotrophic fungus *Botrytis cinerea*, commonly known as grey mould, is one of the world's most devastating plant pathogens. It colonizes a wide range of economically important crops including fruits, vegetables, ornamental plants and cut flowers, and causes major postharvest losses (Jarvis, 1977). *B. cinerea* belongs to the class of Deuteromycetes (imperfect stage), order Moniliales and family Moniliaceae. The fungus predominantly disperses by massive production of grey brown clusters of macroconidia. The perfect stage, found quite rarely in nature, is known as *Botryotinia fuckeliana* (de Bary), belonging to the Ascomycetes, order Helotiales and the family Sclerotiniaceae (Jarvis, 1977).

The fungus requires high relative humidity on the plant surface for germination, temperatures between 15°C and 25°C, damp weather for optimal infection, growth, sporulation and spore release. *B. cinerea* is also active at low temperatures, and can cause problems on vegetables stored for weeks or months at temperatures ranging from 0 - 10°C. Infection rarely occurs at temperatures above 25°C but once the infection occurs, the fungus can grow over a range of 0-35°C (Babadoost, 2000).

The symptoms of *Botrytis* diseases vary greatly depending on the host and plant part attacked. General symptoms include a grey to brown discoloration, water soaking, and a fuzzy whitish grey to tan mould (mycelium and spores) growing on the surface of affected areas. The host tissue becomes soft and rots. The conidiospores produced are the inoculum for the next round of infection. One infection cycle may be completed in 3 to 4 days, depending on the environmental conditions and type of host tissue attacked.

Enzymes and metabolites involved in disease development

Virulence of *B. cinerea* requires a set of enzymes and (secondary) metabolites that serve as weapons for attack, as well as mechanisms to overcome the host defence responses. The pathogen penetrates into the host via wounds, natural openings or even intact surfaces, and rapidly colonizes dead or senescing tissues. To penetrate intact host surface, the fungus has to deal with physical barriers including the cuticle and the epidermal cell wall. During penetration, *B. cinerea* produces and secretes enzymes like cutinases and lipases (Salinas and Verhoeff, 1995; van Kan *et al.*, 1997; Comménil *et al.*, 1998; Gindro and Pezet, 1999; Reis *et al.*, 2005), however, none of these have been shown to be essential in the penetration process.

Cell wall degrading enzymes such as pectinases and cellulases are suggested to act in the breakdown of the cell wall, resulting in tissue destruction and the release of nutrients to the fungus. *B. cinerea* possesses multiple copies of genes encoding these enzymes. At least three genes encoding pectin methylesterases are present in the *B. cinerea* genome. Replacement of the *Bcpme*1 and *Bcpme*2 genes, either separately or in combination, in the strain B05.10, yielded mutants that were fully virulent on tomato and grapevine leaves. *In vitro* growth assays demonstrated that pectin demethylation by these two enzymes is not important for growth on highly methylated pectin (Kars *et al.*, 2005b). There are six endopolygalacturonase (*Bcpg*) genes in the *B. cinerea* genome (Wubben *et al.*, 1999) and these genes are expressed differentially during infection in several hosts (ten Have *et al.*, 2001). Five BcPG enzymes were produced in a heterologous host and shown to possess macerating and necrosis-inducing activity (Kars *et al.*, 2005). Deletion of *Bcpg*1 and *Bcpg*2 resulted in strong reduction in virulence on different hosts (ten Have *et al.*, 2001; Kars *et al.*, 2005a).

Once *B. cinerea* has penetrated the plant surface, it kills the underlying epidermal host cells before the hyphae invade (Clark and Lorbeer, 1976). *B. cinerea* culture filtrates contain low molecular weight compounds with phytotoxic activity, named botrydial and botcinic acid (Calvo *et al.*, 2002; Pinedo *et al.*, 2008). Botrydial has been demonstrated to accumulate in *B. cinerea*-infected leaves (Deighton *et al.*, 2001). The *Bcbot1* gene encoding a P450 monooxygenase is essential in the biosynthesis of this metabolite. Mutants of three different *B. cinerea* strains lacking a functional copy of the *Bcbot1* gene did not produce any botrydial (Siewers *et al.*, 2005). *Bcbot1* deletion mutants were tested for their virulence on different host plants. *Bcbot1* deletion mutants in strains ATCC 58025 and SAS56 did not show any alteration in virulence, because they were still producing botcinic acid. By contrast, mutants in strain T4, which is not able to produce botcinic acid, displayed a significant reduction in virulence on bean leaves, tomato leaves and tomato fruits. Therefore, botrydial must be regarded as a strain-dependent virulence factor (Siewers *et al.*, 2005).

Necrotrophs by definition cause plant cell death, which triggers a spectrum of local and systemic defense responses in the host plant, some of which include an oxidative burst and the production of antifungal metabolites and proteins. *B. cinerea* must be able to evade such plant defences to successfully colonize the host. The presence of an array of enzymes capable of metabolizing hydrogen peroxide such as intra- and extracellular peroxidases, superoxide dismutase (SOD) and catalase (Gil-ad *et al.*, 2000), shows that *B. cinerea* is equipped to cope with active oxygen species produced by the host during early stages of infection (Schouten *et*

al., 2002a). A BcSOD1-deficient mutant showed reduced virulence on French bean and an enhanced accumulation of extracellular hydrogen peroxide was observed at the plant-pathogen interface (Rolke *et al.*, 2004). Furthermore, enzymes like laccases have been reported to be involved in the detoxification of phenolic compounds (Adrian *et al.*, 1998; Mayer *et al.*, 2001). *B cinerea* is able to protect itself from the action of resveratrol, a phytoalexin from grapevine leaves, by its detoxification through laccases (Adrian *et al.*, 1998). Schouten (2002a) however reported that resveratrol is not toxic to *B. cinerea* by itself, but the resveratrol oxidation product (named viniferin) generated by the action of laccase BcLCC2 is the active compound that causes the toxicity. However, deletion of the laccase genes *Bclcc*1 and *Bclcc*2 in strain B05.10 did not affect the virulence on several hosts (Schouten *et al.*, 2002b). Recently it became clear that the *B cinerea* genome contains eleven laccase-encoding genes (www.broad.mit.edu), suggesting that they may have overlapping functions (Schouten *et al.*, 2008). Another mechanism by which *B. cinerea* manages to deal with growth-inhibiting compounds is by the activity of ATP-binding cassette (ABC) transporters and Major Facilitator (MFS) proteins that mediate the efflux of a range of antifungal compounds. Several studies have been performed on the role of transporter proteins in conferring resistance against industrial fungicides, plant defense compounds and a natural antibiotic produced by *Pseudomonas fluorescens* (Vermeulen *et al.*, 2001; Schoonbeek *et al.*, 2003; Schouten *et al.*, 2008a).

NLPs

The Necrosis and Ethylene-inducing Protein Nep1 was purified from culture filtrates of *Fusarium oxysporum* f.sp. *erythroxyli* (Bailey, 1995) and was the first representative of what is currently known to be a large family of necrosis-inducing proteins. Nep1-Like Proteins (NLPs) are present in a spectrum of microorganisms including bacteria, actinomycetes, oomycetes and fungi but not in higher organisms (Pemberton and Salmond, 2004). NLPs are present in Gram-negative and Gram-positive bacteria with saprophytic or pathogenic life styles. In fungi and oomycetes, NLPs are especially present in species interacting with plants, and predominantly in species that display a hemibiotrophic or necrotrophic life style on plants (Qutob *et al.*, 2006). NLPs are secreted proteins that share a conserved heptapeptide motif in the central region of the protein, but they do not contain any previously recognized enzymatic domain. The presence of two or more conserved cysteine residues, predicted to form disulfide bridges, has been used to classify the NLPs in two subgroups. Group I comprises proteins that

harbour only the two first conserved cysteines and group II proteins that contain four cysteines (Gijzen and Nürnberger, 2006). The molecular weight of NLPs ranges from 24 to 26 kDa and they can occur as monomers as well as dimers (Garcia *et al.*, 2007).

Responses to NLPs are restricted to dicotyledonous plants; all monocotyledonous plants or other organisms tested so far are insensitive to NLPs (Bailey, 1995; Keates *et al.*, 1998; Staats *et al.*, 2007; Schouten *et al.*, 2008b). Sensitive plants exposed to NLPs respond by production of H_2O_2, nitric oxide, ethylene, accumulation of transcripts encoding pathogenesis-related proteins, calcium influx, release of phytoalexins, activation of MAP kinases and necrotic lesion formation (Keates *et al.*, 1998; Fellbrich *et al.*, 2002; Bae *et al.*, 2006). Qutob *et al* (2006) demonstrated that NLPs have affinity for lipid bilayers and their phytotoxic activity and specificity for dicots do not require the presence of a cell wall. The mode of action and target site of NLPs in plants however are unknown.

The role of host plant processes in *B. cinerea* pathogenesis

Ethylene is present in trace amounts in nature and it is produced by almost all living organisms, either biologically or chemically by the incomplete combustion of hydrocarbons. The activity of ethylene as a plant hormone was discovered in the nineteenth century (Abeles, 1992). The plant physiologist Nelbujov (1879-1926) observed that etiolated pea seedlings grew horizontally in the laboratory but upright in outside air and he showed that the abnormal growth habit was caused by contaminating illuminating gas. In 1901, he proved that the active principle in illuminating gas was ethylene (Bleecker and Kende, 2000). Since then, the study of ethylene as a plant hormone has been extensive. Ethylene is now known to regulate seed germination, seedling growth, leaf and floral abscission, ripening in climacteric fruits, senescence of plant organs, plant growth. Furthermore it acts as a stress hormone during biotic (pathogen infection) and abiotic (wounding, hypoxia, freezing) stress conditions (Mattoo and Suttle, 1991).

In higher plants, ethylene biosynthesis occurs through the conversion of methionine via S-Adenosyl-L-Methionine, into 1-aminocyclopropane-1-carboxylic acid (ACC) and proceeds to ethylene by oxidation (Fig.1). This process is tightly regulated at the level of ACC synthase (ACS). Both positive and negative feedback regulation of ethylene biosynthesis have been reported in different plant species (Kende, 1993; Nakatsuka *et al.*, 1998; Barry *et al.*, 2000). For example, in tomato, Le-ACS2 and Le-ACS4 are positively regulated, and Le-ACS6 is negatively regulated by ethylene synthesized during fruit ripening (Nakatsuka *et al.*, 1998).

Once ethylene is synthesized, it is perceived by membrane-localized receptors that are homologous to the bacterial two-component histidine kinases (Bleecker and Kende, 2000). *Arabidopsis thaliana* contains five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) that act as negative regulators of ethylene responses and they are inactivated by ethylene binding (Guo and Ecker, 2004). Transduction of the ethylene signal is achieved through a series of phosphorylations that are carried out by a cascade of MAP kinases, resulting in the activation of transcription factors that bind to promoters of ethylene-responsive genes.

An increase in ethylene production by plants occurs as an early response to pathogen infection (Boller, 1991). Ethylene treatment of plants increases either susceptibility or resistance, depending on the plant-pathogen interaction and environmental conditions. Elad (1993) showed that covering pre-inoculated tomato plants with polyethylene bags in order to accumulate ethylene, promoted *B. cinerea* infection. On the other hand, pre-treatment of tomato plants with exogenous ethylene prior to inoculation, resulted in an increased, partial, resistance to *B. cinerea* (Díaz *et al.*, 2002). The complex physiological responses to ethylene in different tissues and during development, as well as the positive and negative feedback mechanisms make it difficult to generalise. For example, ripening makes tomato fruit more susceptible to *B. cinerea* and the ripening is stimulated by ethylene (Cantu *et al.*, 2008). However, ethylene is not required for the increased susceptibility to *B. cinerea* during fruit ripening (Cantu *et al.*, 2009). Thus, ethylene stimulates *B. cinerea* disease in an indirect way.

Cell death in plant-pathogen interactions

Plant cell death can occur during the interaction with pathogens that attempt to colonize the host (Greenberg, 1997). Cell death processes may occur by at least two distinct pathways: one that resembles apoptosis that is observed in programmed cell death (PCD) in animal systems and one that resembles necrotic cell death.

Apoptosis is an active process that includes the fragmentation of DNA into so-called DNA ladders, membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation with nucleic acids found in membrane-bound vessels (apoptotic bodies) and cytoplasmic condensation. Some of the hallmarks for apoptotic cell death have been observed in plant-pathogen interactions during the occurrence of the hypersensitive response (Heath, 1998). The PCD-eliciting mycotoxins AAL toxin or fumonisin B1 (FB1) (Gilchrist *et al.*, 1995; Gilchrist, 1997; 1998) have been used as models for the study of plant cell death in pathogen response pathways. AAL toxin is secreted by the tomato pathogen *Alternaria alternata* f. sp.

lycopersici, while the structurally related toxin FB1 is secreted by the maize pathogen *Fusarium moniliforme.* These toxins induce apoptosis-like cell death in tomato. DNA ladders were observed during cell death in toxin-treated tomato protoplasts and leaflets.

Necrosis in plants is caused by extrinsic factors, such as mechanical damage or phytotoxic accumulation of specific molecules after a stress event. Thus, plant cell death through necrosis is considered a "passive" process. Swelling is the defining feature of the morphological changes during necrosis. Swelling is due to the cell losing the ability to osmoregulate, resulting in water and ions flooding into the cell and culminating in cellular lysis (Lennon *et al.*, 1991). Generation of ROS, ATP depletion and mitochondrial and chloroplast dysfunction are also characteristic of necrosis. Several fungal toxins are known to cause necrosis in plant. For example two naphthazarin phytotoxins (dihydrofusarubin and isomarticin) produced by *Fusarium solani* caused cell necrosis in veins, plasmolysis or collapse of spongy mesophyll cells, collapse of phloem, depletion of starch, swelling of chloroplasts and disruption of cellular organization in leaves of rough lemon seedlings (Achor *et al.*, 1993).

The most widely studied cell death response in plants is the hypersensitive response (HR), which occurs when a biotrophic pathogen produces an effector that is recognized by a host plant with a corresponding resistance gene, resulting in Effector-Triggered Immunity (ETI). HR resembles PCD in animals and results in the killing of host cells at the site of attempted infection. Cells are sacrificed at the point of pathogen entry to avoid further spreading of (hemi)-biotrophic microbes; however this strategy is not effective against necrotrophic microbes since cell death facilitates the infection by necrotrophs (Govrin and Levine, 2000). Many studies have been performed on the role of plant genes in HR and disease symptom development. Such studies were supported by the finding of lesion mimic mutants, such as *Arabidopsis* mutant *acd5*, that spontaneously forms disease-like lesions (Greenberg *et al.*, 2000; Pilloff *et al.*, 2002), and shows more severe disease symptoms and allows increased growth of *P. syringae*. The *Acd5* gene encodes a ceramide kinase (CERK) that is induced during *P. syringae* infection (Liang *et al.*, 2003). Ceramides are bioactive lipids that activate apoptosis in animals (Hannun and Obeid, 2002), while its phosphorylated derivative (the product of the CERK reaction) can partially block PCD in *Arabidopsis* protoplasts (Greenberg and Yao, 2004). Ceramides are part of the sphingolipid family of bioactive lipids (Hannun and Obeid, 2002). Some fungal pathogens secrete cerebrosides, which are derived from sphingolipids that induce HR-like cell death in rice (Koga *et al.*, 1999). Additionally, a number of fungal pathogens secrete related mycotoxins that cause PCD and disrupt

sphingolipid metabolism (Abbas *et al.*, 1994). Transient expression of *cpr22* (constitutive expresser of PR 22) induces cell death in *Nicotiana benthamiana* leaves (Yoshioka *et al.*, 2006). This cell death resembles the HR seen in an incompatible plant-pathogen interaction. A microscopic analysis of *cpr22*-induced cell death revealed strong similarities to pathogen-induced as well as developmental PCD (Fukuda, 2000; Lam, 2004) including retraction of the plasma membrane from the cell wall, vesicle formation and degradation of the tonoplast.

Anti-apoptotic genes have been identified in viruses, animals and humans. The transgenic expression of several of these genes (Op-IAP, CED-9, Bcl-2, Bcl-xL,) in tobacco plants conferred heritable disease resistance to necrotrophic fungi, including *B. cinerea* and *Sclerotinia sclerotiorum* (Dickman et al., 2001).

Ethylene production and perception by microorganisms

Microorganisms can synthesize ethylene by one of three possible pathways. A few fungi produce ethylene from methionine via ACC, alike the pathway described for plants (Figure 1A) (Sharon *et al.*, 2004). In the second pathway, α-keto-γ-(methylthio)butyric acid (KMBA), resulting from deamination of L-methionine, is converted to ethylene by enzymatic conversion (Figure 1B) or by spontaneous oxidation of KMBA in the presence of light (Yang, 1969). In the third pathway, 2-oxoglutarate is converted to ethylene by the ethylene-forming-enzyme (Figure 1C) (Fukuda *et al.*, 1986). The second and third pathways occur much more frequently in microorganisms than the first one.

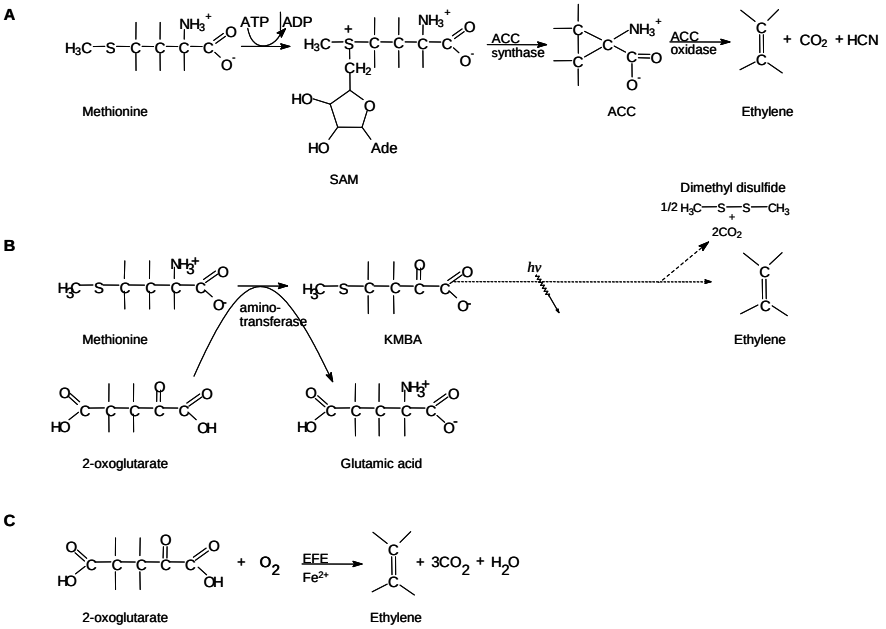


Fig. 1. Biosynthetic pathways in microorganisms for synthesis of ethylene using either methionine (A), KMBA (B) or 2-oxoglutarate (C) as precursors.

Microorganisms cannot only produce ethylene, they can also respond to it. It has been reported for several fungi that ethylene may stimulate conidial germination, germ tube elongation and appressorium formation (Kepczynska, 1994; Sharon *et al.*, 2004). The phenomenon of ethylene perception by microorganisms has been study mainly in fungi but there is no description of the molecular mechanisms involved in the perception.

Outline of the thesis

The aim of the research presented in this thesis was to obtain insight in the roles that ethylene production and perception, both by the pathogen and the plant, plays in the interaction between *B. cinerea* and crop plants, using tomato as a model. Furthermore, functional analysis was performed of two *B. cinerea* necrosis-inducing NLPs, called BcNEP1 and BcNEP2, with emphasis on their role in virulence and their mode of action.

Chapter 2 describes studies on the effects of ethylene on *B. cinerea* either *in vitro* or during pathogenesis. Lesion development on tomato genotypes with a reduced or an enhanced ethylene production level was monitored. Furthermore, reports that the transcript levels of certain *B. cinerea* genes are induced by exposure to ethylene (Chagué *et al.*, 2006), led us to characterize the role of the fungal histidine kinase BcHHK5, which structurally resembles plant ethylene receptors.

Chapter 3 describes the functional analysis of the two NLPs from *B. cinerea*. Expression of *Bcnep* genes and production of the corresponding proteins during infection was investigated. Single knock out mutants were made for *Bcnep*1 and *Bcnep*2 and their role in virulence was tested. Additionally, experiments were conducted to study whether ethylene induced in plants during *B. cinerea* infection results from a response to BcNEP proteins.

Chapter 4 investigates whether the conserved heptapeptide motif, the disulfide bonds, as well as posttranslational modification motifs in the BcNEP proteins influence their necrosis-inducing activity. Transient agro-infiltration assays were performed in *Nicotiana benthamiana* and *N. tabacum* to express site-directed mutant proteins.

Chapter 5 describes a study on the role of the plant in the response to BcNEP proteins. The inhibition of necrosis-inducing activity by polyclonal antibodies raised against BcNEP proteins was analysed. Genetic tools and pharmacological inhibitors were used to elucidate plant pathways required for the necrosis-inducing activity of BcNEP proteins.

Chapter 6 provides a general discussion of the thesis.

REFERENCE LIST

Abbas, H. K., Tanaka, T., Duke, S. O., Porter, J. K., Wray, E. M., Hodges, L., Sessions, A. E., Wang, E., Merrill Jnr, A. H. and Riley, R. T. (1994). "Fumonisin- and AAL-toxin-induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases." Plant Physiology **106**(3): 1085-1093.

Abeles, F. B. (1992). Ethylene in Plant Biology. New York, New York Academic Press.

Achor, D. S., Nemec, S. and Baker, R. A. (1993). "Effects of *Fusarium solani* naphthazarin toxins on the cytology and ultrastructure of rough lemon seedlings." Mycopathologia **123**(2): 117-126.

Adrian, M., Rajaei, H., Jeandet, P., Veneau, J. and Bessis, R. (1998). "Resveratrol oxidation in *Botrytis cinerea* conidia." Phytopathology **88**(5): 472-4776.

Babadoost, M. (2000). Gray mold rot or *Botrytis* blight of vegetables Plant disease., Department of Crop Sciences, University of Illinois**:** 5.

Bae, H., Kim, M. S., Sicher, R. C., Bae, H. J. and Bailey, B. A. (2006). "Necrosis- and ethylene-inducing peptide from *Fusarium oxysporum* induces a complex cascade of transcripts associated with signal transduction and cell death in Arabidopsis." Plant Physiology **141**(3): 1056-1067.

Bailey, B. A. (1995). "Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*." Phytopathology **85**(10): 1250-1255.

Barry, C. S., Llop-Tous, M. I. and Grierson, D. (2000). "The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato." Plant Physiology **123**(3): 979-986.

Bleecker, A. B. and Kende, H. (2000). "Ethylene: A gaseous signal molecule in plant." Annual Review of Cell and Developmental Biology **16**: 1-18.

Boller, T. (1991). Ethylene in pathogenesis and disease resistance. The plant hormone ethylene. Mattoo, A. K. and Suttle, J. C. Boca Raton, Florida, CRC Press**:** 293-314.

Calvo, A. M., Wilson, R. A., Bok, J. W. and Keller, N. P. (2002). "Relationship between secondary metabolism and fungal development." Microbiology and Molecular Biology Reviews **66**(3): 447-459.

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M. and Powell, A. L. T. (2008). "The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*." Proceedings National Academy of Sciences United States of America **105**(3): 859-864.

Cantu, D., Blanco-Ulate, B., Yang, L., Labavitch, J. M., Bennett, A. B. and Powell, A. L. T. (2009). "Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires NOR but not RIN or Ethylene." Plant Physiology **150**(3): 1434-1449.

Chagué, V., Danit, L. V., Siewers, V., Schulze-Gronover, C., Tudzynski, P., Tudzynski, B. and Sharon, A. (2006). "Ethylene sensing and gene activation in *Botrytis cinerea*: a missing link in ethylene regulation of fungus-plant interactions?" Molecular Plant-Microbe Interaction **19**: 33-42.

Clark, C. A. and Lorbeer, J. W. (1976). "Comparative histopathology of *Botrytis squamosa* and *B. cinerea* on onion leaves." Phytopathology **66**: 1279-1289.

Comménil, P., Belingheri, L. and Dehorter, B. (1998). "Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*." Physiological and Molecular Plant Pathology **52**(1): 1-14.

Deighton, N., Muckenschnabel, I., Colmenares, A. J., Collado, I. G. and Williamson, B. (2001). "Botrydial is produced in plant tissues infected by *Botrytis cinerea*." Phytochemistry **57**(5): 689-692.

Díaz, J., ten Have, A. and van Kan, J. A. L. (2002). "The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*." Plant Physiology **129**(3): 1341-1351.

Elad, Y. (1993). "Regulators of ethylene biosynthesis or activity as a tool for reducing susceptibility of host plant tissues to infection by *Botrytis cinerea*." Netherlands Journal of Plant Pathology **99**(3): 105-113.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Engelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M. and Nürnberger, T. (2002). "NPP1, a Phytophthora-associated trigger of plant defense in parsley and Arabidopsis." Plant Journal **32**(3): 375-390.

Fukuda, H. (2000). "Programmed cell death of tracheary elements as a paradigm in plants." Plant Molecular Biology **44**(3): 245-253.

Fukuda, H., Fujii, T. and Ogawa, T. (1986). "Preparation of a cell-free ethylene forming system from *Penicillium digitatum*." Agricultural Biology and Chemistry. **50**: 977-981.

Garcia, O., Macedo, J. A. N., Tibúrcio, R., Zaparoli, G., Rincones, J., Bittencourt, L. M. C., Ceita, G. O., Micheli, F., Gesteira, A., Mariano, A. C., Schiavinato, M. A., Medrano, F. J., Meinhardt, L. W., Pereira, G. A. G. and Cascardo, J. C. M. (2007). "Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*." Mycological Research **111**(4): 443-455.

Gijzen, M. and Nürnberger, T. (2006). "Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa." Phytochemistry **67**(16): 1800-1807.

Gil-ad, N. L., Bar-Nun, N., Noy, T. and Mayer, A. M. (2000). "Enzymes of *Botrytis cinerea* capable of breaking down hydrogen peroxide." FEMS Microbiology Letters **190**(1): 121-126.

Gilchrist, D. G. (1997). "Mycotoxins reveal connections between plants and animals in apoptosis and ceramide signaling." Cell Death and Differentiation **4**(8): 689-698.

Gilchrist, D. G. (1998). "Programmed cell death in plant disease: The purpose and promise of cellular suicide." Annual Review of Phytopathology **36**: 393-414.

Gilchrist, A., Wang, H. and Bostock, R. M. (1995). "Sphingosine-related mycotoxins in plant and animal diseases." Canadian Journal of Botany **73**: 459-467Gindro, K. and Pezet, R. (1999). "Purification and characterization of a 40.8-kDa cutinase in ungerminated conidia of *Botrytis cinerea* Pers.: Fr." FEMS Microbiology Letters **171**(2): 239-243.

Govrin, E. M. and Levine, A. (2000). "The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*." Current Biology **10**(13): 751-757.

Greenberg, J. T. (1997). "Programmed cell death in plant-pathogen interactions." Annual Review of Plant Biology **48**: 525-545.

Greenberg, J. T. and Yao, N. (2004). "The role of regulation of programmed cell death in plant-pathogen interactions." Cellular Microbiology **6**(3): 201-211.

Greenberg, J. T., Silverman, F. P. and Liang, H. (2000). "Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the arabidopsis mutant acd5." Genetics **156**: 341-350.

Guo, H. and Ecker, J. R. (2004). "The ethylene signaling pathway: new insights." Current Opinion in Plant Biology **7**(1): 40-49.

Hannun, Y. A. and Obeid, L. M. (2002). "The ceramide-centric universe of lipid-mediated cell regulation: Stress encounters of the lipid kind." Journal of Biological Chemistry **277**(29): 25847-25850.

Heath, M. C. (1998). "Apoptosis, programmed cell death and the hypersensitive response " European Journal of Plant Pathology **104**(2): 117-124.

Jarvis, W. R. (1977). Botryotinia and Botrytis species: taxonomy, physiology, and pathogenicity. . Otawa, Research Branch Canada Department of Agriculture.

Kars, I., Krooshof, G. H., Wagemakers, L., Joosten, R., Benen, J. A. E. and van Kan, J. A. L. (2005a). "Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*." Plant Journal **43**(2): 213-225.

Kars, I., McCalman, M., Wagemakers, L. and van Kan, J. A. L. (2005b). "Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme*1 and *Bcpme*2 are dispensable for virulence of strain B05.10." Molecular Plant Pathology **6**(6): 641-652.

Keates, S. E., Loewus, F. A., Helms, G. L. and Zink, D. L. (1998). "5-O-(α-D-galactopyranosyl)-D-glycero-pent-2-enono-1,4-lactone: Characterization in the oxalate-producing fungus, *Sclerotinia sclerotiorum*." Phytochemistry **49**(8): 2397-2401.

Kende, H. (1993). "Ethylene biosynthesis." Annual Review of Plant Physiology and Plant Molecular Biology **44**(1): 283-307.

Kepczynska, E. (1994). "Involvement of ethylene in spore germination and mycelial growth of *Alternaria alternata*." Mycological Research **98**(1): 118-120.

Koga, J., Yamauchi, T., Shimura, M., Ogawa, N., Oshima, K., Umemura, K., Kikuchi, M. and Ogasawara, N. (1999). "Cerebrosides A and C, sphingolipid elicitors of hypersensitive cell death and phytoalexin accumulation in rice plants." Journal of Biological Chemistry **273**(48): 31985-31991.

Lam, E. (2004). "Controlled cell death, plant survival and development." Nature Reviews Molecular Cell Biology **5**(4): 305-315.

Lennon, S. V., Martin, S. J. and Cotter, T. G. (1991). "Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli." Cell Proliferation **24**(2): 203-214.

Liang, H., Yao, N., Song, J. T., Luo, S., Lu, H. and Greenberg, J. T. (2003). "Ceramides modulate programmed cell death in plants." Genes and Development **17**(21): 2636-2641.

Mattoo, A. K. and Suttle, J. C. (1991). The Plant Hormone Ethylene. Boca Raton, Florida, CRC Press.

Mayer, A. M., Staples, R. C. and Gil-ad, N. L. (2001). "Mechanisms of survival of necrotrophic fungal plant pathogens in hosts expressing the hypersensitive response." Phytochemistry **58**(1): 33-41.

Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y. and Inaba, A. (1998). "Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, l-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening." Plant Physiology **118**(4): 1295-1305.

Pemberton, C. L. and Salmond, G. P. C. (2004). "The Nep1-like proteins - A growing family of microbial elicitors of plant necrosis." Molecular Plant Pathology **5**(4): 353-359.

Pilloff, R. K., Devadas, S. K., Enyedi, A. and Raina, R. (2002). "The Arabidopsis gain-of-function mutant dll1 spontaneously develops lesions mimicking cell death associated with disease." Plant Journal **30**: 61-70.

Pinedo, C., Wang, C. M., Pradier, J. M., Dalmais, B., Choquer, M., Le Pêcheur, P., Morgant, G., Collado, I. G., Cane, D. E. and Viaud, M. (2008). "Sesquiterpene synthase from the botrydial biosynthetic gene cluster of the phytopathogen Botrytis cinerea." ACS Chemical Biology **3**(12): 791-801.

Qutob, D., Kemmerling, B., Brunner, F., Kufner, I., Engelhardt, S., Gust, A. A., Luberacki, B., Seitz, H. U., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M. and Nürnberger, T. (2006). "Phytotoxicity and innate immune responses induced by Nep1-like proteins." Plant Cell **18**(12): 3721-3744.

Reis, H., Pfiff, S. and Hahn, M. (2005). "Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*." Molecular Plant Pathology **6**(3): 257-267.

Rolke, Y., Liu, S., Quidde, T., Williamson, B., Schouten, A., Weltring, K. M., Siewers, V., Tenberge, K. B., Tudzynski, B. and Tudzynski, P. (2004). "Functional analysis of H2O2-generating systems in *Botrytis cinerea*: The major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable." Molecular Plant Pathology **5**(1): 17-27.

Salinas, J. and Verhoeff, K. (1995). "Microscopical studies of the infection of gerbera flowers by Botrytis cinerea." European Journal of Plant Pathology **101**(4): 377-386.

Schoonbeek, H. J., van Nistelrooy, J. G. M. and de Waard, M. A. (2003). "Functional analysis of ABC transporter genes from *Botrytis cinerea* identifies BcatrB as a transporter of eugenol." European Journal of Plant Pathology **109**(9): 1003-1011.

Schouten, A., Tenberge, K. B., Vermeer, J., Stewart, J., Wagemakers, L., Williamson, B. and van Kan, J. A. L. (2002a). "Functional analysis of an extracellular catalase of *Botrytis cinerea*." Molecular Plant Pathology **3**(4): 227-238.

Schouten, A., Wagemakers, L., Stefanato, F. L., van der Kaaij, R. M. and van Kan, J. A. L. (2002b). "Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase." Molecular Microbiology **43**(4): 883-894.

Schouten, A., Maksimova, O., Cuesta-Arenas, Y., van den Berg, G. and Raaijmakers, J. M. (2008a). "Involvement of the ABC transporter BcAtrB and the laccase BcLCC2 in defence of *Botrytis cinerea* against the broad-spectrum antibiotic 2,4-diacetylphloroglucinol." Environmental Microbiology **10**(5): 1145-1157.

Schouten, A., van Baarlen, P. and van Kan, J. A. L. (2008b). "Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells." New Phytologist **177**(2): 493-505.

Sharon, A., Elad, Y., Barakat, R. and Tudzynski, P. (2004). Phytohormones and *Botrytis*. *Botrytis*: Biology, Pathology and Control. Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. Dordrecht, the Netherlands, Kluwer Academic Publishers**:** 163-179.

Siewers, V., Viaud, M., Jimenez-Teja, D., Collado, I. G., Gronover, C. S., Pradier, J. M., Tudzynski, B. and Tudzynski, P. (2005). "Functional analysis of the cytochrome P450 monooxygenase gene bcbot1 of *Botrytis cinerea* indicates that botrydial is a strain-specific virulence factor." Molecular Plant-Microbe Interactions **18**(6): 602-612.

Staats, M., van Baarlen, P., Schouten, A., van Kan, J. A. L. and Bakker, F. T. (2007). "Positive selection in phytotoxic protein-encoding genes of *Botrytis* species." Fungal Genetics and Biology **44**(1): 52-63.

Stergiopoulos, I. and de Waard, M. A. (2002). "Activity of azole fungicides and ABC transporter modulators on *Mycosphaerella graminicola*." Journal of Phytopathology **150**(6): 313-320.

ten Have, A., Breuil, W. O., Wubben, J. P., Visser, J. and van Kan, J. A. L. (2001). "*Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues." Fungal Genetics and Biology **33**(2): 97-105.

van Kan, J. A. L., van't Klooster, J. W., Wagemakers, C. A. M., Dees, D. C. T. and van der Vlugt-Bergmans, C. J. B. (1997). "Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato." Molecular Plant-Microbe Interactions **10**(1): 30-38.

Vermeulen, T., Schoonbeek, H. and de Waard, M. A. (2001). "The ABC transporter BcatrB from *Botrytis cinerea* is a determinant of the activity of the phenylpyrrole fungicide fludioxonil." Pest Management Science **57**(5): 393-402.

Wubben, J. P., Mulder, W., ten Have, A., van Kan, J. A. and Visser, J. (1999). "Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*." Applied and Environmental Microbiology **65**(4): 1596-1602.

Yang, S. F. (1969). "Further studies on ethylene formation from alpha-keto-gamma-methylthiobutyric acid or beta-methylthiopropionaldehyde by peroxidase in the presence of sulfite and oxygen." Journal of Biological Chemistry **244**(16): 4360-4365.

Yoshioka, K., Moeder, W., Kang, H. G., Kachroo, P., Masmoudi, K., Berkowitz, G. and Klessig, D. F. (2006). "The chimeric Arabidopsis CYCLIC NUCLEOTIDE-GATED ION CHANNEL11/12 activates multiple pathogen resistance responses." Plant Cell **18**(3): 747-763.

Chapter 2

Ethylene perception by *Botrytis cinerea* does not affect pathogenesis on tomato

Chapter 2

Ethylene perception by *Botrytis cinerea* does not affect pathogenesis on tomato

Yaite Cuesta, Ester Dekkers and Jan van Kan

SUMMARY

Ethylene regulates several developmental processes in plants and plays an important role in plant-pathogen interactions. Chagué *et al*. (2006) reported that exposure of *Botrytis cinerea* to ethylene *in vitro* leads to reduction of growth and the induction of expression of two genes, one of which encodes a phytotoxic protein. These results led to the hypothesis that *B. cinerea* for its infection takes advantage of ethylene released by the plant. We did not observe the previously reported growth reduction upon exposure to ethylene. We investigated possible effects of ethylene on *B. cinerea* during pathogenesis by monitoring lesion development on tomato genotypes with either a reduced or an enhanced ethylene production level. The lesion sizes on mutant genotypes and their respective wild type progenitors did not differ.

The genome of *B. cinerea* contains a gene encoding a histidine kinase receptor, BcHHK5, which structurally resembles ETR1, the best characterized ethylene receptor in plants. Gene replacement mutants were made to study the function of the *Bchhk5* gene. The mutants were neither affected in growth *in vitro* nor in virulence. Furthermore, the expression of the two genes reported by Chagué *et al*. (2006) to be ethylene-responsive was not influenced by exposure to ethylene in the *B. cinerea* wild type strain nor in a Δ*Bchhk5* mutant. Altogether we obtained no evidence that *B. cinerea* indeed senses ethylene, nor that the *Bchhk5* gene product acts as an ethylene receptor.

INTRODUCTION

Ethylene perception has been studied in detail in plants. In *Arabidopsis thaliana*, ethylene sensing and signaling occurs in the endoplasmic reticulum through a family of five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) that share similarity with bacterial two component phosphorelay signaling systems (Chang and Stadler, 2001). The receptors function as negative regulators of ethylene responses and are inactivated by ethylene binding (Guo and Ecker, 2004). ETR1 is the best characterized ethylene receptor; mutations in the *A. thaliana etr*1 gene result in an ethylene-insensitive phenotype, indicating that ETR1 must be a dominant receptor (Bleecker *et al.*, 1988). Other proteins downstream in the signaling cascade can integrate signals from other plant hormones, like jasmonic acid, to modulate the response to biotic and abiotic stress. Hundreds of *A. thaliana* genes are induced or repressed by ethylene (Alonso *et al.*, 2003).

A well known effect of ethylene on plant growth is the triple response of etiolated dicotyledonous seedlings. This response is characterized by the inhibition of hypocotyl and root cell elongation, radial swelling and exaggerated curvature of the apical hook (Ecker, 2004). This morphological response allowed rapid screening of mutant populations based on ethylene response defects. Multiple mutations affecting ethylene perception have been identified. In *A. thaliana*, a series of mutants (*etr1*, *ein1, ein2, ein3, ctr1*) has been obtained that are characterized by an altered ethylene response, sometimes leading to changes in developmental processes (Bleecker *et al.*, 1988; Gúzman and Ecker, 1990; Kieber *et al.*, 1993). In tomato, mutants affected in ethylene responses have been identified but their phenotypes only become evident during fruit ripening and flower abscission. The natural tomato mutant Never Ripe (*Nr*) is insensitive to ethylene (Lanahan *et al.*, 1994) and presumably lacks the feedback mechanism that normally abolishes ethylene production once high concentrations are reached. As a consequence, the *Nr* mutant overproduces ethylene. The tomato *Nr* mutant does not display a triple response and shows only little differences in morphology and development, other than a late flower abscission and an incomplete fruit ripening (Lanahan *et al.*, 1994). The severity of the fruit ripening defect caused by the *Nr* mutation in tomato varies between genetic backgrounds (J. Díaz & J. van Kan, unpublished).

In order to block or reduce ethylene production, different approaches have been reported. The expression in a transgenic plant of an antisense construct of the tomato ACC oxidase gene reduced ethylene production and extended the shelf life of tomato fruit after harvest (Hamilton *et al.*, 1990). Tomato plants expressing a bacterial ACC deaminase, which

degrades the ethylene precursor ACC, showed a reduced ethylene biosynthesis throughout the vegetative parts of the plant and the fruit (Klee *et al.*, 1991).

In fungi, there are few reports of ethylene perception. Ethylene produced by ripening climacteric fruit induces spore germination and appressorium formation in *Colletotrichum gloeosporioides* and *C. musae*, but not in other *Colletotrichum* species that normally infect non-climacteric fruit (Flaishman and Kolattukudy, 1994). In *Aspergillus nidulans*, aflatoxin production and ascus development are inhibited by ethylene in a dose-dependent manner (Roze *et al.*, 2004). Chagué *et al*. (2006) have previously reported that the radial growth of *Botrytis cinerea in vitro* is affected by ethylene and the transcript levels of two genes, *Bcspl*1 and *Bchsp30*, are induced at 24 h after exogenous ethylene application. The *Bcspl*1 gene (Kunz *et al.*, 2006) is homologous to a gene from *Phaeosphaeria nodorum* that encodes a phytotoxic protein produced during infection on wheat (Hall *et al.*, 1999). The *Bchsp*30 gene encodes a heat shock protein (Chagué *et al.*, 2006), which in *S. cerevisiae* is induced by heat shock, ethanol and weak acid (Panaretou and Piper, 1992; Piper *et al.*, 1997).

B. cinerea infection induces the expression in the host plant of ethylene biosynthetic genes, ACC synthase and ACC oxidase (Benito *et al.*, 1998; Diaz *et al.*, 2002). A correlation was reported between the level of ethylene produced by flower petals and leaves from different rose cultivars and the severity of grey mould symptoms (Elad and Volpin, 1988). In strawberry, *B. cinerea* establishes a primary infection that stays quiescent until the initiation of fruit ripening, which coincides with a burst of ethylene synthesis (Williamson, 1994). Effects of ethylene on disease development can be caused by direct action of the gas on the pathogen or by indirect action via modifications in the host metabolism, which predispose the plant to fungal invasion (Brown and Burns, 1998). We set out to evaluate the hypothesis by Chagué *et al*. (2006) that ethylene may act as a signal for *B. cinerea* to begin the colonization on weakened and senescent tissues. Responses to ethylene were studied *in vitro* and *B. cinerea* transformants were generated in which we attempted to alter ethylene perception. Furthermore tomato mutant lines with altered ethylene production levels were inoculated with *B. cinerea*. We found no evidence that ethylene is important for *B. cinerea* to colonize tomato.

RESULTS

Ethylene does not affect *B. cinerea in vitro* growth

The effect of ethylene on the growth of *B. cinerea* strain B05.10 was determined by inoculating mycelium plugs on agar plates. The cultures were grown in sealed desiccators

either in ethylene-free atmosphere or in the presence of ethylene at a concentration of 200 ppm. The radial growth of mycelia in the presence of ethylene was similar to that of the control (Table 1). The experiment was repeated several times, in the presence of different concentrations of ethylene ranging from 0.1 to 200 ppm, and in three different media. In all experiments, growth rates in the presence of ethylene did not differ from that in ethylene-free air (data not shown).

Table 1. Effect of ethylene on radial growth of *B. cinerea* strain B05.10.

	Colony radius (mm ± standard deviation; n=3)		
	24 h	65 h	90 h
Ethylene (200 ppm)	6 ± 1.4	26.5 ± 3.5	37.5 ± 0.7
Control	6 ± 0	24.5± 0.7	35.5± 2.1

Ethylene production by plants does not affect *B. cinerea* lesion size

To investigate whether ethylene produced by the host plant affects disease development, *B. cinerea* was inoculated on detached leaves from mutant tomato lines that have altered levels of ethylene production, i.e. the transgenic line UC8338, an ethylene non-producer and *Nr*, a natural ethylene-insensitive mutant that overproduces ethylene. After 72 h, the lesion sizes on line UC8338 and *Nr* were similar and did not differ from the lesion sizes in the corresponding wild type control lines (Figure 1). These observations suggest that the levels of ethylene produced by the host plant do not affect *B. cinerea* lesion size.

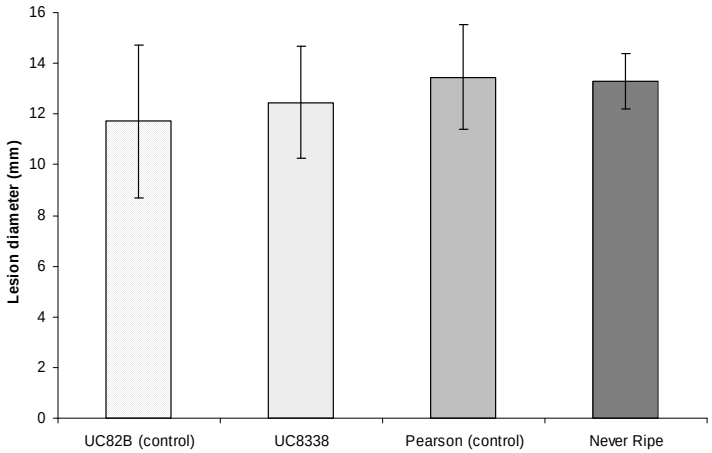


Fig. 1. Lesion diameters of *B. cinerea* inoculated on detached leaves from mutant and wild type tomato lines at 72 hpi. Diameters (given in mm) are the average of 20 inoculation points per genotype; the standard deviation is indicated by an error bar. The experiment was repeated 3 times with similar results.

The *Bchhk*5 gene encodes a protein homologous to the ethylene receptor ETR1

The genome of *B. cinerea* contains a family of 20 genes encoding histidine kinase receptors, belonging to two-component phosphorelay signaling systems (Catlett *et al.*, 2003). One of the *B. cinerea* histidine kinases, named BcHHK5 (BC1G_08461), is the only family member that contains transmembrane domains (Catlett *et al.*, 2003) and shares structural features with *Arabidopsis thaliana* ETR1, the best characterized ethylene receptor in plants (Figure 2). Most notable structural similarities of the BcHHK5 protein to ETR1 are the presence of an N-terminal hydrophobic region with three predicted membrane-spanning domains, followed by a histidine kinase domain and a C-terminal response regulator domain (Figure 2). Furthermore, the second membrane-spanning domain of ETR1 contains a Cysteine residue at position 65 which is essential for ethylene binding (Bleecker *et al.*, 1988; Chang *et al.*, 1993). A cysteine residue is also present in the second membrane-spanning domain of BcHHK5 at position 66. These features would make BcHHK5 a candidate to be involved in ethylene binding.

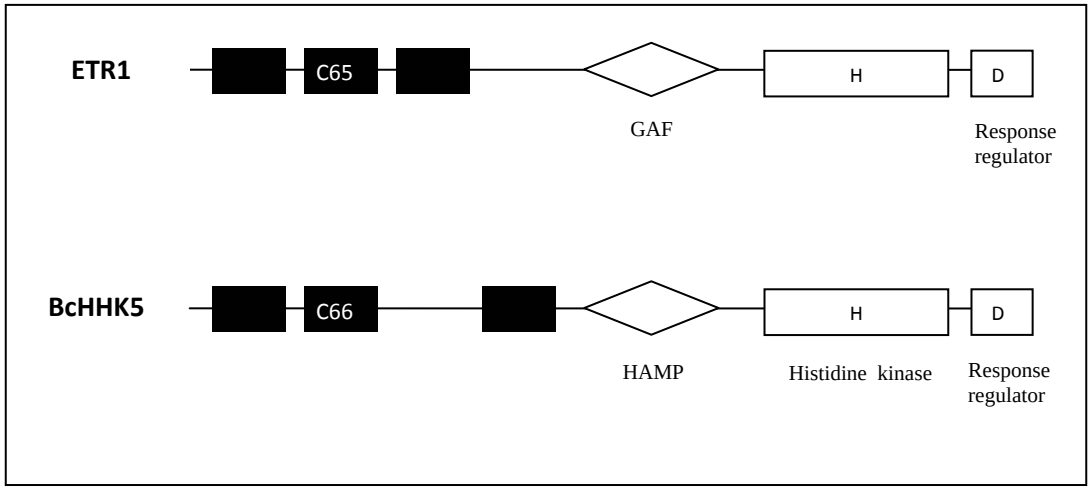
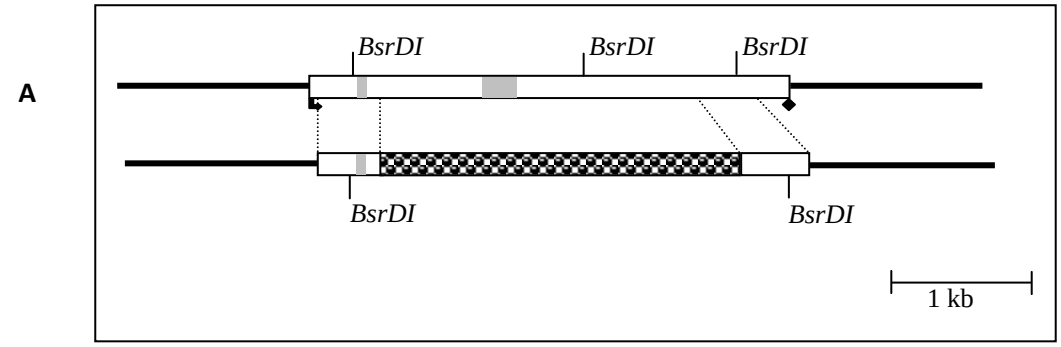


Fig. 2. Structural similarity of *A. thaliana* ETR1 and BcHHK5. The black boxes are membrane-spanning domains. The conserved Cysteine (C) residue in the second membrane-spanning domain is indicated. Conserved Histidine (H) residues in the kinase domain and aspartic acid (D) residues involved in phosphorelay signaling in the regulatory domain are indicated. The GAF domain, which in some proteins is involved in cGMP binding (Aravind and Ponting, 1997) has an unknown function in ETR1 (Chang and Stadler, 2001). The HAMP domain is found in bacterial sensor and chemotaxis proteins, and in eukaryotic histidine kinases (Aravind and Ponting, 1999).

In order to study the role of the *Bchhk*5 gene in virulence and in ethylene perception, gene replacement mutants were made by homologous integration of a hygromycin resistance cassette (Figure 3A). Three independent mutants were obtained. Southern analysis after digestion with *BsrDI* showed that *BsrDI* fragments of 1.2 kb and 1.7 kb in the wild type were replaced by a 3.1 kb fragment in the mutant, as expected when correct homologous recombination has occurred (Figure 3B).



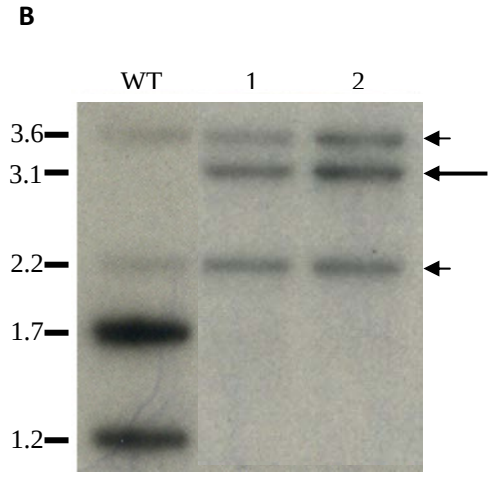


Fig. 3. Gene replacement of the *Bchhk*5 gene. **A:** Organization of the *Bchhk*5 locus before and after integration of a hygromycin selection marker cassette by homologous recombination. The translation start (arrow) and stop codons (diamond), the introns (grey boxes) and restriction sites used for Southern analysis are indicated. The dotted box represents the hygromycin selection cassette. **B**. Southern analysis of DNA from the wild type B05.10 (WT) and two independent Δ*Bchhk*5 transformants (1, 2) digested with *BsrDI*. The large arrow indicates the recombinant fragment. The two small arrows indicate the *BsrDI* fragments of 2.2 kb and 3.6 kb, which border the *Bchhk*5 gene.

The phenotypic behavior of knock out mutants was analyzed. The growth rate and appearance of the Δ*Bchhk*5 mutants were indistinguishable from that of the wild type B05.10 and were not affected by the presence of ethylene (not shown). Virulence assays of Δ*Bchhk*5 mutants on detached tomato leaves did not show any difference in lesion sizes (Table 2) or in the appearance of symptoms when compared with wild type B05.10 (not shown).

Table 2. Lesion diameter by *B.cinerea* B05.10 **and Δ*Bchhk*5 mutants on tomato genotypes at 72 hpi.**

	Lesion diameter (mm ± standard deviation, n=32)			
	Control	Ethylene non-producer mutant	Control	Ethylene over-producer mutant
	UC82B	UC8338	Pearson	Never Ripe
B05.10	17.8 ± 1.4	17.5 ± 1.6	17.3 ± 2	17.9 ± 1.5
Δ*Bchhk*5	17.1 ± 1.5	17.3 ± 1.4	16 ± 0.8	16.9 ± 1.3

Expression analysis of *Bcspl*1 and *Bchsp*30 in wild type and Δ*Bchhk5* mutants

The expression of the ethylene-inducible genes *Bcspl1 and Bchsp30* (Chagué *et al*., 2006) was analyzed in wild type strain B05.10 and in one of the Δ*Bchhk*5 mutants in the absence or presence of ethylene during 48 h (Figure 4). The level of actin (*Bcact*1) transcript in the wild type and the Δ*Bchhk*5 mutant strain decreased between 24 h and 48 h in both cultures, presumably related to reduced growth of the fungus due to medium depletion. Transcript levels of *Bcspl*1 increased very strongly between 24 and 48 h both in the wild type and the Δ*Bchhk*5 mutant. At 24 h, *Bcspl*1 transcript levels in the Δ*Bchhk*5 mutant culture grown in the presence of ethylene were slightly lower as compared with the culture grown in air. This decrease was not observed in the wild type culture in this experiment, but it was observed in at least one biological replication (not shown). The *Bchsp*30 transcript level in the wild type strain slightly decreased between 24 and 48 h in absence of ethylene, but slightly increased in the presence of ethylene. By contrast the *Bchsp*30 transcript level in the Δ*Bchhk*5 mutant slightly decreased between 24 and 48 h in the presence of ethylene, but remained constant in the absence of ethylene. The *Bchsp*30 transcript profiles were consistently observed in other experiments (not shown).

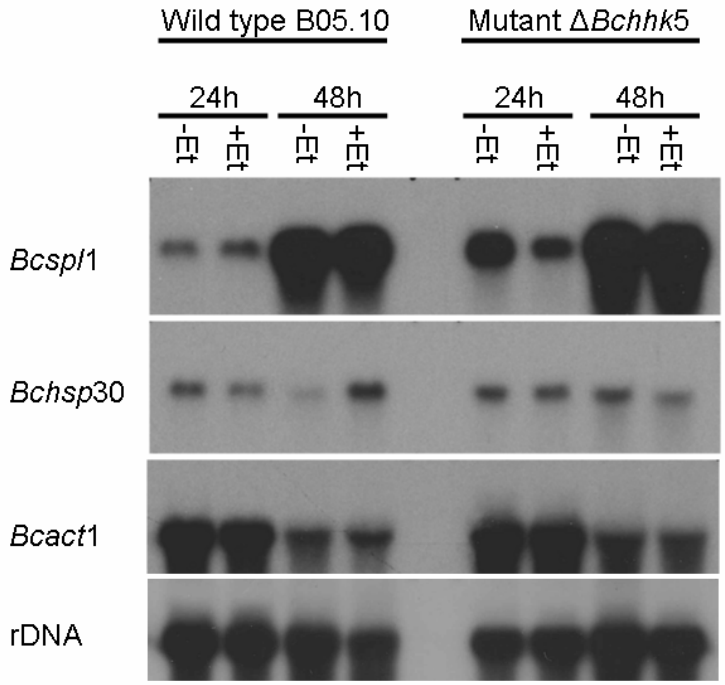


Fig. 4. *Bcspl*1 and *Bchsp*30 expression in *B. cinerea* wild type B05.10 and a Δ*Bchhk*5-mutant during growth in ethylene-free atmosphere (-) or in the presence of 200 ppm ethylene (+). Hybridization intensity with the actin probe *Bcact*1 is a measure for the amount of mRNA in the total RNA sample. Hybridization with an rDNA probe was used as measure for equal lading.

DISCUSSION

We aimed to unravel the role of ethylene during the interaction of *B. cinerea* with tomato plants, and specifically to elucidate whether ethylene influences the fungus. Our results showed that ethylene, in a range of concentrations and on different media, did not affect to any discernible degree the radial growth or the mycelium morphology of *B. cinerea* strain B05.10 *in vitro*. This result contrasted with the report by Chagué *et al.* (2006), that the application of 200 ppm ethylene to the same strain reduced the mycelium surface by 24-30 %. The experiment was repeated several times in the same way as described by Chagué *et al.* (2006) and we never observed any growth reduction.

Experiments were conducted *in planta* to study whether ethylene production by the host plant affects disease development, by inoculating detached leaves from tomato mutant plants with altered ethylene production with *B. cinerea*. The lesion diameters on the ethylene over-producing mutant *Nr* were similar to those on the ethylene non-producing mutant UC8338 and on their respective wild type lines. These results are in agreement with those reported by Diaz *et al.* (2002), who used the same tomato genotypes, but inoculated intact 3 week-old plants instead of detached leaves. We conclude that even if *B. cinerea* were able to sense ethylene produced by the host, it does not affect disease development to any detectable degree. Interestingly pre-treatment of tomato plants with exogenous ethylene prior to inoculation with *B. cinerea* partially reduced their susceptibility (Diaz *et al.*, 2002), suggesting that ethylene responses affect predisposition of tomato to *B. cinerea* infection.

The genome of *B. cinerea* contains the *Bchhk*5 gene, encoding a protein that shows structural similarity to *A. thaliana* ETR1, the best characterized ethylene receptor in plants. The BcHHK5 protein is the only of the 20 *B. cinerea* histidine kinases that contains membrane-spanning regions in the N-terminal part of the protein. Furthermore BcHHK5 contains a cysteine residue in the second membrane-spanning domain, in a position that is very similar to the cysteine residue which, in ETR1, is essential for ethylene binding (Bleecker *et al.*, 1988; Chang *et al.*, 1993). Virulence assays and gene expression studies in Δ*Bchhk*5 mutant did not provide any indication that the gene product is involved in ethylene perception or important for virulence of *B. cinerea*. Lesion diameters caused by Δ*Bchhk*5 mutants were similar to those of the wild type recipient strain and there were no differences in lesion diameters between the tomato genotypes tested, regardless of whether or not they differed in their capacity to produce ethylene.

The observed expression profiles of the presumed ethylene-inducible genes *Bchsp*30 and *Bcspl*1 (Figure 4) were not in agreement with the profiles previously published by Chagué *et al.* (2006), who reported a strong (at least 10-fold at 24 h) increase in *Bchsp*30 transcript in ethylene-treated cultures of strain B05.10 and a moderate (approx. 2- to 5-fold) induction of *Bcspl*1 transcript at 24 and 48 h. In our experiments, the effects of ethylene treatment were small and in some cases a decrease rather than an increase was observed. The *Bcspl*1 transcript level dramatically increased between 24 and 48 h, regardless of the presence of ethylene. The concomitant reduction of actin mRNA between 24 and 48 h was indicative of cessation of growth, possibly related to starvation caused by nutrient depletion in the medium.

Taken together, our results do not support the earlier report by Chagué *et al.* (2006) that *B. cinerea* is able to sense ethylene and induce the expression of *Bchsp*30 and *Bcspl*1. The reasons for this discrepancy remain unclear. Secondly, we have not found any indications that the *Bchhk5* gene product acts as an ethylene receptor, in spite of its structural similarity to the *A. thaliana* ethylene receptor ETR1. Thirdly, inoculations of *B. cinerea* on various tomato genotypes with altered ethylene production levels have not provided any indication that ethylene production by the plant influences disease development to any visually detectable degree. Most reported effects of ethylene on grey mould development are related to flowers or fruit (El Kazzaz *et al.*, 1983; Elad, 1988; Kepczynska, 1993; Govrin and Levine, 2000). Much more than leaves, flowers and fruit undergo tremendous physiological changes by exposure to ethylene. Disassembly of tomato fruit cell walls during ripening by a cooperative action of expansins and polygalacturonases contributes to fruit softening, thereby increasing the fruit susceptibility to *B. cinerea* (Cantu *et al.*, 2008). We propose that the stimulation of grey mould disease by ethylene treatment exclusively results from ethylene-induced senescence and ripening processes in the host and not from effects of ethylene on the pathogen.

MATERIALS AND METHODS

Plant material

The tomato (*Solanum lycopersicum*) lines used in this experiment were cv. Moneymaker, cv. Pearson, the homozygous mutant *Nr/Nr* in the cv. Pearson background, the transgenic line UC8338, expressing a bacterial ACC deaminase and its non-transgenic progenitor UC82B (Klee *et al.*, 1991). All plants were grown in potting soil for 6 weeks in the greenhouse at 23°C with a 16 h photoperiod, in soil supplemented with nutrients (ten Have *et al.*, 1998).

Fungal growth conditions

Botrytis cinerea wild-type haploid strain B05.10 was used for all experiments. To grow mycelium or isolate conidia, malt extract agar plates (Difco) were inoculated with conidia and incubated at 20°C. Plates, which were completely covered with mycelium, were placed under near-UV light for 16 h to induce sporulation. Conidia were harvested from sporulating plates one week later, using sterile water. The suspension was filtered through glasswool, conidia were pelleted by centrifugation at 800 rpm during 5 minutes and re-suspended in sterile water.

Culture growth for RNA analysis

$5x10^6$ conidia of wild type and transformed *B. cinerea* isolates were inoculated in 25 ml potato dextrose broth medium in 150 ml bottles that were sealed with an aluminium lid with rubber septum. Ethylene was injected through the septum to a final concentration of 200 ppm and the culture was incubated for 24 h at 20°C in a rotary shaker at 180 rpm. A sample was taken; ethylene was added again to 200 ppm. Incubation was continued for 24 h and another sample was taken. The mycelium was separated from culture filtrate over Miracloth (Calbiochem), collected in tubes and rapidly submerged in liquid nitrogen and freeze dried.

Ethylene treatment

Mycelia plugs of 3 mm in diameter from wild type strain B05.10 and Δ*Bchhk*5 mutants were inoculated on plates containing malt extract agar (Oxoid), potato dextrose agar (Oxoid) or oatmeal agar (Sigma) and placed in a 10 L desiccator. Ethylene was injected through a sealed rubber lid to a final concentration of 200 ppm. The control was placed under the same conditions in a desiccator in ethylene-free atmosphere. The colony diameter was measured every day over four days, until the colony reached the edge of the Petri dish. After opening the desiccator for colony diameter measurement, ethylene was readjusted to 200 ppm.

Construction of the gene replacement cassette

Three fragments were generated (5'-HHK5, 3'-HHK5 and the hygromycin selection marker cassette) and individual fragments were joint by a single step overlap-extension PCR. Based on genomic DNA sequences, two sets of primers were designed to generate 5'HHK5 and 3' HHK5 fragments (Table 3). The 5' HHK5 fragment (500 bp) was amplified using primers HHK5_5for and HHK5_5SOE. The 3' HHK5 fragment (500 bp) was amplified using primers HHK5_3SOE and HHK5_3rev. The HHK5_5SOE and HHK5_3SOE primers contain an

extension of around 20 nucleotides (underlined in Table 3) which are complementary to the primers that amplified the hygromycin cassette from vector pLOB1. Approximately 50-100 ng of genomic DNA from the strain B05.10 was used as template in 50 µl PCR reactions.

The hygromycin cassette (2700 bp) - abbreviated as HYG- consists of the hygromycin B phosphotransferase (*hph*) gene from *E. coli* under control of the OliC promoter from *Aspergillus nidulans* and a terminator fragment from *B. cinerea*. HYG was amplified using primers 20 and 21 (Table 3) and as template 20 ng of a HYG-containing plasmid. The PCR conditions were as described by Kars *et al.* (2005). The three PCR products were analyzed by gel electrophoresis and joint to a single fragment by overlap extension PCR using nested primers HHK5-5Nfor and HHK5-3Nrev. The resulting fragment was cloned into PCR-BluntII TOPO® vector and transformed in One Shot®TOP10 competent *E.* coli (Invitrogen).

Table 3. Primers used to generate the gene replacement fragments.

Gene	Fragment	Name	Sequence
*Bchhk*5	5'HHK5	HHK5_5for	GGCCTGGTTGTCCTGGTGGCTG
		HHK5_5SOE	TACTAACAGATATCAAGCTTCGCCTAGGAAAGCGGGCTGG
	3'HHK5	HHK5_3SOE	GGGTACCGAGCTCGAATTCCTCCCAGTTAGCAGGCCTTATGGG
		HHK5_3rev	GCCTCCTGCCCATCCTTGGCGACC
	Construct	HHK5_N5	CCATTGATTGTGCTCGCTCTCGCTACC
		HHK5_N3	GGCGACCACCACGTCATACACGTCTTC
cassette	HYG	20	GAATTCGAGCTCGGTACCC
		21	AAGCTTGATATCTGTTAGTA

Underlined sequences indicate primer extensions complementary to the primers that amplified the hygromycin cassette.

Transformation of *B. cinerea*

Protoplast preparation, transformation, selection of transformants and single spore purification of homokaryotic mutants were performed as described (Kars *et al.*, 2005).

Southern analysis

Genomic DNA from 4-day-old cultures was isolated with a Puregene DNA purification kit (Gentra systems) from monospore isolates of transformants and the control B05.10. The DNA yield was determined by electrophoresis. 1.5 µg of genomic DNA from Δ*Bchhk*5 mutants and B05.10 was digested with *BsrDI*, size separated on a 1% agarose gel and blotted onto Hybond-N^+ (Amersham) as described (Sambrook *et al.*, 1989). Blots were hybridized in

Church buffer at 65°C for 48 h in the presence of probe labeled with α-^{32}P-dCTP (Amersham) and a Prime-a-Gene labeling kit according to manufacturer's manual (Promega). The blots were washed twice in 2X SSC/0.5 % (w/v) SDS and once in 0.5XSSC/0.5% (w/v) SDS at 65°C. Autoradiograms were made using Kodak Scientific Imaging film X-OMAT AR with an intensifying screen at -80°C overnight. The probe used for Southern blots was a 3.5 kb fragment containing the hygromycin cassette flanked by 500 bp from the *Bchhk*5 gene.

RNA analysis

Total RNA was isolated using TRIzol (LifeTechnologies) according to the manufacturer's recommendations. Electrophoresis under denaturing conditions, blotting and hybridization were performed as described previously (Prins *et al.*, 2000). Fragments of the *Bcspl*1, *Bchsp*30 and *BcactA* genes, used as probes in RNA hybridization, were obtained by PCR amplification from *B. cinerea* genomic DNA using primer combinations listed in Table 4.

Table 4. Primers used to generate *Bcspl*1, *Bchsp*30 and *BcactA* fragments used as probes.

Gene	Name	Sequence
*Bcspl*1	Bcspl1- for	ATGCAATTCCCAACTCTCGC
	Bcspl1- rev	AAGCACTCTTATCGACTTGGGCG
*Bchsp*30	Bchsp30- for	GTCTTTCTTCCCACGACACTACA
	Bchsp30- rev	GGTGATTTTGCGGCCTTCTTGC
BcactA	BcactA- for	CCCAATCAACCCAAAGTCCAACAG
	BcactA- rev	CCACCGCTCTCAAGACCCAAGA

Infection assay

Conidia of sporulating *B. cinerea* cultures, wild-type strain B05.10 and Δ*Bchhk*5 mutants were harvested, resuspended in Gamborg's B5 medium (Duchefa), supplemented with 10 mM glucose and 10 mM potassium phosphate, pH 6.0 (10^6 conidia/ml). The conidial suspension was applied in 3 droplets of 2 µl each onto tomato leaflets on both sides of the central vein. The 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 100 %. The boxes were placed at 20°C with a diurnal cycle of 16 h light and 8 h darkness. At 72 h post-inoculation, the diameter of the spreading lesions was measured. Statistical analysis was performed using Student's *t*-test (two-tailed distribution, two-sample unequal variance) on each leaf.

REFERENCE LIST

Alonso, J. M., Stepanova, A. N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F. M. and Ecker, J. R. (2003). "Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis." Proceedings of the Natural Academy of Sciences of the United States of America **100**: 2992-7.

Aravind, L. and Ponting, C. P. (1997). "The GAF domain: an evolutionary link between diverse phototransducing proteins." Trends in Biochemical Sciences **22**: 458-9.

Aravind, L. and Ponting, C. P. (1999). "The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins." FEMS Microbiology Letters **176**: 111-6.

Benito, E. P., ten Have, A., van 't Klooster, J. W. and van Kan, J. A. L. (1998). "Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea* " European Journal of Plant Pathology **104**: 207-220.

Bleecker, A. B., Estelle, M. A., Somerville, C. and Kende, H. (1988). "Insensitivity to Ethylene Conferred by a Dominant Mutation in *Arabidopsis thaliana*." Science **241**: 1086-1089.

Brown, E. G. and Burns, J. K. (1998). "Enhanced activity of abscission enzymes predisposes oranges to invasion by *Diplodia natalensis* during ethylene degreening." Postharvest Biology and Technology **14**: 217-227.

Cantu, D., Vicente, A. R., Greve, L. C., Dewey, F. M., Bennett, A. B., Labavitch, J. M. and Powell, A. L. (2008). "The intersection between cell wall disassembly, ripening, and fruit susceptibility to *Botrytis cinerea*." Proceedings of Natural Academy of Sciences of the United States of America **105**: 859-64.

Catlett, N. L., Yoder, O. C. and Turgeon, B. G. (2003). "Whole-genome analysis of two-component signal transduction genes in fungal pathogens." Eukaryotic Cell **2**: 1151-61.

Chagué, V., Danit, L. V., Siewers, V., Schulze-Gronover, C., Tudzynski, P., Tudzynski, B. and Sharon, A. (2006). "Ethylene sensing and gene activation in *Botrytis cinerea*: a missing link in ethylene regulation of fungus-plant interactions?" Molecular Plant-Microbe Interaction **19**: 33-42.

Chang, C., Kwok, S. F., Bleecker, A. B. and Meyerowitz, E. M. (1993). "Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators." Science **262**: 539-44.

Chang, C. and Stadler, R. (2001). "Ethylene hormone receptor action in Arabidopsis." Bioessays **23**: 619-27.

Diaz, J., ten Have, A. and van Kan, J. A. (2002). "The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*." Plant Physiology **129**: 1341-51.

El Kazzaz, M. K., Sommer, N. F. and Kader, A. A. (1983). "Ethylene effects on in vitro and in vivo growth of certain postharvest fruit-infecting fungi." Phytopathology **73**: 998-1001.

Elad, Y. (1988). "Involvement of ethylene in the disease caused by *Botrytis cinerea* on rose and carnation flowers and the possibility of control." Annals of Applied Biology **113**: 589-598.

Elad, Y. and Volpin, H. (1988). "The involvement of ethylene, and calcium in gray mold of pelargonium, Ruscus, and rose plants." Phytoparasitica **16**: 119-132.

Flaishman, M. A. and Kolattukudy, P. E. (1994). "Timing of fungal invasion using host's ripening hormone as a signal." Proceedings of the Natural Academy of Sciences of the United States of America **91**: 6579-83.

Govrin, E. M. and Levine, A. (2000). "The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*." Current Biology **10**: 751-7.

Guo, H. and Ecker, J. R. (2004). "The ethylene signaling pathway: new insights." Current Opinion in Plant Biology **7**: 40-9.

Gúzman, P. and Ecker, J. R. (1990). "Exploiting the triple response of Arabidopsis to identify ethylene-related mutants." Plant Cell **2**: 513-23.

Hall, N., Keon, J. P. R. and Hargreaves, J. A. (1999). "A homologue of a gene implicated in the virulence of human fungal diseases is present in a plant fungal pathogen and is expressed during infection " Physiological and Molecular Plant Pathology **55**: 69-73.

Hamilton, A. J., Lycett, G. W. and D., G. (1990). "Antisense gene that inhibits synthesis of the hormone ethylene in transgenic plants " Nature **346**: 284-286.

Kars, I., McCalman, M., Wagemakers, L. and van Kan, J. A. L. (2005). "Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme*1 and *Bcpme*2 are dispensable for virulence of strain B05.10." Molecular Plant Pathology **6**: 641-652.

Kepczynska, E. (1993). "Involvement of ethylene in the regulation of growth and development of the fungus *Botrytis cinerea* Pers. ex. Fr." Plant Growth Regulation **13**: 65-69.

Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. and Ecker, J. R. (1993). "CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases." Cell **72**: 427-41.

Klee, H. J., Hayford, M. B., Kretzmer, K. A., Barry, G. F. and Kishore, G. M. (1991). "Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants." Plant Cell **3**: 1187-93.

Kunz, C., Vandelle, E., Rolland, S., Poinssot, B., Bruel, C., Cimerman, A., Zotti, C., Moreau, E., Vedel, R., Pugin, A. and Boccara, M. (2006). "Characterization of a new, nonpathogenic mutant of *Botrytis cinerea* with impaired plant colonization capacity." New Phytologist **170**: 537-50.

Lanahan, M. B., Yen, H. C., Giovannoni, J. J. and Klee, H. J. (1994). "The never ripe mutation blocks ethylene perception in tomato." Plant Cell **6**: 521-30.

Panaretou, B. and Piper, P. W. (1992). "The plasma membrane of yeast acquires a novel heat-shock protein (hsp30) and displays a decline in proton-pumping ATPase levels in response to both heat shock and the entry to stationary phase." European Journal of Biochemistry **206**: 635-40.

Piper, P. W., Ortiz-Calderon, C., Holyoak, C., Coote, P. and Cole, M. (1997). "Hsp30, the integral plasma membrane heat shock protein of *Saccharomyces cerevisiae*, is a stress-inducible regulator of plasma membrane H(+)-ATPase." Cell Stress Chaperones **2**: 12-24.

Prins, T. W., Wagemakers, L., Schouten, A. and van Kan, J. A. L. (2000). "Cloning and characterization of a glutathione S-transferase homologue from the plant pathogenic fungus *Botrytis cinerea*." Molecular Plant Pathology **1**(3): 169-178.

Roze, L. V., Calvo, A. M., Gunterus, A., Beaudry, R., Kall, M. and Linz, J. E. (2004). "Ethylene modulates development and toxin biosynthesis in aspergillus possibly via an ethylene sensor-mediated signaling pathway." Journal of Food Protection **67**: 438-47.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: a laboratory manual. Cold Spring Harbor, Cold Spring Harbor Press.

ten Have, A., Mulder, W., Visser, J. and van Kan, J. A. L. (1998). "The endopolygalacturonase gene *Bcpg*1 is required for full virulence of *Botrytis cinerea*." Molecular Plant-Microbe Interaction **11**: 1009-1016.

Williamson, B. (1994). Latency and quiescence in survival and success of fungal plant pathogens. Ecology of plant pathogens. J. P. Blakeman and B. Williamson. Oxford, UK, CAB International**:** 187-207.

Chapter 3

Expression and functional analysis of NLP-encoding genes of *Botrytis cinerea*

Chapter 3

Expression and functional analysis of NLP-encoding genes of *Botrytis cinerea*

Yaite Cuesta, Alexander Schouten, Beatrice Uwumukiza and Jan van Kan

SUMMARY

Nep1-like proteins (NLPs) have been described in bacteria, oomycetes and fungi and have been proposed to act as phytotoxins in dicotyledonous plants. The *Botrytis cinerea* genome contains two genes encoding NLPs, named *Bcnep*1 and *Bcnep*2. The genes are differentially expressed during infection of tomato and *Nicotiana benthamiana* leaves. *Bcnep*1 is predominantly expressed during the formation of primary lesions, whereas the expression of *Bcnep*2 starts at the onset of lesion expansion and increases with the fungal biomass. Single knock-out mutants of either *Bcnep*1 or *Bcnep*2 gene showed no reduction of virulence on tomato or *N. benthamiana*. Ethylene emission by leaves inoculated with Δ*Bcnep* mutants was not significantly different from the parental wild type strain. From this study we conclude that BcNEP proteins are not essential in the infection process of *B. cinerea*.

INTRODUCTION

The main determinant of *Botrytis cinerea* to be a successful necrotroph is the production of secreted metabolites and proteins, able to kill the host (Durán-Patrón *et al.*, 2000; Colmenares *et al.*, 2002; Kars and van Kan, 2004; Reino *et al.*, 2004). *B. cinerea* isolates can produce two chemically different phytotoxic metabolites (botrydial and botcinic acid) and there are differences in the types and amounts of phytotoxins produced by individual isolates (Reino *et al.*, 2004). Deletion of a cytochrome P450 gene, named *Bcbot*1, led to a deficiency in botrydial production. The mutation resulted in a reduction in virulence in strain T4, but not in strains B05.10 and ATCC 58025 (Siewers *et al.*, 2005). This was explained by the observation that strains B05.10 and ATCC 58025 still produce botcinolide whereas T4 does not produce botcinolide, suggesting functional overlap between the phytotoxins.

The *B. cinerea* genome sequence revealed the presence of a number of genes encoding phytotoxic proteins, including a protein designated as SPL1 (Kunz *et al.*, 2006) and two paralogous proteins, BcNEP1 and BcNEP2, members of the NLP protein family (Staats *et al.*, 2007; Schouten *et al.*, 2008). Both proteins induce necrosis when infiltrated into leaves of dicot plants (Figure 1), and when expressed by agro-infiltration (Schouten *et al.*, 2008).

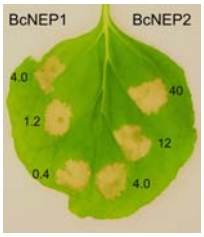


Fig. 1. Response of *Nicotiana benthamiana* leaf infiltrated with purified BcNEP1 (left hand side of the leaf) and BcNEP2 (right hand side of the leaf), expressed in *Pichia pastoris*. The concentrations applied range from 0.4 to 4µM for BcNEP1 and from 4 to 40 µM for BcNEP2 as indicated. Control leaves infiltrated with 10 mM phosphate buffer, pH 7, showed no symptoms. (From Schouten *et al.*, 2008)

NLPs, first discovered in *Fusarium oxysporum* f.sp. *erythroxyli*, are present strictly in bacterial, fungal and oomycete microbes, including pathogens and non-pathogens (Bailey, 1995; Gijzen and Nürnberger, 2006). Purified NLPs in low concentrations can induce callose apposition, accumulation of reactive oxygen species and ethylene, activation of genes involved in stress and defence responses (Veit *et al.*, 2001; Qutob *et al.*, 2002; Pemberton and Salmond, 2004) and at higher concentrations induce localized cell death. NLPs are only phytotoxic to dicotyledonous plant cells (Gijzen and Nürnberger, 2006; Staats *et al.*, 2007). In spite of all these observations the importance of NLPs in pathogenesis remains elusive. Disruption of *nep*1 in *F. oxysporum* f.sp. *erythroxyli* did not affect its ability to cause wilting on coca plants (Bailey *et al.*, 2002). The only evidence of a role of NLPs in virulence is in the soft-rot bacteria *Erwinia carotovora* subsp. *carotovora* (syn. *Pectobacterium carotovorum*) and *E. carotovora* subsp. *atroseptica.* Disruption of nip_{ecc} and nip_{eca} genes reduced virulence

on potato (Pemberton *et al.*, 2005). Virulence of *P. carotovorum* mutants could partially be restored by complementation with NLPs from the oomycetes *Phytophthora parasitica* and *Pythium aphanidermatum* (Ottmann *et al.*, 2009). Gene expression studies have demonstrated that NLP genes are differentially expressed during infection. In *Phytophthora sojae*, a hemibiotroph that causes root and stem rot on soybean, the gene encoding PsojNIP is expressed exclusively during late stages of the infection, corresponding with the transition to the necrotrophic phase (Qutob *et al.*, 2002). *Moniliophthora perniciosa*, a basidiomycete that causes witches broom disease of cacao, possesses two NLP genes. *Mpnep*1 is expressed in the biotrophic and saprophytic phases whereas *Mpnep*2 is expressed only in the biotrophic phase (Garcia *et al.*, 2007). The aim of this study was to investigate the expression pattern of *Bcnep* genes during colonization and their contribution to the virulence of *B. cinerea* on different hosts. We generated mutant strains deficient in *Bcnep*1 and *Bcnep*2 and tested the virulence of mutants on tomato and *Nicotiana benthamiana* leaves.

RESULTS

BcNEP1 and BcNEP2 are differentially expressed during infection

Bcnep transcripts and the release of BcNEP proteins in culture medium could not be detected during growth of *B. cinerea* B05.10 *in vitro* in any medium tested (not shown). Expression of *Bcnep* genes during infection was investigated on detached tomato leaves. Leaflets inoculated with *B. cinerea* were collected in a time course. Total RNA was extracted, separated on gel, blotted onto a nylon membrane and hybridized with *Bcnep*1 or *Bcnep*2 cDNA as probes (Figure 2). *Bcnep*1 transcript was detected as early as 8 h post inoculation (hpi) and its level increased until 48 hpi, corresponding with the formation of primary lesions and the subsequent lag period that occurs in tomato leaf infection (Benito *et al.*, 1998). *Bcnep*1 transcript levels declined from 56 hpi onwards. Transcripts of *Bcnep*2 were first detected around 40 hpi and increased over time with lesion expansion, which starts between 40 and 64 hpi (Benito *et al*., 1998). The transcript profile of *Bcnep*2 was similar to that of *Bcact*1 (not shown), used as a measure of *B. cinerea* biomass during infection (Benito *et al.*, 1998).

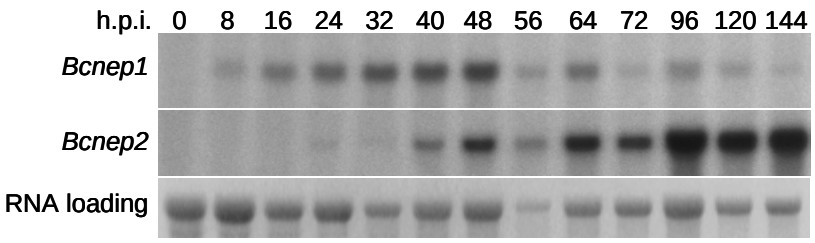


Fig. 2. *Bcnep*1 and *Bcnep*2 expression during tomato leaf infection by *B. cinerea*. Samples were taken at different times until 144 hpi. Blots were hybridized with *Bcnep*1 or *Bcnep*2 cDNA. The lower panel shows an image of one of the rRNA bands in the ethidium bromide-stained gel.

Gene replacement

Single gene knock-out mutants were generated by transforming *B. cinerea* with PCR fragments containing a hygromycin cassette (2.4 kb) flanked at both sides by ± 400 bp from the target gene. A total of five and nine transformants were obtained for *Bcnep*1 and *Bcnep*2, respectively. Hygromycin-resistant colonies were screened by PCR to detect transformants in which homologous recombination had occurred. Recombinants were subjected to monospore isolation to resolve heterokaryons and Southern hybridization performed on genomic DNA from single-spore transformants. For each gene, one independent mutant was obtained in which homologous recombination had occurred and no additional ectopic integrations were detected. A 5.8 kb *EcoRI* fragment, containing the *Bcnep*1 gene in wild type strain B05.10, was replaced by 5.8 kb and 2.4 kb fragments in the mutant. A 5.9 kb *Eco*RI fragment in the wild type strain, containing the *Bcnep*2 gene, was replaced by 5.3 kb and 3.0 kb fragments in the mutant (Figure 3). A 2.1 kb *Hind*III fragment, containing the *Bcnep*1 gene in wild type strain B05.10, was replaced by 1.4 kb and 3.1 kb fragments in the mutant (not shown). A 5.5 kb *Hind*III fragment in the wild type strain, containing the *Bcnep*2 gene, was replaced in the mutant by 0.8 kb and 7.1 kb fragments (not shown). An additional ectopic insertion was detected in mutants Δ*Bcnep1-2* and *ΔBcnep2-1* after digestion with *Hind*III (not shown).

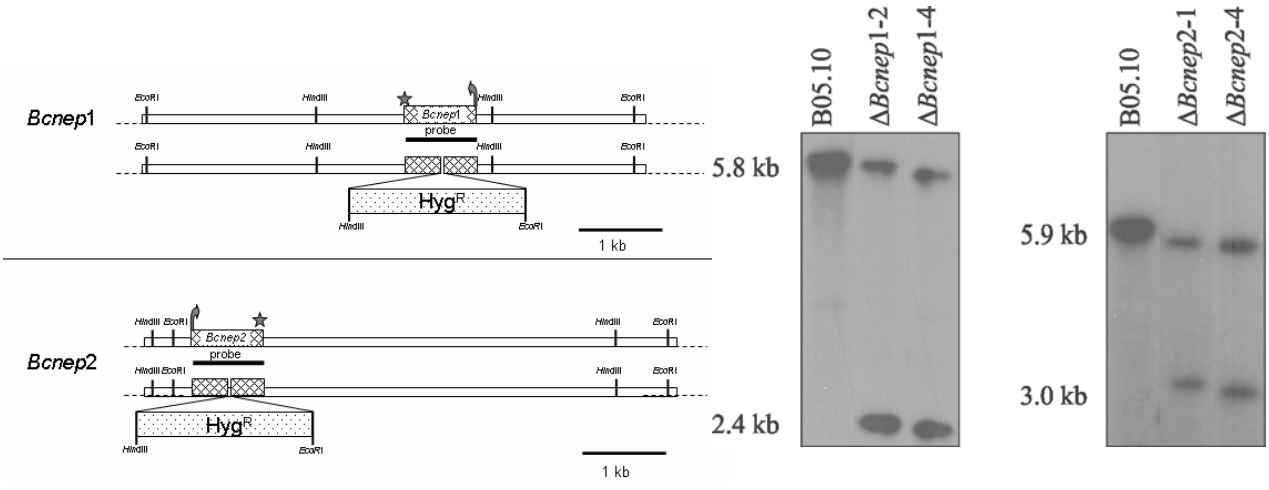


Fig. 3. A: Organization of the *Bcnep*1 and *Bcnep*2 locus before and after disruption by integration of a hygromycin selection marker by homologous recombination. The translation start codons (arrow) and stop codons (diamond), as well as the *Eco*RI and *Hin*dIII restriction sites used for Southern analysis are indicated. The dotted box represents the hygromycin marker cassette.
B. Southern analysis of the Δ*Bcnep*1 and Δ*Bcnep*2 mutants. Genomic DNA from the wild type strain B05.10 and the mutants (Δ*Bcnep1-2*, Δ*Bcnep1-4*, Δ*Bcnep2-1* and Δ*Bcnep2-4*) was digested with *Eco*RI, and hybridized with cDNA from *Bcnep*1 or *Bcnep*2 genes.

Protein detection

Total protein was extracted from *N. benthamiana* leaves inoculated with B05.10, Δ*Bcnep*1 or Δ*Bcnep*2 mutant strains at 24, 48 and 72 hpi. Infection on *N.benthamiana* progressed faster than on tomato leaves; the lag phase reported by Benito *et al*. (1998) to occur on tomato between 16 and 48 hpi was not observed on *N. benthamiana*, and the lesions expanded from 16 hpi onwards. Proteins were concentrated and analyzed by Western blot using polyclonal antibodies against BcNEP1 and BcNEP2. Purified BcNEP proteins (200 ng) and total protein from non-inoculated leaves were used as controls. BcNEP1 was detected only in the samples collected at 24 hpi from leaves inoculated with the wild type strain B05.10 and the Δ*Bcnep*2 mutant. The protein band detected in these samples had an apparent molecular weight of 75 kDa, whereas BcNEP1 produced in *P. pastoris* has an apparent molecular weight of 27 kDa. No BcNEP1 protein was detected at any time point in the samples isolated from leaves inoculated with the Δ*Bcnep*1 mutant. BcNEP2 was detected in samples collected at 48 and 72 hpi from leaves inoculated with B05.10 and the Δ*Bcnep*1 mutant and this protein had the same apparent molecular weight as the purified BcNEP2, produced in *P. pastoris* (Figure 4). No BcNEP2 protein was detected at any time point in the samples isolated from leaves inoculated with the Δ*Bcnep*2 mutant.

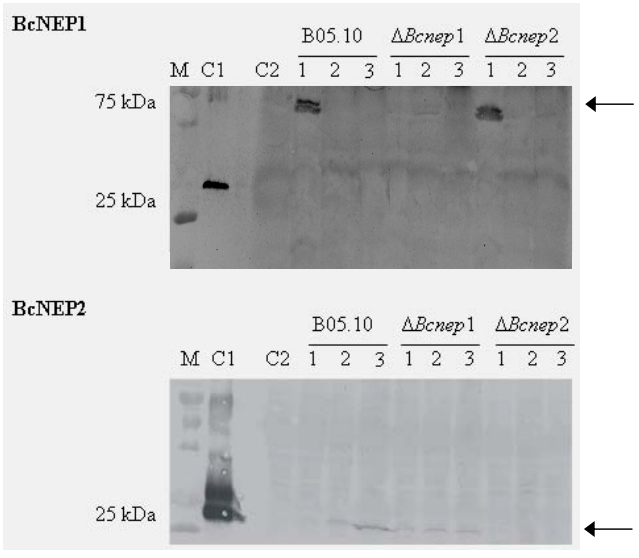


Fig. 4. Immunological detection of BcNEP proteins during colonization of *Nicotiana benthamiana* leaves (indicated by the arrow). Total protein was extracted from *N. benthamiana* leaves at 1, 2 and 3 dpi with a conidial suspension from B05.10, Δ*Bcnep*1-4 and Δ*Bcnep*2-4 mutant strains. Control lanes marked as C1 contain purified protein (200 ng), while control lanes marked C2 contain total protein from uninoculated leaves. The lanes marked as M contain a molecular size standard. Arrows indicate positions of proteins detected on the blot.

Virulence assay

Conidial suspensions of B05.10, Δ*Bcnep*1 or Δ*Bcnep*2 strains were inoculated on detached tomato and *N. benthamiana* leaves and infection progress was monitored over several days. Both mutants formed primary lesions at the same rate as the wild type strain B05.10. Lesion diameters caused by the mutants were not significantly different from those caused by the wild type in both plants at 72 hpi (Figure 5, Table 1) or any other time point (not shown).

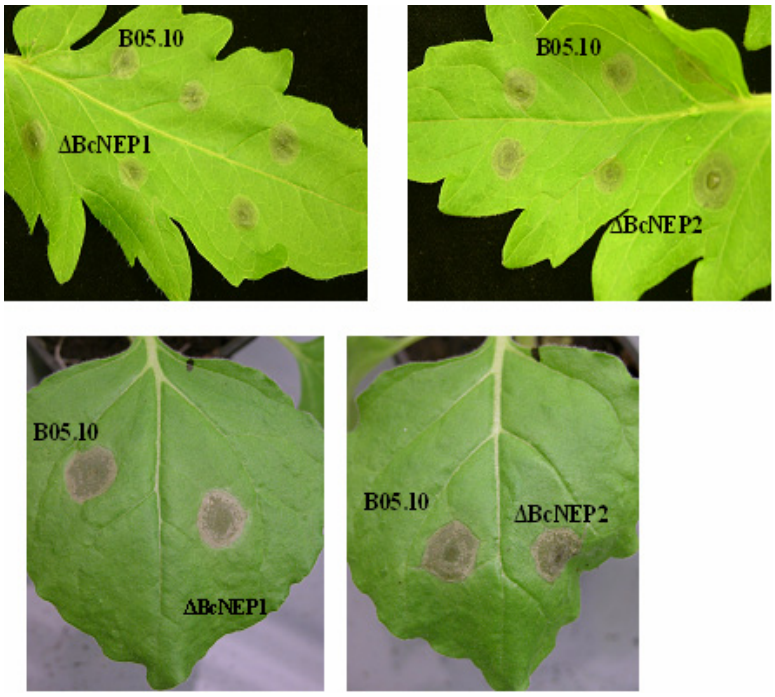


Fig. 5. Virulence assays. The wild type strain B05.10 and the mutants Δ*Bcnep*1-4 and *ΔBcnep*2-4 were inoculated on detached tomato leaves (Moneymaker Cf4) **(A)**, or on *N. benthamiana* **(B)**.

Table 1. **Diameter of lesions by *B. cinerea* wild type and mutant strains on tomato and *Nicotiana benthamiana* leaves (sizes given in mm ± standard deviation).**

	B05.10	Δ*Bcnep*1-4	B05.10	Δ*Bcnep*2-4
Tomato (n= 26)	14.5 ± 1.3	15.1± 1.3	14.8± 1.4	15.6 ± 1.3
N. benthamiana (n= 14)	12.4 ± 1.9	12.4 ± 2.7	13.2 ± 1.8	13.1 ± 2.6

Ethylene production by leaves inoculated by Δ*Bcnep* mutant strains

B. cinerea-infected plants release large amounts of the plant hormone ethylene. In order to evaluate whether ethylene induced during *B. cinerea* infection is a consequence of a response to BcNEP proteins, detached *N. benthamiana* leaves were inoculated either with the wild type strain B05.10, Δ*Bcnep*1 or Δ*Bcnep*2 mutants and placed in bottles at high humidity. At various times the bottles were closed for 1 h and ethylene production was measured, over a

time span of five days (Figure 5, Table 2). At 16 hpi, ethylene was detected even though no disease symptoms were visible yet; at this time point, the ethylene production following inoculation with wild type strain B05.10 was twice as high as the level following inoculation with the mutants. By 24 hpi, the leaves inoculated with all three strains produced similar amounts of ethylene. At 48 hpi, disease symptoms became evident and ethylene production increased rapidly, reaching maximum levels at 72 hpi. At this time point, more than 50% of the leaf surface was colonized and lesions started to converge. At later time points, lesion growth rate declined and ethylene production decreased. At 120 hpi, the leaf surfaces were completely colonized and only low levels of ethylene were produced. Leaves inoculated only with PDB showed a transient ethylene production and never reached levels above 2 nl/ h/ leaf.

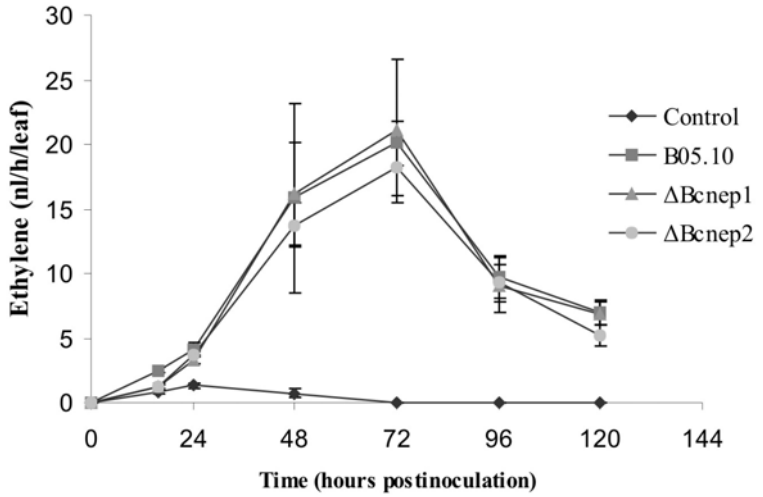


Fig. 5. Ethylene production induced by *B. cinerea* wild type strain and mutants on *N. benthamiana* leaves. Five droplets of 2 µl of conidial suspension from each strain (wild type B05.10, mutants Δ*Bcnep*1-2 and Δ*Bcnep*2-4) were inoculated on *N. benthamiana* leaves. Leaves inoculated with PDB medium were used as control. The amount of ethylene produced in one hour was measured at different time points using gas chromatography. The means of four replicates are shown. Error bars represent standard errors of the means. On the fifth day, leaf surfaces were almost completely colonized by *B. cinerea.*

Table 2. Ethylene production rates in *N. benthamiana* inoculated with *B. cinerea* wild type and mutant strains (given in nl per hour per infiltrated leaf).

Strain / hpi	Ethylene (nl/ h/leaf, n=4)						
	0	16	24	48	72	96	120
Control[a]	0	0.8	1.4	0	0	0	0
B05.10	0	2.5	4.1	15.8	20.2	9.8	7.0
Δ*Bcnep1*	0	1.2	3.3	16.1	21.1	9.1	6.9
Δ*Bcnep2*	0	1.3	3.8	13.7	18.3	9.3	5.3

[a] leaves inoculated with PDB medium.

DISCUSSION

The presence of NLP-encoding gene families in phytopathogenic fungi and oomycetes (Qutob *et al.*, 2006) suggests that NLPs may play a role in the plant–pathogen interaction, especially for pathogens that have a hemibiotrophic or necrotrophic lifestyle. In this study we report the expression and functional analysis of two NLP-encoding genes in *B. cinerea*, designated *Bcnep*1 and *Bcnep*2. The expression patterns of *Bcnep* genes suggest that they may play different roles during pathogenesis. Transcripts of *Bcnep*1 were detected very early after inoculation, coinciding with the formation of primary lesions whereas transcripts of *Bcnep*2 were detected from the onset of lesion expansion and increased with the fungal biomass. Interestingly, the *Bcnep*1 promoter region contains multiple sequence motifs that, in *Aspergillus nidulans*, act as binding sites for transcription factor AbaA, which is crucial for the initiation of conidia development in this fungus (Andrianopoulos and Timberlake, 1994). The genome of *B. cinerea* contains a gene homologous to *A. nidulans Aba*A, but its role in regulating the expression of the *Bcnep* genes remains to be studied.

Immunological detection demonstrated the transient presence of BcNEP1 in *B. cinerea*-infected *N. benthamiana* leaves at 1 dpi, whereas BcNEP2 accumulated during disease progression at 2 and 3 dpi (Figure 4). The timing of protein accumulation in *N. benthamiana* (Fig. 4) slightly differed from the timing of mRNA accumulation in tomato (Fig. 2) due to differences in disease progress between the hosts used. *B. cinerea* infection in *N. benthamiana* appears to skip the lag phase that was observed in tomato (Benito *et al*., 1998) and immediately proceeds from primary lesion induction to the stage of lesion expansion. Remarkably, in protein samples collected from *B. cinerea*-infected *N. benthamiana*, BcNEP1 migrated as a protein with an apparent molecular weight of 75 kDa, while BcNEP1 produced in *P. pastoris* has an apparent molecular weight of 27 kDa, which is in agreement with the amino acid sequence. One of the two NLPs from *Moniliophthora perniciosa*, MpNLP1, was also reported to show aberrant migration in non-denaturing protein gels due to its ability to form dimers or trimers (Garcia *et al*., 2007). Oligomerization was suggested to be mediated by intermolecular cysteine bridges as the MpNLP1 protein migrated as a 25 kDa protein in denaturing gels (Garcia *et al*., 2007). The aberrant migration of BcNEP1 cannot be due to intermolecular cysteine bridges, as protein samples from *B. cinerea*-infected *N. benthamiana* were denatured prior to electrophoresis and separated on denaturing gels. It is unknown whether the aberrant migration of BcNEP1 is due to a posttranslational modification that specifically occurs in *B. cinerea* but not in *P. pastoris*, or by a plant-mediated modification.

The fact that *Bcnep*1 was expressed very early during infection could indicate that the BcNEP1 protein is involved in the initial breakdown of plant membranes and in triggering plant cell death during primary lesion development (Schouten *et al.*, 2008). *Bcnep*2 transcript levels increased from the initiation of the lesion expansion phase onwards and followed the increase of fungal biomass, suggesting that BcNEP2 could be responsible for killing plant cells at the plant-fungus interface in the expanding lesions. The observed expression profile of the *Bcnep* genes during pathogenesis would predict that Δ*Bcnep*1 mutants might possibly be disturbed or delayed in primary lesion formation, whereas Δ*Bcnep*2 mutants might be affected in the lesion expansion rate. Gene replacement of *Bcnep*1 or *Bcnep*2, however, showed that these genes are not important for either early or late stages of the infection process. Single mutants did not show any reduction in virulence when compared with the parental strain on *N. benthamiana* and tomato plants. Attempts to generate double mutants were unsuccessful for unknown reasons. The loss of BcNEP-mediated necrotizing activity in the mutants could be compensated by the production of phytotoxic secondary metabolites (e.g. botrydial and botcinic acid) or other phytotoxic proteins (Deighton *et al.*, 2001; Brito *et al.*, 2006; Tani *et al.*, 2006), which could be masking any phenotype that Δ*Bcnep* mutants might display.

Ethylene emission by leaves inoculated with Δ*Bcnep* mutants or the wild type strain was not significantly different. Therefore, we conclude that BcNEP1 and BcNEP2 do not significantly contribute to the induction of ethylene production during *B. cinerea* infection.

MATERIAL AND METHODS

Northern blot analysis

Total RNA was extracted from *B. cinerea*-inoculated tomato leaves that were harvested at different intervals following inoculation by spraying a suspension of conidia (10^6/ml) of *B. cinerea* strain B05.10 onto detached leaves as described (Benito *et al.*, 1998). RNA from uninoculated leaves was used as control. Leaves frozen in liquid nitrogen were disintegrated in a 12 ml centrifuge tube by vortexing with a pre-cooled spatula in liquid nitrogen at high speed. To the powder, 1 ml Trizol was added and incubated for 1 h at room temperature and inverted regularly. The mixtures were centrifuged at 14000 rpm for 10 min to precipitate non-dissolved material and polysaccharides. The supernatant was transferred to clean tubes, mixed with 0.2 ml chloroform, shaken for 2 to 3 min at room temperature and centrifuged at 14000 rpm for 15 min. After centrifugation the aqueous phase containing total RNA was transferred to a 1.5 ml tube and 0.5 ml iso-propanol was added to precipitate RNA. The tubes were

inverted several times, incubated 10 min at room temperature and centrifuged at 14000 rpm. Pellets were washed twice with 1 ml 75% (v/v) ethanol. The centrifugation between washes was at 8000 rpm for 5 min. The pellets were air dried and dissolved in 0.2 ml RNAse free water and incubated for 10 min at 55°C. All centrifugation steps were performed at 4°C. Northern blotting and hybridization were performed as described (Prins *et al.*, 2000). cDNA fragments from *Bcnep*1 and *Bcnep*2 genes were used as probes.

Generation of gene replacement mutants

B. cinerea mutants in which the *Bcnep*1 or the *Bcnep*2 gene was disrupted were generated as described by Kars *et al.* (2005) with some modifications. Three fragments were generated (5'NEP, 3'NEP and the hygromycin selection marker cassette), and the individual fragments were joint by a single step overlap-extension PCR. Two sets of primers were designed to generate 5'NEP and 3'NEP fragments (Table 3). The 5'NEP1 fragment (807 bp) was amplified using NEP1_5for and NEP1_5SOE primers. The 3'NEP1 fragment (791 bp) was amplified using NEP1_3SOE and NEP1_3rev. The 5'NEP2 fragment (850 bp) was amplified using the primer pair NEP2_5for and NEP2_5SOE and the 3'NEP2 fragment (964 bp) was amplified using the primer pair NEP2_3SOE and NEP2_3rev. The NEP1_5SOE, NEP1_3SOE, NEP2_5SOE and NEP2_3SOE primers contain an extension of around 20 nt (underlined in Table 3) complementary to primers that amplified the hygromycin cassette.

Approximately 50-100 ng of genomic DNA from *B. cinerea* strain B05.10 was used as template in 50 µl PCR reactions, using 2.6 U Expand High Fidelity polymerase (Roche), 1X Expand High Fidelity buffer, 0.2 mM of each dNTP, and 1 µmol of each specific primer (Sigma Aldrich). The hygromycin cassette (2403 bp) - abbreviated as HYG- used as selection marker contains the hygromycin B phosphotransferase (*hph*) gene from *Escherichia coli* under the control of the OliC promoter and the TrpC terminator from *Aspergillus nidulans.* HYG was amplified in a similar reaction using primers 20a and 30 (Table 3) and as template 20 ng of a HYG-containing plasmid. The PCR conditions for all reactions were as follows: 1 cycle of 94ºC for 2 min; 10 cycles of 94 ºC for 15 sec, 50 ºC for 30 sec and 68 ºC for 3 min; 20 cycles of 94 ºC for 15 sec, 50 ºC for 30 sec and 68 ºC for 3 min increasing with 5 sec/ cycle, and a final extension at 68 ºC for 7 min. The PCR products were analyzed by agarose gel electrophoresis and purified using GFX PCR purification kit (Amersham Pharmacia).

The respective 5'NEP and 3'NEP fragments were joint with HYG by overlap extension PCR using nested primers (Table 3). The PCR conditions were as described above, and 40 ng of

each purified product were used as templates. The resulting amplified fragments were 3210 bp and 3188 bp respectively and were cloned into PCR-Blunt II TOPO® vector and transformed in One Shot®TOP10 chemically competent *E.* coli (Invitrogen).

Botrytis cinerea protoplast preparation, transformation, selection of transformants and single spore purification of heterokaryotic transformants to obtain homokaryotic mutants were performed as described by Kars *et al.* (2005).

Table 3. Primers used to generate the gene replacement fragments.

Gene	Fragment	Primer name	Primer sequence
*Bcnep*1	5'NEP1	NEP1_5for	GGTCCATCGCATGTCGTATTCGGA
		NEP1_5SOE	GCGCGCCGAGAGAGAAGCTTCATAGGTTTGGCCCTTGCTC
	3'NEP1	NEP1_3SOE	TCCCCGGGTACCGAGCTCGAATTCGGCTGGTCTGGAGGTCGCTATGG
		NEP1_3rev	CAATGCGGAGGACCGCGCGTGGTG
	Construct	NEP1_n5	CTATCCTCGCTGCTGCTGCAGCAG
		NEP1_n3	GAGGTTGTTGTTGAAGTTTGCGTC
*Bcnep*2	5'NEP2	NEP2_5for	GGACCCGCTTGCAATCTATTGTCTCC
		NEP2 5-SOE	GCGCGCCGAGAGAGAAGCTTGACCGGTACTGCTGCTGCAACC
	3'NEP2	NEP2_3SOE	TCCCCGGGTACCGAGCTCGAATTCGGATCAAACTACGCCATCATGTAC
		NEP2_3rev	GATCTGAATTGCGGTAGCAGGGGG
	Construct	NEP2_n5	GGTCTTGGCATCTACAGTCATTGCC
		NEP2_n3	GTAGCCTTCGCAAGATTGTCTGTG
Cassette	HYG	20a	GAATTCGAGCTCGGTACCCGGGGA
		30	AAGCTTCTCTCTCGGCGCG

Underlined sequences are primer extensions complementary to primers that amplified the hygromycin cassette.

Southern analysis

Genomic DNA was isolated with GenElute™ Plant Genomic DNA Miniprep Kit (Sigma) from transformants and wild type strain B05.10. 1.5 µg genomic DNA was digested with *EcoRI*, separated on a 1% agarose gel and blotted onto Hybond-N^+ (Amersham) as described (Sambrook *et al.*, 1989). Blots were hybridized in 0.25M Na-phosphate, pH 7.2, 1 mM EDTA, 7% SDS, 1% BSA (Church and Gilbert, 1984) at 65°C for 48 h in the presence of a probe, radioactively labeled with α-^{32}P-dCTP (Amersham) and a from *Bcnep*1 and *Bcnep*2 genes were used as probes. The blots were washed twice in 2X SSC/0.5 % (w/v) SDS and once in 0.5XSSC/0.5% (w/v) SDS at 65°C. Autoradiograms were made using Kodak Scientific Imaging film X-OMAT AR with intensifying screen at -80°C overnight.

Virulence assay of mutants

Conidia of sporulating *B. cinerea* cultures of wild type strain B05.10, Δ*Bcnep*1 and Δ*Bcnep*2 mutants were harvested and suspended in 12 g/l of Potato Dextrose Broth medium (10^6 conidia/ml). The conidial suspension was applied in 3 droplets of 2 µl each onto both halves of the tomato leaflets on opposite sides of the central vein. The compound leaves were incubated with their stem inserted in wet florist's foam oasis, in closed plastic boxes with a transparent lid to generate high humidity. The boxes were placed at 20°C with a diurnal cycle of 16 h light and 8 h darkness. The disease development was followed every day. At 72 h post-inoculation, the diameter of the spreading lesions was measured. Statistical analysis was done by Student's *t*-test (two-tailed distribution, two-sample unequal variance).

Ethylene induction in *Nicotiana benthamiana*

Four detached leaves of *N. benthamiana* (5 week-old) were inoculated each with five droplets of 2 µl of conidia suspended in 12 g/l PDB (10^6 conidia/ml) of B05.10, Δ*Bcnep*1 or Δ*Bcnep*2 mutants. Leaves inoculated with PDB medium were used as control. Every inoculated leaf was dried at room temperature and subsequently inserted into opened 60 ml bottles. Bottles were placed into boxes containing wet filter paper and covered with plastic lids to generate high relative humidity. Air samples were taken at 0, 16, 24, 48, 72, 96 and 120 hpi for ethylene measurements. At each time point, the bottles were taken from the boxes and closed with rubber tight caps for exactly 1 h. Subsequently, 2 ml air samples were taken from each bottle and injected with a syringe into a gas chromatograph (Shimadzu 17A). Then, bottles were opened and placed back into the boxes until the next sampling.

To calculate the amount of ethylene produced per hour per leaf in each treatment, the average of ethylene concentrations from each treatment was multiplied by a calibration factor and by the volume of the bottles. The calibration factor was calculated from the average of several ethylene measurements from pure ethylene of known concentration.

Acknowledgements

Prof. Ernst Woltering and Dianne Somhorst (Food & Biobased Research Institute, Wageningen UR, The Netherlands) are acknowledged for supplying ethylene and for providing the GC facilities for ethylene measurements.

REFERENCE LIST

Andrianopoulos, A. and Timberlake, W. E. (1994). "The *Aspergillus nidulans aba*A gene encodes a transcriptional activator that acts as a genetic switch to control development." Molecular Cell Biology **14**(4): 2503-2515.

Bae, H., Bowers, J. H., Tooley, P. W. and Bailey, B. A. (2005). "NEP1 orthologs encoding necrosis and ethylene inducing proteins exist as a multigene family in *Phytophthora megakarya*, causal agent of black pod disease on cacao." Mycological Research **109**: 1373-1385.

Bailey, B. A. (1995). "Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of erythroxylum-coca." Phytopathology **85**: 1250-1125.

Bailey, B. A., Apel-Birkhold, P. C. and Luster, D. G. (2002). "Expression of Nep1 by *Fusarium oxysporum* f.sp. *erythroxyli* after gene replacement and overexpression using polyethylene glycol-mediated transformation." Phytopathology **92**: 833-841.

Benito, E. P., ten Have, A., van 't Klooster, J. W. and van Kan, J. A. L. (1998). "Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea* " European Journal of Plant Pathology **104**: 207-220.

Brito, N., Espino, J. J. and Gonzalez, C. (2006). "The endo-beta-1,4-xylanase xyn11A is required for virulence in Botrytis cinerea." Molecular Plant-Microbe Interaction **19**(1): 25-32.

Church, G. M. and Gilbert, W. (1984). "Genomic sequencing." Proceedings of Natural Academy of Sciences of the United States of America **81**(7): 1991-1995.

Colmenares, A. J., Aleu, J., Duran-Patron, R., Collado, I. G. and Hernandez-Galan, R. (2002). "The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*." Journal of Chemical Ecology **28**(5): 997-1005.

Deighton, N., Muckenschnabel, I., Colmenares, A. J., Collado, I. G. and Williamson, B. (2001). "Botrydial is produced in plant tissues infected by *Botrytis cinerea*." Phytochemistry **57**(5): 689-692.

Durán-Patrón, R., Hernández-Galán, R. and Collado, I. G. (2000). "Secobotrytriendiol and Related Sesquiterpenoids: New Phytotoxic Metabolites from *Botrytis cinerea*." Journal of Natural Products **63**: 182-184.

Garcia, O., Macedo, J. A., Tiburcio, R., Zaparoli, G., Rincones, J., Bittencourt, L. M., Ceita, G. O., Micheli, F., Gesteira, A., Mariano, A. C., Schiavinato, M. A., Medrano, F. J., Meinhardt, L. W., Pereira, G. A. and Cascardo, J. C. (2007). "Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*." Mycological Research **111**: 443-455.

Gijzen, M. and Nürnberger, T. (2006). "Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa." Phytochemistry **67**(16): 1800-1807.

Kars, I. and van Kan, J. A. L. (2004). Extracellular Enzymes and Metabolites Involved in Pathogenesis of Botrytis. In". Botrytis: Biology, Pathology and Control". Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. Dordrecht, the Netherlands, Kluwer Academic Press**:** 99-118.

Kars, I., McCalman, M., Wagemakers, L. and van Kan, J. A. L. (2005). "Functional analysis of *Botrytis cinerea* pectin methylesterase genes by PCR-based targeted mutagenesis: *Bcpme*1 and *Bcpme*2 are dispensable for virulence of strain B05.10." Molecular Plant Pathology **6**(6): 641-652.

Kunz, C., Vandelle, E., Rolland, S., Poinssot, B., Bruel, C., Cimerman, A., Zotti, C., Moreau, E., Vedel, R., Pugin, A. and Boccara, M. (2006). "Characterization of a new, nonpathogenic mutant of *Botrytis cinerea* with impaired plant colonization capacity." New Phytologist **170**(3): 537-550.

Pemberton, C. L. and Salmond, G. P. C. (2004). "The Nep1-like proteins - a growing family of microbial elicitors of plant necrosis." Molecular Plant Pathology **5**: 353-359.

Pemberton, C. L., Whitehead, N. A., Sebaihia, M., Bell, K. S., Hyman, L. J., Harris, S. J., Matlin, A. J., Robson, N. D., Birch, P. R., Carr, J. P., Toth, I. K. and Salmond, G. P. (2005). "Novel quorum-sensing-controlled genes in *Erwinia carotovora* subsp. *carotovora*: identification of a fungal elicitor homologue in a soft-rotting bacterium." Molecular Plant-Microbe Interaction **18**(4): 343-353.

Prins, T. W., Wagemakers, L., Schouten, A. and van Kan, J. A. L. (2000). "Cloning and characterization of a glutathione S-transferase homologue from the plant pathogenic fungus *Botrytis cinerea*." Molecular Plant Pathology **1**(3): 169-178.

Qutob, D., Kamoun, S. and Gijzen, M. (2002). "Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy." Plant Journal **32**(3): 361-373.

Qutob, D., Kemmerling, B., Brunner, F., Kufner, I., Engelhardt, S., Gust, A. A., Luberacki, B., Seitz, H. U., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M. and Nürnberger, T. (2006). "Phytotoxicity and innate immune responses induced by Nep1-like proteins." Plant Cell **18**(12): 3721-3744.

Reino, J. L., Hernández-Galán, R., Durán-Patrón, R. and Collado, I. G. (2004). "Virulence–toxin production relationship in isolates of the plant pathogenic fungus *Botrytis cinerea*." Phytopathology **152**: 563-566.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular Cloning: a laboratory manual. Cold Spring Harbor, Cold Spring Harbor Press.

Schouten, A., van Baarlen, P. and van Kan, J. A. (2008). "Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells." New Phytologist **177**(2): 493-505.

Staats, M., van Baarlen, P., Schouten, A., van Kan, J. A. and Bakker, F. T. (2007). "Positive selection in phytotoxic protein-encoding genes of Botrytis species." Fungal Genetics and Biology **44**(1): 52-63.

Tani, H., Koshino, H., Sakuno, E., Cutler, H. G. and Nakajima, H. (2006). "Botcinins E and F and Botcinolide from *Botrytis cinerea* and structural revision of botcinolides." Journal of Natural Products **69**(4): 722-725.

Veit, S., Worle, J. M., Nürnberger, T., Koch, W. and Seitz, H. U. (2001). "A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defense responses in carrot, arabidopsis, and tobacco." Plant Physiology **127**: 832-841.

Chapter 4

Site-directed mutagenesis of *Botrytis cinerea* NLPs to identify amino acids that are essential for necrosis-inducing activity

Chapter 4

Site-directed mutagenesis of *Botrytis cinerea* NLPs to identify amino acids that are essential for necrosis-inducing activity

Yaite Cuesta, Mirjam Dieho, Peter Vredenbregt, Alexander Schouten and Jan van Kan

SUMMARY

Botrytis cinerea produces two phytotoxic proteins that are members of the family of Nep1-Like Proteins (NLPs). These proteins are designated BcNEP1 and BcNEP2, and contain a conserved heptapeptide motif, typical for NLPs, as well as a number of cysteine residues, which are predicted to form disulfide bridges. They also possess several potential posttranslational modification motifs for phosphorylation, N-glycosylation, O-glycosylation. Furthermore there is a potential signal for endocytosis at the C-terminus of BcNEP1. In order to study which amino acid residues and motifs are important for the necrosis-inducing activity of *B. cinerea* NLPs, site directed mutagenesis was performed. Wild type and mutant proteins were expressed in *Nicotiana tabacum* and *N. benthamiana* by means of *Agrobacterium*-mediated transient transformation. Substitution of the heptapeptide motif, or of either of the two cysteine residues in the N-terminal part of the protein completely abolished the necrosis-inducing activity of both BcNEP proteins. Substitution of cysteine residues in the C-terminal part of the protein (one in BcNEP1, two in BcNEP2), as well as the substitution of posttranslational modification motifs or endocytosis signals did not lead to loss of function.

INTRODUCTION

NEP1 like proteins (NLPs) are 24-26 kDa proteins that have been identified in a wide range of microorganisms, including pathogenic and non pathogenic species (Bailey, 1995) All these proteins share a conserved hepta-peptide ('GHRHDWE') in the central region that is not found in other proteins (Pemberton and Salmond, 2004). NLPs are divided into two subgroups, called type I and type II, based on the presence of either two or four conserved cysteine residues (Gijzen and Nürnberger, 2006). Both types may occur in a single species. *Botrytis cinerea* contains two genes, representative of each type, called BcNEP1 and BcNEP2. The overall sequence similarity between both proteins is 39% (Schouten *et al.*, 2008). BcNEP1 contains three cysteine residues and it is predicted that the two most N-terminal cysteine residues, which are also conserved in BcNEP2, form a disulfide bridge (Staats *et al.*, 2007b). Both BcNEP proteins contain a secretion signal peptide and several potential posttranslational modification motifs, but these motifs differ between the two proteins. BcNEP1 contains several *N*-glycosylation motifs but no *O*-glycosylation motifs, whereas BcNEP2 contains exclusively *O*-glycosylation motifs.

The focus of this study was to elucidate whether the heptapeptide motif, the disulfide bonds and posttranslational modifications in the BcNEP proteins are important for their necrosis-inducing activity. Transient agro-infiltration assays were performed in *Nicotiana benthamiana* and *N. tabacum* to express site-directed mutant proteins. The use of *Agrobacterium tumefaciens* infiltration for transient assays (ATTA) has become an established method for studying processes related to gene function, regulation and promoter element analysis. The majority of experiments have been conducted in *N. benthamiana* and *N. tabacum*, which are particularly suited to this method. ATTA has recently been optimized for other species, including, *Lactuca sativa* (lettuce), *L. serriola* (wild lettuce), *Solanum lycopersicum* (tomato) and *Arabidopsis thaliana*. Vegetative tissues have typically been used for agro-infiltration, although tomato fruit and hairy root cultures have also been used (Shang *et al.*, 2007).

ATTA was used to test the effects of amino acid substitutions in BcNEP proteins on their necrosis-inducing activity.

RESULTS

To determine which amino acids are essential for the necrosis-inducing activity of the BcNEP proteins, several constructs were generated to introduce amino acid substitutions in the heptapeptide motif, in the cysteine residues or in potential glycosylation sites (Figure 1).

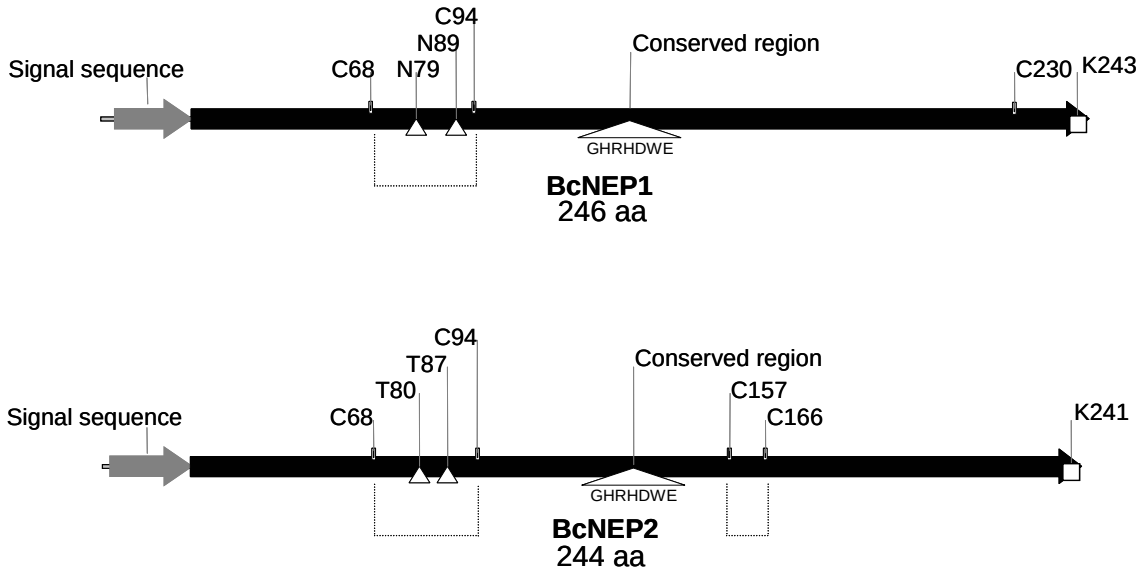


Fig.1. Schematic representation of BcNEP1 and BcNEP2; the amino acid residues targeted for substitution are indicated, dashed lines represent the predicted disulfide bonds.

Two amino acids (DW) in the heptapeptide motif were substituted by alanine residues (AA). Each cysteine residue was individually substituted by serine, which is chemically most closely related to cysteine. Furthermore, constructs were generated in which several cysteines were substituted. The *N*-glycosylation motif NXS/T was present twice at positions N79 and N89 in BcNEP1 while no *N*-glycosylation motif was present in BcNEP2. These two motifs were mutated by substituting the asparagine residue by glutamine. No *O*-glycosylation motif was detected in BcNEP1 while in BcNEP2, *O*-glycosylation motifs are present at T80, T87 and T243. The first two motifs were mutated by substituting residues T80 or T87 by valine.

BcNEP1 and BcNEP2 can enter plant cells and accumulate in the nuclear envelope (Schouten et al., 2008). The sequence motif KARI, at the C-terminus of BcNEP1, was predicted to act as a potential motif for endocytosis (MotifScan) and thus might be important in uptake of the protein into the plant cell. The sequence motif KATF, at the C-terminus of BcNEP2, was not predicted to act as endocytosis motif. We deleted the C-terminal four amino acids from both proteins by introducing stop codons at the positions of residue K243 (BcNEP1) or K241 (BcNEP2). The latter mutation also removed the *O*-glycosylation motif T243 in BcNEP2.

Agrobacterium tumefaciens strains containing constructs encoding wild type or mutant BcNEP1 or BcNEP2 proteins were infiltrated in *N. benthamiana* and *N. tabacum*. As negative controls, *A. tumefaciens* containing the empty vector pMOG800 were infiltrated. The results are presented in Table 1. The wild type BcNEP1-encoding construct caused cell collapse and chlorosis between 24 and 48 h progressing to a severe necrosis at 72 h post-infiltration.

Infiltration of the wild type BcNEP2-encoding construct needed more than 48 h to cause cell collapse which progressed in a patchy necrosis (Schouten *et al*., 2008). Constructs encoding BcNEP1 and BcNEP2 proteins carrying amino acid substitutions in the conserved heptapeptide region, in the C68 residue or the C94 residue did not cause necrotic symptoms in the infiltrated leaves. Constructs encoding BcNEP1 protein carrying a substitution in the C230 residue, or encoding BcNEP2 proteins carrying a substitution in the C157 or C166 residues caused necrotic symptoms similar to the wild type constructs. Constructs encoding BcNEP1 proteins carrying a substitution in the N-glycosylation motifs (N79 and N89), or encoding BcNEP2 proteins carrying a substitution in the O-glycosylation motifs (T80, T87) caused necrotic symptoms similar to the wild type constructs (Figure 2). Constructs encoding truncated proteins in which the four C-terminal amino acids were lacking (KARI in BcNEP1, KATF in BcNEP2) caused necrotic symptoms similar to the wild type constructs.

Table 1. Necrosis on *N. benthamiana* and *N. tabacum* after agro-infiltration of BcNEP constructs.

BcNEP	Substitution	Feature	Necrosis formation
BcNEP1	none	Wild type	+
	GHRHDWE → GHRHAAE	Heptapeptide motif	-
	C68S	Disulfide bonds	-
	C94S		-
	C230S		+
	C68S C94S		-
	C68S C230S		-
	C94S C230S		-
	C68S C94S C230S		-
	N79Q	*N*-glycosylation	+
	N89Q		+
	K243stop	Endocytosis motif	+
BcNEP2	none	Wild type	+
	GHRHDWE → GHRHAAE	Heptapeptide motif	-
	C68S	Disulfide bonds	-
	C94S		-
	C157S		+
	C166S		+
	C68S C94S		-
	T80A	*O*-glycosylation	+
	T87A		+
	K241stop		+

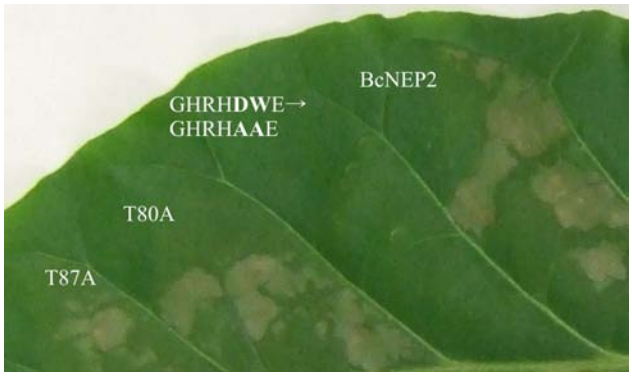


Fig. 2. ATTA to study the effect of amino acid substitutions on the necrosis-inducing activity of BcNEP2. *N. tabacum* leaves were agro-infiltrated, as indicated, with BcNEP2 constructs carrying a substitution in *O*-glycosylation motifs (T80, T87) or in the conserved heptapeptide region GHRHDWE. Symptoms were observed after 2 days.

DISCUSSION

The transient expression of site-directed mutant forms of BcNEP proteins by agroinfiltration in *N. benthamiana* and *N. tabacum* was used to determine whether specific amino acids are essential for the necrosis-inducing activity of these proteins. We confirmed that the conserved heptapeptide motif is essential for necrosis-inducing activity, as was also reported for other NLPs (Ottmann *et al.*, 2009). Furthermore we analysed the role of cysteine residues which are potentially involved in disulfide bond formation, and of asparagine and threonine residues that may be subject to post-translational *N*- and *O*- glycosylation, respectively. Finally, we studied the importance of the four C-terminal amino acids, that in BcNEP1 were predicted to act as a potential signal for endocytosis.

Recently, the crystal structure of NPP1 has been elucidated (Ottmann *et al.*, 2009). The conserved motif GHRHDWE in NLPs was shown to be important for the necrosis-inducing activity and is exposed to the protein surface where it presumably binds Ca^{2+}. Scavenging extracellular calcium by a membrane-impermeable Ca^{2+} chelator, BAPTA, abolished the plasma membrane-disintegrating activity of NLP_{PP} and NLP_{Pcc} from *Phytophthora parasitica* and *Pectobacterium carotovorum*, respectively. Substitution of three amino acid residues in this motif (H101, D104, E106) and another highly conserved residue (D93) in NLP_{PP} and NLP_{Pcc} suggested that these residues are required for the binding of a divalent cation within this cavity (Ottmann *et al.*, 2009). We tested the effects of the ion chelator EDTA (in 1000-fold molar excess) on the necrosis-inducing activity of BcNEP1 and BcNEP2, but observed no effect (not shown). Simultaneous site-directed mutagenesis in BcNEP1 and BcNEP2 on two residues (DW) in the heptapeptide motif, showed that this core is indeed essential for necrosis-inducing activity. Interestingly, non-pathogenic microbes like *Streptomyces coelicolor* and *Bacillus halodurans* produce NEP-like proteins with an intact heptapeptide domain, that nevertheless do not show cytolytic activity (Qutob *et al.*, 2002).

Disulfide bonds stabilize the native conformation of a protein and maintain protein stability by protecting it from oxidative and proteolytic attack in the extracellular environment. The function of some secreted soluble proteins and cell-surface receptors is controlled by cleavage of one or more of their disulfide bonds (Hogg, 2003). The two N-terminal cysteines are conserved among all NLPs (Gijzen, 2006) and have been shown to form a disulfide bond in NLP_{Pya} from *Pythium aphanidermatum* (Ottmann *et al*., 2009). Moreover, they are essential for necrosis-inducing activity of NLP_{PP} from *P. parasitica*. Substitution of either of the two cysteine residues impaired the ability of NLP_{PP} to trigger phytoalexin production and cell death in parsley protoplasts and necrotic lesion formation in tobacco leaves (Fellbrich *et al.*, 2002). The substitution of BcNEP1 and BcNEP2 residues C68 and C94, or substitution of any combination of cysteine residues that included either C68 or C94, resulted in the loss of necrosis-inducing activity (Table 1). It remains to be determined whether the loss of disulfide bridge formation merely reduces protein stability and accumulation, or whether it affects the protein conformation required for recognition by the plant. The cysteine residues C230 (in BcNEP1), and C157 and C166 (in BcNEP2) are not required for necrosis-inducing activity.

Glycosylation has been suggested to play a role in modulating the function of secreted proteins by enhancing stability and solubility, or by protecting against proteolytic attack (Goto, 2007). The predicted structures of BcNEP1 and BcNEP2 (Staats *et al*., 2007b) were compared to the crystal structure of NLP_{Pya} (Ottmann *et al.* 2009). The sequence elements of BcNEP1 or BcNEP2 that contain the glycosylation motifs were predicted to be located on the protein surface and are potentially accessible for glycosylation. Transient expression of BcNEP1, carrying a substitution in the *N*-glycosylation motifs or BcNEP2 carrying a substitution in the *O*-glycosylation motifs, did not show any alteration of necrosis-inducing activity compared with the wild type protein. If BcNEP proteins are glycosylated following agroinfiltration, such a modification seems not to be essential for their biological activity.

In conclusion, besides the conserved heptapeptide domain and the two N-terminal cysteine residues, no residues could be identified that are crucial for activity of BcNEP1 or BcNEP2.

MATERIALS AND METHODS

Plant material

N. benthamiana and *N. tabacum* plants were grown from seeds in a peat-moss mixture (Jiffy) and maintained in a greenhouse at 21°C (day), 19°C (night), 75% humidity and 16h/8h day/night photoperiod under natural light for 4-6 weeks.

Site-directed mutagenesis of *Bcnep* cDNAs and agroinfiltration

Site-directed mutagenesis was performed using as template *Bcnep*1 or *Bcnep*2 cDNA, lacking the signal peptide region (Schouten *et al.*, 2008). cDNA constructs containing the respective mutation were generated by PCR. Forward primers NEP1+65for or Nep2+68for were combined with the corresponding reverse primers that contain the nucleotide substitution to generate the N-terminal fragments. Analogously, C-terminal fragments were generated through the combination of forward primers that contain the nucleotide substitution(s) and reverse primer NEP1+907rev or Nep2+898rev (Table 2).

Table 2. Primers used in site-directed mutagenesis.

Gene	Feature Mutation	Primer name	Primer sequence 5' → 3'
*Bcnep*1		Nep1+65for	ACGCGTCGACAATTGAGGAGAGCACCATTCAAGCTCGCGCC
		Nep1+907rev	TCCCCGCGGCTGACAGGCCAAACTTCCAGATTCTCC
	Heptapeptide DW→ AA	DWfor	CCGTCAC**GCTGCA**GAG**T**ATGTCGTCGCTTGGGTC
		DWrev	CATACTC**TGCAGC**GTGACGGTGACCTCCGACGAC
	Disulfide bridge C→ S	C68for	CACATTGCTCATGGT**TCT**CAACCTACAGTGCCGTTGATGG
		C68rev	CTGTATGGTTG**AGA**ACCATGAGCAATGTGAAGATATGG
		C94for	GTCTCAGCCGGC**TCC**CGTGATCAGAGCAAGGGCCAAACC
		C94rev	CTTGCTCTGATCACG**GGA**GCCGGCTGAGACATTGCCAGTATC
		C230for	GGAAAGGCAAAC**TCC**CCATTCAATGACGCAAACTTC
		C230rev	GCGTCATTGAATGG**GGA**GTTTGCCTTTCCAAAGTTGGTG
	N-glycosylation N→ Q	N79for	GGTAATGGT**CAG**ACCAGTGGTG GACTCCAAG
		N79rev	CACCACTGGT**CTG**ACCATTACCATCAACG
		N89for	GATACTGGC**CAG**GTCTCAGCCG GCTGCCGTG
		N89rev	CGGCTGAGAC**CTG**GCCAGTATCTTGGAGTC
	Truncation endocytosis motif, ΔKARI	K243stop	TCCCCGCGGCT**A**GGCGAGGTTGTTGTTGAAGTTTGCGTC
*Bcnep*2		Nep2+68for	ACGCGTCGACTACACCATCACAACTTGAGTCTCGGG
		Nep2+898rev	TCCCCGCGGCCAATAGACTCCCAGAATATAGCCCCT
	Heptapeptide DW→ AA	DW2for	CACCGTCAC**GCTGCA**GAAGGTGTAATTGTCTGGCT
		DW2rev	CCTTCT**TGCAGC**GTGACGGTGACCAATACCG
	Disulfide bridge C→ S	C68for	CGTAAATGGA**TCC**GTACCATTCCCTGCCGTCGA
		C68rev	GGGAATGGTAC**GGA**TCCATTTACGACTTTAAGGA
		C94for	GTGGT**TCC**AGCAGCAGTACCGGTCAAGTA
		C94rev	GGTACTGCTGCT**GGA**ACCACCATTGCTACTGCCAG
		C157for	CTTAGCCGTTT**TCT**CCTTCCGCCCACGGAGGCTGG
		C157rev	GCGGAAGG**AGA**AACGGCTAAGATGTTGTCGG
		C166for	GGCTGGGAT**TCT**TCCACGGATGGCTATTCCCT
		C166rev	TCCGTGGA**AGA**ATCCCAGCCTCCGTGGGCGGA
	O-glycosylation T→ V	T80for	CGGGTAAC**GTA**GGTGGTGGTTTGTCACCAAC
		T80rev	ACCACCACC**TAC**GTTACCCGATGCATCGACGG
		T87for	GTCACCA**GTA**GGCAGTAGCAATGGTGGTT
		T87rev	GCTACTGCC**TAC**TGGTGACAAACCACCACCTG
	Truncation	K241stop	TCCCCGCGGCT**A**GGCGAGGTTGTTGTTGAAGTTTGCGTC

The underlined sequences correspond to the restriction sites *Sal*I and *Sac*II introduced for cloning purposes. Sequences in bold represent the nucleotides that generate the mutations.

PCR was carried out in volumes of 50 µl containing 1U Taq polymerase (Roche), 0.2 mM of each dNTP, 1X Taq PCR buffer, 1µM of specific primers (Sigma–Aldrich) and 20-50 ng DNA. PCR conditions were: 1 cycle of 95°C for 4 min; 10 cycles at 95°C for 1 min, 50°C for 2 min, 72°C for 4 min; followed by 20 cycles of 95°C for 1 min, 60°C for 2 min, 72°C for 5 min. Finally, each pair of fragments was fused using a single-step overlap extension PCR with the conditions described above but using the primer combination NEP1+65for/ NEP1+907rev or Nep2+68for/ NEP1+907rev. Constructs in which the last four codons are deleted were generated in one step using PCR primers NEP1+65for/K243stop or Nep2+68for/K241 stop. Fragments were extracted from agarose gel with GFX PCR purification kit (Amersham Pharmacia), cloned and characterized as described by Schouten *et al*. (2008). Validated mutant constructs were cloned into binary expression vector pMOG800, transformed into *A. tumefaciens* and used in agroinfiltration as described by Schouten *et al*. (2008).

REFERENCE LIST

Bailey, B. A. (1995). "Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*." Phytopathology **85**(10): 1250-1255.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Engelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M. and Nürnberger, T. (2002). "NPP1, a Phytophthora-associated trigger of plant defense in parsley and Arabidopsis." Plant Journal **32**(3): 375-390.

Garcia, O., Macedo, J. A. N., Tibúrcio, R., Zaparoli, G., Rincones, J., Bittencourt, L. M. C., Ceita, G. O., Micheli, F., Gesteira, A., Mariano, A. C., Schiavinato, M. A., Medrano, F. J., Meinhardt, L. W., Pereira, G. A. G. and Cascardo, J. C. M. (2007). "Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*." Mycological Research **111**(4): 443-455.

Gijzen, M. and Nürnberger, T. (2006). "Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa." Phytochemistry **67**(16): 1800-1807.

Goto, M. (2007). "Protein O-glycosylation in fungi: Diverse structures and multiple functions." Bioscience, Biotechnology and Biochemistry **71**(6): 1415-1427.

Hogg, P. J. (2003). "Disulfide bonds as switches for protein function." Trends in Biochemical Sciences **28**: 210-214.

Ottmann, C., Luberacki, B., Küfner, I., Koch, W., Brunner, F., Weyand, M., Mattinen, L., Pirhonen, M., Anderluh, G., Seitz, H. U., Nürnberger, T. and Oecking, C. (2009). "A common toxin fold mediates microbial attack and plant defense." Proceedings of the National Academy of Sciences of the United States of America **106**(25): 10359-10364.

Pemberton, C. L. and Salmond, G. P. C. (2004). "The Nep1-like proteins - A growing family of microbial elicitors of plant necrosis." Molecular Plant Pathology **5**(4): 353-359.

Qutob, D., Kamoun, S. and Gijzen, M. (2002). "Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy." Plant Journal **32**(3): 361-373.

Schouten, A., van Baarlen, P. and van Kan, J. A. L. (2008). "Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells." New Phytologist **177**(2): 493-505.

Shang, Y., Schwinn, K. E., Bennett, M. J., Hunter, D. A., Waugh, T. L., Pathirana, N. N., Brummell, D. A., Jameson, P. E. and Davies, K. M. (2007). "Methods for transient assay of gene function in floral tissues." Plant Methods **3**(1).

Staats, M., van Baarlen, P., Schouten, A. and van Kan, J. A. L. (2007a). "Functional analysis of NLP genes from *Botrytis elliptica*" Molecular Plant Pathology **8**(2): 209-214.

Staats, M., van Baarlen, P., Schouten, A., van Kan, J. A. L. and Bakker, F. T. (2007b). "Positive selection in phytotoxic protein-encoding genes of *Botrytis* species." Fungal Genetics and Biology **44**(1): 52-63.

Chapter 5

Dissecting the mode of action of phytotoxic proteins of *Botrytis cinerea*

Chapter 5

Dissecting the mode of action of phytotoxic proteins of *Botrytis cinerea*

Yaite Cuesta, Eric Kalkman, Miriam Oses Ruiz, Beatrice Uwumukiza, Alexander Schouten and Jan van Kan

SUMMARY

The contribution of the plant to the necrosis-inducing activity of *Botrytis cinerea* NLPs was investigated. Infiltration of purified BcNEP1 and BcNEP2 into *Nicotiana benthamiana* leaves induced ethylene emission and necrosis in a dose-dependent manner. For BcNEP2 a higher protein concentration was required to induce ethylene emission as compared to BcNEP1. Necrotic symptom development in response to BcNEP1 was generally faster than in response to BcNEP2, indicating that BcNEP1 is more active than BcNEP2. The necrosis-inducing activity of BcNEP1 was independent of light, whereas necrosis-inducing activity of BcNEP2 was transiently compromised when plants were incubated in darkness following infiltration.

Arabidopsis thaliana mutants altered in ethylene, jasmonate or salicylate production or in signalling, as well as tomato plants affected in ethylene production or signalling, developed normal necrotic lesions following infiltration with BcNEP proteins, demonstrating that phytohormone production and hormone signalling pathways are not required for responses to these proteins. Virus-induced gene silencing in *Nicotiana benthamiana* of genes that are required for the necrotic response to an effector from *Phytopthora infestans* did not affect the necrotic response to BcNEP1 or BcNEP2. Pharmacological inhibitors that have been reported to interfere with various cellular processes (endocytosis, cytoskeleton function, vesicle trafficking, apoptotic cell death, necrotic cell death, protein kinase activity and cell energy supply) did not affect the necrotic response of plants to BcNEP1 or BcNEP2. We have not been able to identify any cellular process or pathway in plants that is required for the necrosis-inducing activity of *B. cinerea* NLPs.

INTRODUCTION

The induction by *Botrytis cinerea* of cell death in host plants constitutes an important component of its virulence (van Kan, 2006; Choquer *et al*., 2007) and has been proposed to represent a type of hypersensitive response (HR) (Govrin and Levine, 2000). HR has been extensively studied in the context of resistance responses to biotrophic pathogens triggered by pathogen effectors (Kliebenstein and Rowe, 2008). Plant genes involved in effector recognition and the activation of downstream HR-induced defence responses have been studied in many plant–pathogen interactions (Chisholm *et al*., 2006). HR is accompanied by an oxidative burst, membrane perturbation leading to calcium influx, pH changes, phytoalexin biosynthesis, the induction of pathogenesis-related protein synthesis, and reinforcement of cell walls, all of which processes occur in *B. cinerea*-plant interactions (e.g. van Baarlen *et al*., 2007). HR also leads to an increase in phytohormone biosynthesis. A surge of ethylene production occurs at the onset of tissue necrosis in *B. cinerea*-infected tomato plants (Cristescu *et al.*, 2002). Enzymes secreted by *B. cinerea* may directly generate reactive oxygen species (Rolke *et al*., 2004) and the fungus also secretes phytotoxic metabolites and proteins that induce an oxidative burst in host plants, factors that collectively have been implicated in the initiation of plant cell death by *B. cinerea* (Choquer *et al.*, 2007). Among the phytotoxic, necrosis-inducing proteins produced by *B. cinerea* are Nep1-like proteins (NLPs), named BcNEP1 and BcNEP2 (Staats *et al.*, 2007; Schouten *et al.*, 2008). *Bcnep*1 and *Bcnep*2 genes are expressed during infection of tomato and *Nicotiana benthamiana* leaves, albeit with different temporal expression patterns (Chapter 3). Production of BcNEP1 and BcNEP2 coincides with ethylene emission in *B. cinerea*-infected *N. benthamiana* leaves (Chapter 3).

Microbial effectors that either induce ethylene emission, such as the xylanase EIX from *Trichoderma viride* (Avni *et al*., 1994), or induce HR, such as the AVR proteins of *Cladosporium fulvum* (Stergiopoulos and de Wit, 2009) are proposed to interact with plant receptors and activate signalling cascades that trigger the downstream responses in the host (Ron and Avni, 2004; Chisholm *et al*., 2006). Whether microbial NLPs also act as effectors that are recognized by a receptor and activate a signal transduction pathway that culminates in the activation of cell death pathways, however, was unknown. We aimed to investigate the contribution of the plant to the necrosis- and ethylene-inducing activity of purified *B. cinerea* NLPs. We used tomato, Arabidopsis and *Nicotiana benthamiana* and performed physiological, genetic and pharmacological studies to unravel the contribution of cellular processes in the plant in the response to BcNEP proteins.

RESULTS

Dose-dependent induction of necrosis and ethylene production

Nicotiana benthamiana leaves were infiltrated with BcNEP1 in a concentration range from 0.04 to 1.2 µM, and BcNEP2 from 0.4 to 4 µM. The appearance of symptoms was evident after a few hours and was monitored during the following three days. For BcNEP1, a concentration as low as 0.04 µM was able to induce discoloration in the infiltrated zone after 8 h, whereas BcNEP2 required 24 h and at least 0.4 µM to induce the same type of symptoms (Figure 2). Three days after infiltration, the tissues infiltrated either with BcNEP1 (0.4 µM) or BcNEP2 (4 µM) were completely necrotic. Control leaves infiltrated with phosphate buffer did not show any symptom during the three days (data not shown).

NLPs have been reported to induce expression of genes encoding ethylene biosynthetic enzymes (Qutob *et al.*, 2006). In order to quantify the amount of ethylene that can be induced by BcNEP proteins in plants, different concentrations of BcNEP1 and BcNEP2 were infiltrated into individual *N. benthamiana* leaves. The leaves were subsequently enclosed in bottles with rubber caps and the ethylene concentration in the bottle was monitored every 30 minutes. After each sampling, bottles were opened to release the remnant ethylene and refresh the air. BcNEP1 and BcNEP2 induced ethylene production in a dose-dependent manner (Figure 1). Both BcNEP proteins induced a sharp increase in ethylene production that peaked at 1.5 h after infiltration. BcNEP1 induced ethylene production at the lowest concentration tested (0.04 µM). Interestingly, this concentration of BcNEP1 did not induce any visible symptom in this experiment (not shown). For BcNEP2 the highest concentration tested (4 µM) induced an ethylene production comparable with the highest concentration tested for BcNEP1 (1.2 µM), showing that BcNEP2 is less active or phytotoxic than BcNEP1.

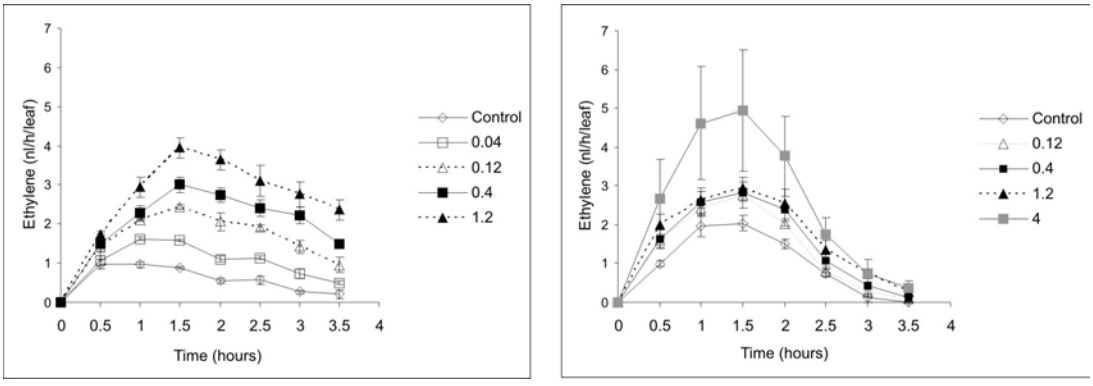


Fig. 1: Ethylene production in *N. benthamiana* following infiltration with BcNEP1 (A) or BcNEP2 (B) at different concentrations (µM). Proteins were infiltrated (100 µl of each concentration) in *N. benthamiana* leaves. 10 mM phosphate buffer, pH 7 was used as control. The amount of ethylene produced during 30 min intervals was measured over a period of 3.5 h. The means of three replicates are shown. Error bars represent standard errors of the means.

Inhibition of necrosis-inducing activity by antibodies

Polyclonal antibodies raised against purified BcNEP1 and BcNEP2 were tested for their ability to inhibit BcNEP-induced necrosis. The antibodies against BcNEP1 showed slight cross-reactivity towards BcNEP2 and vice-versa (not shown). When antibodies were co-infiltrated with BcNEP proteins in different ratios, BcNEP1- and BcNEP2-induced necrosis was reduced when the corresponding antibodies were added at equimolar concentration and abolished when 3-fold or 10-fold excess of antibody was added (Figure 2). The antibody raised against BcNEP1 could not reduce necrosis-inducing activity of BcNEP2 or vice versa.

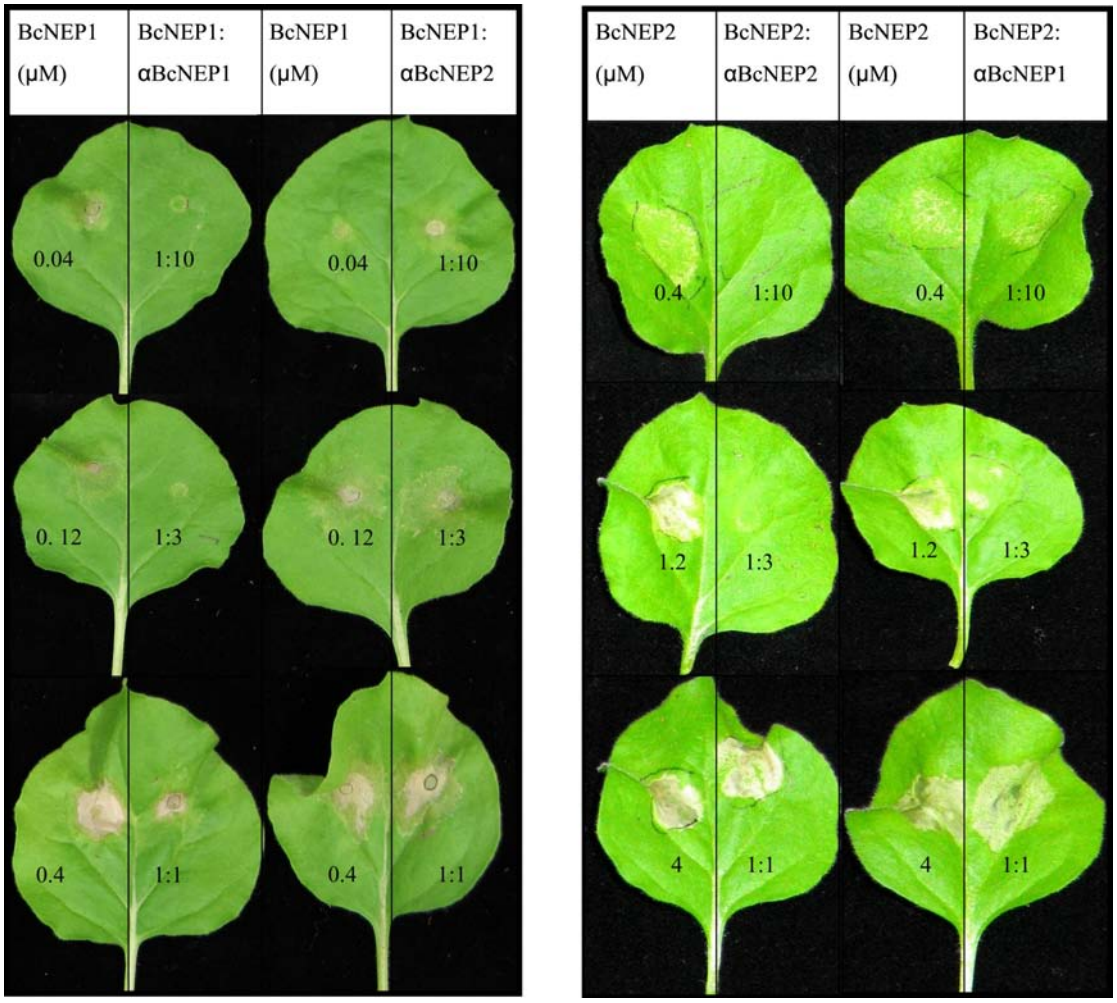


Fig. 2. Effect of co-infiltration of BcNEP proteins with polyclonal antibodies. *N. benthamiana* leaves were infiltrated on the left hand-side of each leaf with different concentrations of the BcNEP protein as indicated (μM). The right hand side of each leaf was infiltrated with a mixture of BcNEP protein (in the same concentration as on the left hand side) and a polyclonal antibody raised either against BcNEP1 or BcNEP2, as indicated at the top of the image. The BcNEP protein and the antibodies were mixed at different molar ratios (1:10, 1:3 or 1:1) as indicated on the right hand side of each leaf.

Necrosis-inducing activity of BcNEP2 is light-dependent

Based on the observation that the induction of necrosis by NLP_{PP} from *Phytophthora parasitica* is an active light-dependent process (Qutob *et al.*, 2006), we also tested whether BcNEP-induced necrosis would be affected by light. *N. benthamiana* plants were placed in total darkness for one day and subsequently infiltrated in a dark room with BcNEP1 or BcNEP2. Immediately after infiltration, one plant infiltrated with each protein was transferred to a growth chamber with light (16 h/day). Other plants were transferred to the growth chamber at one day intervals over a week. Symptoms were evaluated 8 days post infiltration (dpi). As controls, one plant infiltrated with each protein was kept in the growth chamber before and after infiltration, while one plant infiltrated with each protein was kept in darkness before and after infiltration. All the plants infiltrated with BcNEP1 showed similar necrotic symptoms, regardless how long they had been incubated in darkness after infiltration (Figure 3A). In contrast, the response to BcNEP2 was affected by the duration of the incubation in darkness following infiltration. In plants that were transferred from darkness to light at 1, 2 and 3 dpi, the symptoms at 8 dpi were clearly less pronounced (Figure 3B). Remarkably, plants that were transferred from darkness to light at 4 and 5 dpi showed necrotic symptoms comparable to the control plant that was transferred to light immediately after infiltration.

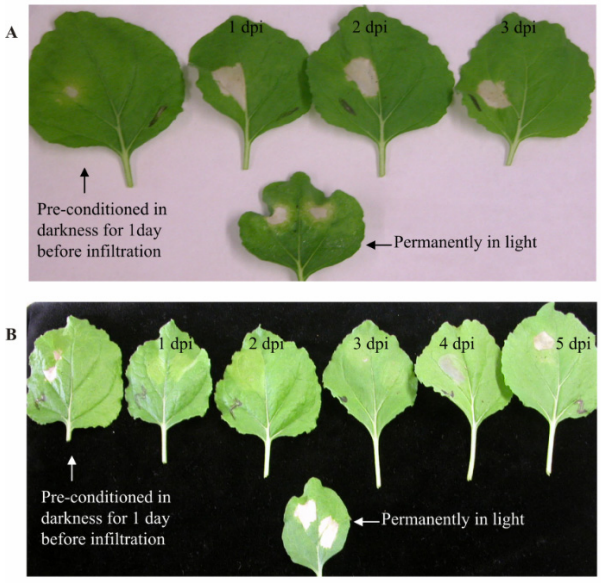


Fig. 3. Effect of light on the necrosis-inducing activity of BcNEP1 (A) and BcNEP2 (B). *N. benthamiana* plants were preconditioned in total darkness for one day and subsequently infiltrated in a dark room with BcNEP1 or BcNEP2. One plant infiltrated with each protein was immediately transferred to a growth chamber. Other plants were transferred to the growth chamber at one day intervals. As a control, one plant infiltrated with each protein was kept in permanent light before and after infiltration.

This experiment was repeated two more times; for each time point, BcNEP proteins were infiltrated in three plants and in several leaves per plant. The results were not always consistent between leaves and plants. The trend was in all cases that the response to BcNEP2 was reduced in the first days of incubation in darkness, whereas plants that were incubated in darkness for longer times showed typical necrotic responses similar to the controls.

Is ethylene required for the response to BcNEP proteins?

In order to determine whether ethylene production or perception by the plant is required for the necrosis-inducing activity of BcNEP proteins, tomato mutants that are altered in ethylene biosynthesis or perception were tested (Figure 4). Different concentrations of BcNEP1 were infiltrated into the leaves of UC8338, an ethylene non-producing transgenic tomato that expresses ACC deaminase. The non-transgenic progenitor UC82B was tested as control. BcNEP1 was infiltrated into the leaves in a concentration range of 0.04-0.4 µM. BcNEP1 induced necrosis in a dose-dependent manner in both tomato lines. At the lowest concentration, the response was restricted to the zone where the syringe was placed on the leaf, even though the area that was infiltrated with the protein sample extended well beyond this zone. No important difference was observed between mutants and wild type in severity of symptom development (Figure 4), nor in timing (not shown).

A similar experiment was conducted to study whether ethylene perception is required for the necrosis-inducing activity. In this case, the tested plants were Never Ripe (*Nr*) and its wild type progenitor Pearson. *Nr* is a natural mutant that carries a mutation in the ethylene receptor LeETR1, making the plant less sensitive to ethylene (Lanahan *et al.*, 1994). No important differences were observed between *Nr* and its wild type progenitor Pearson (Figure 4).

In addition, 1-methylcyclopropene (MCP), which irreversibly binds to the ethylene-binding domain of ethylene receptors (Blankenship and Dole, 2003) was used to inactivate the entire set of (five) ethylene receptors present in tomato (Bleecker *et al.*, 1998).

Half of the leaves were pre-treated with 1-methylcyclopropene (MCP), while the other half of the leaves remained untreated. MCP-treated and untreated leaves were infiltrated with BcNEP1 at concentrations of 0.04 and 0.12 µM. No differences were observed between treatments or between plant genotypes. All leaves showed very similar necrotic symptoms as observed in the previous experiment with the transgenic UC8338 genotype.

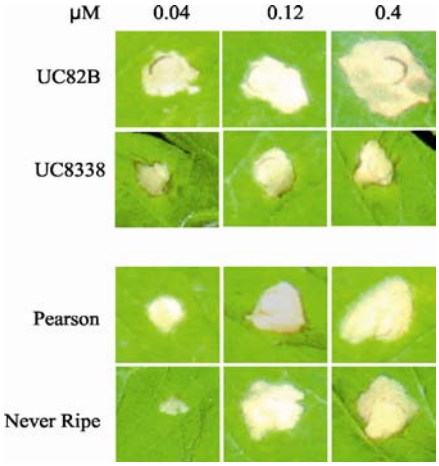


Fig. 4. Role of ethylene in the necrosis-inducing activity of BcNEP1. Tomato mutants that are altered in ethylene biosynthesis (UC8338) or perception (Never Ripe) and their respective wild types were infiltrated with BcNEP1 at different concentrations as indicated (µM). Symptoms were recorded after 5 days.

Plant pathways involved in the response to BcNEP proteins

In order to investigate whether different defense-related pathways play a role in the necrosis-inducing activity of BcNEP proteins, leaves of Arabidopsis mutant genotypes were infiltrated with BcNEP1 (0.04 and 0.12 µM) or BcNEP2 (1.2 µM). Genotypes tested included the ethylene-insensitive mutants *ein*2, *ein*3, *etr*1; the jasmonate-insensitive mutant *jin*4; the salicylate-dependent defense pathway mutants *mpk*4, *npr*1 and NahG; the camalexin-deficient mutant *pad*3. All mutant plants tested developed necrotic lesions similar to the progenitor line Col-0 at all NLP concentrations tested (0.04 and 0.12 BcNEP1; 1.2 µM BcNEP2).

Gabriëls et al. (2006) described tomato genes that are involved in responses to effectors. Silencing these genes by Tobacco Rattle Virus-based VIGS constructs severely compromised the necrotic response to *Phytopthora infestans* INF1, as well as the resistance gene-mediated hypersensitive response to *Cladosporium fulvum* effector AVR4 (Gabriels *et al.*, 2006). To study whether the plant genes required for these responses are also involved in the necrotic response to BcNEP1, VIGS constructs were introduced into *N. benthamiana* using TRV-based vectors in *A. tumefaciens* along with three control constructs (Table 1). Symptoms of viral infection were observed 12 days post agroinfiltration and resembled phenotypes described by Gabriels *et al*. (2006). At this time, the plants were either infiltrated with *A. tumefaciens* containing a construct encoding the *P. infestans* protein INF1 or with purified BcNEP1 protein. Plants that were first inoculated with TRV:LeHsp90-1, TRV:nGTPase and TRV:rL19 displayed a strongly compromised necrotic response to INF1 (score= 1). Plants infected with TRV:EDS1, TRV:NRC1, TRV:MEK2, TRV:RAR1, TRV:SGT1 and TRV:NDR1 showed less pronounced symptom development in response to INF1 (score= 2). BcNEP1 induced severe necrosis in the entire infiltrated area in leaves of all plants in 3-5 days (Score =3; Figure 5). No differences were observed between BcNEP1 concentrations used, 0.04 and 0.12 µM (not shown).

Fig. 5. Responses of TRV-inoculated (silenced) *Nicotiana benthamiana* plants to phytotoxic proteins were scored from 3 to 1, with 3 = full necrosis, 2= chlorosis with mild necrosis, 1 = chlorosis.

Table 1. Response in TRV-inoculated plants to INF1 and BcNEP1.

Construct [a]	Phenotype [b]	INF1 [c]	BcNEP1 [d]
TRV:EDS1	Severe viral symptoms	2	3
TRV:NRC1	Compact/ Fragile	2	3
TRV:MEK2	Severe viral symptoms	2	3
TRV:RAR1	Severe viral symptoms	2	3
TRV:SGT1	Stunted/ branched	2	3
TRV:NDR1	None	2	3
TRV:LeHsp90-1	Stunted	1	3
TRV:nGTPase	Compact/ Curly leaves	1	3
TRV:rL19	None	1	3
TRV:Cf4	None	3	3
TRV:00	None	3	3

[a] VIGS construct inoculated in young plants
[b] Phenotype in TRV-inoculated plants at moment of effector infiltration
[c] Response to INF1 in TRV-inoculated plants, scored on scale 1-3 as shown in Fig. 5
[d] Response to BcNEP1 in TRV-inoculated plants, scored on scale 1-3 as shown in Fig. 5

Pharmacological analysis of processes involved in the response to BcNEP proteins

To determine which cellular processes are required for the necrosis-inducing activity of BcNEP proteins, *N. benthamiana* leaves were pre-treated with several inhibitors and then infiltrated with BcNEP1 protein. Inhibitors (Table 4) were selected that have been reported to interfere in endocytosis, cytoskeleton function, vesicle trafficking, apoptotic cell death, necrotic cell death, protein kinase activity and cell energy supply. Of the inhibitors tested, all except dynasore and ikarugamycin (both inhibitors of endocytosis in mammals) were tested and confirmed to be effective in plants. A concentration range of each inhibitor was infiltrated in *N. benthamiana* leaves to determine whether they caused any visible phytotoxic effect in the leaves. For each inhibitor, the highest concentration that did not cause any visible damage was either infiltrated simultaneously with BcNEP1 (0.04 μM or 0.12 μM) into the same area, or the inhibitor was infiltrated 3 h or 8 h prior to infiltration with BcNEP1. Of all inhibitors tested, only dynasore (>100 μM) was able to reduce the necrosis-inducing activity of BcNEP1 (0.04 μM) when compared with the control (Figure 6). Dynasore was not able to reduce the necrosis-inducing activity of recombinant NLP_{PP} from *Phytopthora parasitica* (Figure 6), nor was it able to reduce the necrosis-inducing activity of BcNEP1 at 0.12 μM or of BcNEP2 at any concentration tested (up to 4 μM, not shown).

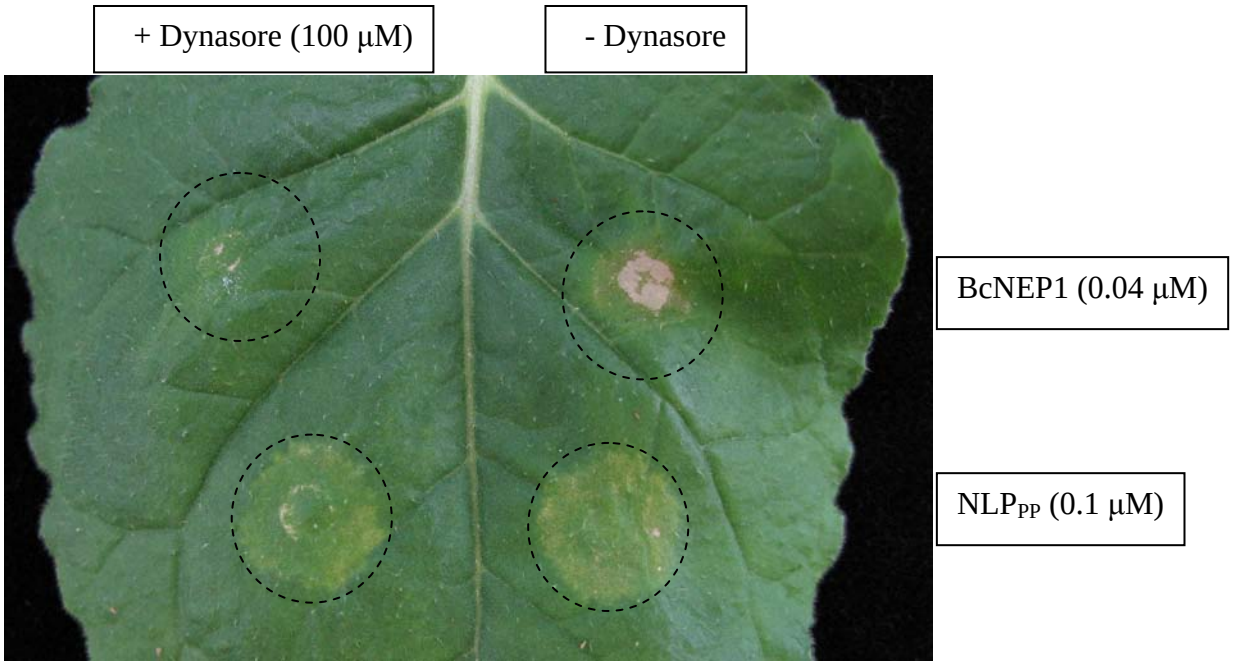


Fig 6. Effects of the dynamin-mediated endocytosis inhibitor Dynasore on response of *Nicotiana benthamiana* leaves to NLPs. BcNEP1 was infiltrated on both sides of the central vein at 0.04 μM in phosphate buffer, pH 7. Recombinant NLP_{PP} was infiltrated on both sides of the central vein at 0.1 μM in phosphate buffer, pH 7. In the left side of the leaf, Dynasore (100 μM) was co-infiltrated with the protein. The dotted circle marks the area of the leaf which was infiltrated.

DISCUSSION

Here, we studied the contribution of the plant in the necrosis-inducing activity of BcNEP proteins in an attempt to unravel their mode of action and/or molecular target(s). Both BcNEP1 and BcNEP2 were able to induce necrosis and ethylene production in dose-dependent manner, but their dose-response profiles were different. The fact that BcNEP1 is able to cause necrosis in shorter time than BcNEP2, and the differences in the amount of protein that is required to induce ethylene production, lead us to conclude that BcNEP1 is more phytotoxic than BcNEP2. The transcription profile described in Chapter 3 shows that *Bcnep*1 is transiently expressed during the first 40 h of the infection process, while *Bcnep*2 expression is first detected when the infection is already established and the lesion is expanding. This observation suggested that these proteins may have different functions or have a similar function albeit at different stages of the infection.

Remarkably, necrosis-inducing activity of BcNEP1 was independent of light, whereas necrosis-inducing activity of BcNEP2 was compromised when the protein was infiltrated in leaves of dark-adapted plants and the infiltrated plants were kept in darkness. Light-dependent necrosis-inducing activity was also reported for other NLPs (Qutob et al., 2006), the ToxA

protein from *Pyrenophora tritici* (Sarpeleh *et al.*, 2008), as well as the sphingoid-like AAL toxin (Gechev *et al.*, 2004). All these toxins may require an active photosynthetic apparatus to cause necrosis. Chloroplasts play a major role in ROS-dependent lipid peroxidation occurring during the HR (Mur *et al.*, 2009). Liu *et al.* (2007) reported that the pathogen-responsive NtMEK2-SIPK/Ntf4/WIPK cascade plays an active role in promoting ROS generation in chloroplasts by inhibiting the carbon fixation reaction, which can create a situation of excess excitation energy in plants under illumination. In the absence of light, chloroplast-generated ROS is inhibited and cell death delayed. It remains unclear why the reduced response to BcNEP2 in dark-adapted plants was transient.

Plant hormone signaling pathways are important key regulators in plant cell death processes and defense against microbial pathogens. Ethylene and jasmonate (JA) are important in determining disease severity caused by a range of necrotrophic microorganisms, while salicylate (SA) is important for resistance against biotrophs (Glazebrook *et al.*, 1996). Protoplasts from Arabidopsis mutants in phytohormone biosynthesis or signaling pathways were less responsive to the toxin Fumonisin B1 (Asai *et al.*, 2000). Arabidopsis mutants altered in ethylene, JA and SA production or signalling, as well as tomato mutants altered in ethylene production or signalling, when infiltrated with BcNEP proteins, showed necrosis development indistinguishable from the wild type progenitor. Moreover plants pre-treated with MCP in which all the ethylene receptors are irreversibly inactivated, developed necrosis, demonstrating that ethylene perception is not required for responses to BcNEP proteins.

None of the Arabidopsis mutants tested showed any alteration in sensitivity to BcNEP1 or BcNEP2 as compared to the wild type, as was also reported by Qutob *et al*. (2006) for responses to NLP_{PP}. Also the silencing of several genes, previously shown in *N. benthamiana* to be required for the necrotic response to *P. infestans* protein INF1 (Gabriels *et al*., 2006) did not affect the response to BcNEP1. Silencing of *hsp*90, *mek*2, *nrc*1, *rar*1 and *sgt*1 in *N. benthamiana* compromised the necrosis-inducing activity of the *P.infestans* elicitor INF1, but the plants still displayed an unaltered response to BcNEP1.

Previously, it was reported that BcNEP proteins can induce a combination of apoptotic and necrotic cell death mechanisms in tomato cell cultures (Schouten *et al*., 2008). BcNEP1 and BcNEP2 have been found to associate *in vivo* with cell membranes and even accumulate in the nuclear envelope (Schouten *et al.*, 2008). To further study their mode of action, pharmacological inhibitors were co-infiltrated with BcNEP proteins. Of all tested compounds, only dynasore was able to consistently reduce the phytotoxic effect of BcNEP1. Dynasore

acts by blocking coated vesicle formation and thereby inhibits endocytotic pathways known to depend on dynamin. Dynasore was reported to partially protect human cells against the action of Shiga-toxin (Romer *et al.*, 2007). The observed partial inhibition of BcNEP1-mediated necrosis induction suggested that BcNEP1 may enter the plant cell by a dynamin-mediated endocytosis pathway. However, dynasore was not effective when higher concentrations of BcNEP1 were applied and it was totally ineffective against BcNEP2 and NLP_{PP}. Moreover, a different endocytosis inhibitor, ikarugamycin, did not influence necrosis-inducing activity of BcNEP1. It should be noted that dynasore and ikarugamycin have been successfully used in animal systems, but have not yet been reported to be functional in plants. We have not verified that these compounds indeed affect the same process(es) in plants as they were reported to inhibit in the organism for which they were previously developed.

In conclusion, the mode of action of BcNEP proteins remains unresolved. We were not able to identify any cellular process or pathway that is required for their necrosis-inducing activity. Our results are consistent with the mechanism recently proposed by Küfner *et al.* (2009), that NLPs act as pore-forming cytolytic toxins that permeate and disintegrate membranes without the need for active recognition by putative receptors in the plant cell.

MATERIALS AND METHODS

Plant material

N benthamiana plants were grown from seeds in a peat-moss mixture (Jiffy) and maintained in a greenhouse at 21°C (day), 19°C (night), 75% humidity and 16h/8h day/night photoperiod under natural light for 4-6 weeks. Transgenic *N. benthamiana* plants containing the *Cf-4* resistance gene from tomato against *C. fulvum* were grown for four weeks under greenhouse conditions at 22 °C under a 12/12 h light/ dark regime with 200 watt mercury vapor lamps. The plants were agroinfiltrated and kept in a climate chamber at 22 °C and 70% of RH.

The tomato (*Solanum lycopersicum*) lines used were cv. Moneymaker, cv. Pearson, the homozygous mutant *Nr* in the cv. Pearson background, the transgenic line UC8338, expressing a bacterial ACC deaminase and its non-transgenic progenitor UC82B (Klee *et al.*, 1991). All plants were grown in potting soil as described by ten Have *et al.* (1998).

Eight mutant genotypes of *A. thaliana* were used to investigate whether these genes were involved in the necrotic response of the plant to BcNEP proteins (Table 2). Ten 4-5 week old plants of each mutant were tested. As controls wild type Columbia (*Col-0*) and Landsberg *erecta* (*Ler*) were used, both of which are susceptible to *B. cinerea* (Thomma *et al.*, 1998).

Table 2. Arabidopsis mutant genotypes used in this study

Plants	Feature	Reference
Col-0	Wild type	
Ein2	Ethylene signaling pathway	Alonso (1999)
Ein3	Ethylene signaling pathway	Chao *et al.*(1997)
Etr1	Ethylene signaling pathway	Chang *et al.*(1993)
Jin4	Jasmonate insensitive	Berger *et al.*(1996)
Mpk4	MAP kinase involved in SA pathway	Sundaresan *et al.*(1995)
NahG	SA depletion	Delaney *et al.*(1994)
Npr1	No PR protein accumulation	Chao *et al.*(1997)
Pad3	Camalexin synthesis	Zhou *et al.*(1999)

Protein preparation and infiltration

Heterologous expression, purification and quantification of BcNEP1 and BcNEP2 were described by Schouten *et al.* (2008). From a BcNEP1 (4 µM) stock solution, dilutions (1.2, 0.4, 0.12, 0.04 µM) were prepared in 10mM KPi buffer, pH 7. From BcNEP2 (40 µM), dilutions (4, 1.2, 0.4, 0.12 µM) were prepared in the same buffer. Tomato, *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves were infiltrated with a 1 ml needle-less syringe on their abaxial side with protein dilutions mentioned above or with 10mM KPi buffer, pH 7.

Measurement of ethylene production

Detached *N. benthamiana* leaves were infiltrated each with 100 µl of either BcNEP1 or BcNEP2 at the concentrations mentioned above or with 10mM KPi buffer, pH 7 using a 1 ml syringe on the abaxial surface. Leaves infiltrated with the buffer were used as control. Subsequently each leaf was placed into a 30 ml bottle containing 2ml tap water (to avoid wilting) and the bottles were sealed with rubber caps. Ethylene-air samples were extracted every 30 minutes with a 2 ml syringe and injected in a gas chromatograph (Shimadzu 17A). After each sampling, bottles were opened during 5 minutes to release the remnant ethylene and closed again. The experiment was terminated after 3.5 h. Each experiment contained three biological replicates per treatment and was performed three times. To calculate the amount of ethylene produced per leaf in each treatment (nl/ l/ leaf), the average of ethylene concentrations from each treatment was multiplied by a calibration factor and by the volume of the bottles. The calibration factor was calculated from the average of several ethylene measurements from pure ethylene of known concentration.

Antibody inhibition

From a BcNEP1 (4μM) stock solution, dilutions (0.04, 0.12, 0.4 μM) were prepared in 10mM KPi buffer, pH 7. For BcNEP2 (40 μM) the dilutions (0.4, 1.2, 4 μM) were prepared in the same buffer. Purified IgG fractions of polyclonal antisera (made in rabbits) against purified recombinant BcNEP1 and BcNEP2 (Schouten *et al.*, 2008) were prepared by Eurogentec (Maastricht, The Netherlands). BcNEP protein dilutions were mixed with antibody in a molar ratio of 1:10, 1:3, 1:1 in 10mM KPi buffer, pH 7, and incubated at 37 ºC for 15 min. The mixture was cooled at room temperature for 5 min and infiltrated into *N. benthamiana* leaves. BcNEP dilutions without antibody served as controls.

Gene silencing

Tobacco rattle virus (TRV) was used for silencing genes involved in HR (Gabriels *et al.*, 2006). cDNA fragments of the genes tested originated from different plants (Table 3). Transgenic *Cf4-N. benthamiana* plants were infiltrated as described (van der Hoorn *et al.*, 2001) with *A. tumefaciens* strain MOG101 carrying binary vector pBintra6 and the TRV recombinants in 1:1 ratio. Ten days post-infiltration, purified BcNEP1 protein (0.04 μM) was infiltrated in the lower side of the 5^{th} or 6^{th} leaf. *A. tumefaciens* strain MOG101 containing binary plasmid pInf1 (Kamoun *et al.*, 2003) was used to transiently express *P. infestans* INF1.

Table 3. Plasmid constructs used for Virus-Induced Gene Silencing (VIGS)

Construct	Viral vector	Origin of insert	Reference
TRV:EDS1	pYL156	*N. tabacum*	Ekengren *et al.* (2003)
TRV:NRC1	pTV00	Tomato	Gabriels *et al.* (2006)
TRV:MEK2	pTV00	*N. tabacum*	Abd-el-Haliem (unpublished)
TRV:RAR1	pTV00	*N. tabacum*	Abd-el-Haliem (unpublished)
TRV:SGT1	pTV00	*N. benthamiana*	Peart *et al.* (2002)
TRV:NDR1	pYL156	*N. benthamiana*	Ekengren *et al.* (2003)
TRV:LeHSP90-1	pTV00	Tomato	Gabriels *et al.* (2006)
TRV:nGTPase	pTV00	Tomato	Gabriels *et al.* (2006)
TRV:rL19	pYL156	Tomato	Abd-el-Haliem (unpublished)
TRV:Cf4	pTV00	Tomato	van der Hoorn *et al* (2000)
TRV:00	pTV00	-	Ratcliff *et al.*(2001)
TRV:1	pBintra6	-	Ratcliff *et al.*(2001)

Dark treatment

N. benthamiana plants (6 weeks-old) were placed in a closed cabinet inside a dark room for 24 h. Subsequently, leaves were infiltrated with BcNEP1 (0.4 μM) or BcNEP2 (4μM) in the dark room under a red candescent lamp (15 W). The infiltration was performed in multiple leaves of each plant. One plant infiltrated with each protein was taken out immediately and placed in a growth chamber. Other plants were transferred to the growth chamber at one day intervals. As a control, one plant infiltrated with each protein was kept in constant light before and after infiltration. Symptoms were scored 8 days after infiltration.

Pharmacological inhibition assays

All inhibitors (Table 4) were purchased from Sigma, with exception of Ac-DEVD-CHO (Promega) and Ikarugamycin (BioRes Labs). The dilutions were infiltrated in the abaxial side of the leaves from *N. benthamiana* plants (4 week-old). The highest inhibitor concentration, at which no phytotoxic effects were observed at 24 h after infiltration, was selected for further experiments. A new set of leaves were infiltrated with the selected dilutions and 3 h later, BcNEP1 (0.04 or 0.12 μM) was infiltrated, partially overlapping the inhibitor infiltrated zone.

Table 4. Pharmacological inhibitors used in this study

Inhibitor	Target	Stock	Concentrations tested	Solvent
BDM	Cytoskeleton function	0.5 M	20, 30, 40, 60 mM	Water
Brefeldin A	Vesicle trafficking	10mM	10,36 μM	DMSO
Staurosporine	Protein kinase	1mM	0.5, 1, 1.5, 2 μM	DMSO
Ac-DEVD-CHO	Apoptotic cell death	10 mM	1 μM	DMSO
E-64	Apoptotic cell death	10 mM	25 μM	DMSO
Ikarugamycin	Endocytosis	10 mM	10, 20, 50, 100 μM	DMSO
$LaCl_3$	Apoptotic cell death	1M	2, 5, 10, 20, 30, 40 mM	Water
NEC-1	Necrotic cell death	300 mM	10, 20, 30 μM	DMSO
Oligomycin	Cell energy supply	20 mM	50, 200, 500 nM, 1, 2, 5, 10, 100, 200 μM	CH3OH
Na-Vanadate	Cell energy supply	0.5 M	50, 100, 200, 300, 400μM	Water
Latrunculin B	Cytoskeleton function	2.5 mM	10, 20, 50 μM	DMSO
Dynasore	Endocytosis	29.4 mM	10, 80, 200, 300 μM	DMSO

MCP treatment

Leaves from different tomato genotypes were detached and their petioles inserted in wet florist foam. The leaves were placed in sealed glass containers (1 l) with a rubber septum, through which gaseous MCP was injected to a final concentration of 100 nl/l. After 4 h

exposure to MCP, the leaves were placed into open plastic containers for 1 h to remove excess MCP and then infiltrated with 100 µl BcNEP1 of each concentration mentioned above. As control, detached leaves were incubated in closed containers without addition of MCP and treated as above. The experiment was performed twice.

Acknowledgements

Dr. Isabell Küfner and Prof. Thorsten Nürnberger (ZPMB, Univerisyt of Göttingen, Germany) are acknowledged for providing the recombinant NLP_{PP} produced in *E. coli*. Prof. Ernst Woltering and Dianne Somhorst (Food & Biobased Research Institute, Wageningen UR, The Netherlands) are acknowledged for supplying ethylene and MCP, and for providing the GC facilities for ethylene measurements. Ahmed Abd-el-Haliem (Laboratory of Phytopathology, Wageningen University) is acknowledged for providing the *Agrobacterium tumefaciens* strains with gene silencing constructs.

REFERENCE LIST

Able, A. J. (2003). "Role of reactive oxygen species in the response of barley to necrotrophic pathogens." Protoplasma **221**(1-2): 137-143.

Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J. R. (1999). "EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis." Science **284**(5423): 2148-2152.

Asai, T., Stone, J. M., Heard, J. E., Kovtun, Y., Yorgey, P., Sheen, J. and Ausubel, F. M. (2000). "Fumonisin B1-induced cell death in arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways." Plant Cell **12**(10): 1823-1836.

Avni A, Bailey B. A., Mattoo, A. K. and Anderson, J. D. (1994) "Induction of ethylene biosynthesis in *Nicotiana tabacum* by a *Trichoderma viride* xylanase is correlated to the accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase transcripts." Plant Physiology **106**: 1049-1055.

Bailey, B. A. (1995). "Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*." Phytopathology **85**(10): 1250-1255.

Berger, S., Bell, E. and Mullet, J. E. (1996). "Two Methyl Jasmonate-Insensitive Mutants Show Altered Expression of AtVsp in Response to Methyl Jasmonate and Wounding." Plant Physiology **111**(2): 525-531.

Blankenship, S. M. and Dole, J. M. (2003). "1-Methylcyclopropene: A review." Postharvest Biology and Technology **28**(1): 1-25.

Bleecker, A. B., Esch, J. J., Hall, A. E., Rodriguez, F. I. and Binder, B. M. (1998). "The ethylene-receptor family from Arabidopsis: structure and function." Philosophical Transactions of the Royal Society B Biological Sciences **353**(1374): 1405-1412.

Chang, C., Kwok, S. F., Bleecker, A. B. and Meyerowitz, E. M. (1993). "Arabidopsis ethylene-response gene *ETR*1: similarity of product to two-component regulators." Science **262**(5133): 539-544.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. and Ecker, J. R. (1997). "Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins." Cell **89**(7): 1133-1144.

Chisholm, S. T., Coaker, G., Day, B. and Staskawicz, B.J. (2006) "Host-microbe interactions: Shaping the evolution of the plant immune response." Cell **124**: 803-814.

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J. M., Simon, A. and Viaud, M. (2007). "*Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen." FEMS Microbiology Letters **277**(1): 1-10.

Cristescu, S. M., De Martinis, D., Te Lintel Hekkert, S., Parker, D. H. and Harren, F. J. (2002). "Ethylene production by *Botrytis cinerea* in vitro and in tomatoes." Applied and Environmental Microbiology **68**(11): 5342-5350.

Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994). "A central role of salicylic acid in plant disease resistance." Science **266**(5188): 1247-1250.

Ekengren, S. K., Liu, Y., Schiff, M., Dinesh-Kumar, S. P. and Martin, G. B. (2003). "Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato." Plant Journal **36**(6): 905-917.

Furman-Matarasso, N., Cohen, E., Du, Q., Chejanovsky, N., Hanania, U. and Avni, A. (1999). "A point mutation in the ethylene-inducing xylanase elicitor inhibits the β-1-4-endoxylanase activity but not the elicitation activity." Plant Physiology **121**(2): 345-351.

Gabriels, S. H., Takken, F. L., Vossen, J. H., de Jong, C. F., Liu, Q., Turk, S. C., Wachowski, L. K., Peters, J., Witsenboer, H. M., de Wit, P. J. and Joosten, M. H. (2006). "CDNA-AFLP combined with functional analysis reveals novel genes involved in the hypersensitive response." Molecular Plant Microbe Interactions **19**(6): 567-576.

Garcia, O., Macedo, J. A. N., Tibúrcio, R., Zaparoli, G., Rincones, J., Bittencourt, L. M. C., Ceita, G. O., Micheli, F., Gesteira, A., Mariano, A. C., Schiavinato, M. A., Medrano, F. J., Meinhardt, L. W., Pereira, G. A. G. and Cascardo, J. C. M. (2007). "Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*." Mycological Research **111**(4): 443-455.

Gechev, T. S., Gadjev, I. Z. and Hille, J. (2004). "An extensive microarray analysis of AAL-toxin-induced cell death in Arabidopsis thaliana brings new insights into the complexity of programmed cell death in plants." Cellular and Molecular Life Sciences **61**(10): 1185-1197.

Gilchrist, D. G. and Grogan, R. G. (1976). "Production and nature of a host-specifc toxin from *Alternaria alternata* f. sp. lycopersici." Phytopathology **66**: 165-171.

Glazebrook, J., Rogers, E. E. and Ausubel, F. M. (1996). "Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening." Genetics **143**(2): 973-982.

Govrin, E. M. and Levine, A. (2000). "The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*." Current Biology **10**(13): 751-757.

Kamoun, S., Hamada, W. and Huitema, E. (2003). "Agrosuppression: a bioassay for the hypersensitive response suited to high-throughput screening." Molecular Plant Microbe Interactions **16**(1): 7-13.

Klee, H. J., Hayford, M. B., Kretzmer, K. A., Barry, G. F. and Kishore, G. M. (1991). "Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants." Plant Cell **3**(11): 1187-1193.

Kliebenstein, D. J. and Rowe, H. C. (2008). "Ecological costs of biotrophic versus necrotrophic pathogen resistance, the hypersensitive response and signal transduction." Plant Science **174**: 551-556.

Küfner, I., Ottmann, C., Oecking, C. and Nürnberger, T. (2009) "Cytolytic toxins as triggers of plant immune response." Plant Signalling and Behaviour **4**: 977-979.

Lanahan, M. B., Yen Hsiao, C., Giovannoni, J. J. and Klee, H. J. (1994). "The Never ripe mutation blocks ethylene perception in tomato." Plant Cell **6**(4): 521-530.

Liu, Y., Ren, D., Pike, S., Pallardy, S., Gassmann, W. and Zhang, S. (2007). "Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade." Plant Journal **51**(6): 941-954.

Lund, S. T., Stall, R. E. and Klee, H. J. (1998). "Ethylene regulates the susceptible response to pathogen infection in tomato." Plant Cell **10**(3): 371-382.

Markham, J. E. and Hille, J. (2001). "Host-selective toxins as agents of cell death in plant-fungus interactions." Molecular Plant Pathology **2**(4): 229-239.

Mur, L. A., Lloyd, A. J., Cristescu, S. M., Harren, F. J., Hall, M. A. and Smith, A. R. (2009). "Biphasic ethylene production during the hypersensitive response in Arabidopsis: a window into defense priming mechanisms?" Plant Signal Behaviour **4**(7): 610-613.

Peart, J. R., Lu, R., Sadanandom, A., Malcuit, I., Moffett, P., Brice, D. C., Schauser, L., Jaggard, D. A., Xiao, S., Coleman, M. J., Dow, M., Jones, J. D., Shirasu, K. and Baulcombe, D. C. (2002). "Ubiquitin ligase-associated protein SGT1 is required for host and nonhost disease resistance in plants." Proceedings of Natural Academy of Sciences of the United States of America **99**(16): 10865-10869.

Qutob, D., Kemmerling, B., Brunner, F., Kufner, I., Engelhardt, S., Gust, A. A., Luberacki, B., Seitz, H. U., Stahl, D., Rauhut, T., Glawischnig, E., Schween, G., Lacombe, B., Watanabe, N., Lam, E., Schlichting, R., Scheel, D., Nau, K., Dodt, G., Hubert, D., Gijzen, M. and Nürnberger, T. (2006). "Phytotoxicity and innate immune responses induced by Nep1-like proteins." Plant Cell **18**(12): 3721-3744.

Ratcliff, F., Martin-Hernandez, A. M. and Baulcombe, D. C. (2001). "Tobacco rattle virus as a vector for analysis of gene function by silencing." Plant Journal **25**(2): 237-245.

Rolke, Y., Liu, S., Quidde, T., Williamson, B., Schouten, A., Weltring, K. M., Siewers, V., Tenberge, K. B., Tudzynski, B. and Tudzynski, P. (2004). "Functional analysis of H2O2-generating systems in *Botrytis cinerea*: The major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable." Molecular Plant Pathology **5**(1): 17-27.

Romer, W., Berland, L., Chambon, V., Gaus, K., Windschiegl, B., Tenza, D., Aly, M. R., Fraisier, V., Florent, J. C., Perrais, D., Lamaze, C., Raposo, G., Steinem, C., Sens, P., Bassereau, P. and Johannes, L. (2007). "Shiga toxin induces tubular membrane invaginations for its uptake into cells." Nature **450**: 670-675.

Ron, M. and Avni, A. (2004) "The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato." Plant Cell **16**: 1604-1615.

Sarpeleh, A., Wallwork, H., Tate, M. E., Catcheside, D. E. A. and Able, A. J. (2008). "Initial characterisation of phytotoxic proteins isolated from *Pyrenophora teres*." Physiological and Molecular Plant Pathology **72**(1-3): 73-79.

Schouten, A., van Baarlen, P. and van Kan, J. A. L. (2008). "Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells." New Phytologist **177**(2): 493-505.

Staats, M., van Baarlen, P., Schouten, A., van Kan, J. A. L. and Bakker, F. T. (2007). "Positive selection in phytotoxic protein-encoding genes of *Botrytis* species." Fungal Genetics and Biology **44**(1): 52-63.

Stergiopoulos, I., and de Wit P. J. G. M. (2009). "Fungal effectors." Annual Review of Phytopathology **47**: 233-263.

Stone, J. M., Heard, J. E., Asai, T. and Ausubel, F. M. (2000). "Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (fbr) Arabidopsis mutants." Plant Cell **12**(10): 1811-1822.

Sundaresan, V., Springer, P., Volpe, T., Haward, S., Jones, J. D., Dean, C., Ma, H. and Martienssen, R. (1995). "Patterns of gene action in plant development revealed by enhancer trap and gene trap transposable elements." Genes Development **9**(14): 1797-1810.

ten Have, A., Mulder, W., Visser, J. and van Kan, J. A. L. (1998). "The endopolygalacturonase gene *Bcpg*1 is required to full virulence of *Botrytis cinerea*." Molecular Plant-Microbe Interactions **11**(10): 1009-1016.

Thomma, B. P., Eggermont, K., Penninckx, I. A., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. and Broekaert, W. F. (1998). "Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens." Proceeding of Natural Academy of Sciences of the United States of america **95**(25): 15107-15111.

van der Hoorn, R. A., Laurent, F., Roth, R. and de Wit, P. J. (2000). "Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr9/Cf-9-induced and Avr4/Cf-4-induced necrosis." Molecular Plant Microbe Interactions **13**(4): 439-446.

van Kan J. A. L (2006) "Licensed to kill: the lifestyle of a necrotrophic plant pathogen." Trends in Plant Sciences **11**: 247-253.

Zhou, N., Tootle, T. L. and Glazebrook, J. (1999). "Arabidopsis *PAD*3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase." Plant Cell **11**(12): 2419-2428.

Chapter 6

General discussion

Chapter 6

General discussion

Senescence and ripening processes in plant tissues are often stimulated by ethylene, and may result in an increase of susceptibility to pathogens, which may cause enormous losses in the post-harvest chain of a wide variety of horticultural products. Total post-harvest losses of fresh produce world-wide are on average estimated to exceed 30%. After harvest, fruits as well as flowers become increasingly sensitive to ethylene. Low exogenous ethylene concentrations or stress conditions (dark storage, desiccation, pathogen attack) can induce auto-catalytic ethylene production and senescence that results in product loss. Ethylene-induced plant defence is hardly active at this stage since microbial attack is no longer threatening to the survival of the individual, as long as seeds remain unaffected. Harvested products can be highly susceptible to a range of post-harvest pathogens. Among the most important post-harvest problems are diseases caused by the fungal plant pathogen *Botrytis cinerea*. This fungus can infect at least 235 plant species, including a range of economically important crops (Jarvis, 1977). The aim of this research was to obtain insight in the role of the phytohormone ethylene in the interaction of plants with *B. cinerea,* using tomato as a model. Furthermore, functional analysis of *B. cinerea* Nep1-Like Proteins (NLPs) was performed.

At the onset of this research there were reports that *B. cinerea* can produce ethylene, as well as perceive and respond to ethylene. *B. cinerea* can produce ethylene in culture from α-keto-γ-(methylthio)butyric acid (KMBA), produced by deamination of L-methionine (Qadir *et al.*, 1997; Chagué *et al.*, 2002), however the amount of ethylene produced by the fungus *in planta* was reported to be below the detection level (Cristescu *et al.*, 2002). It was postulated that production of ethylene by *B. cinerea* might weaken plant tissue and thereby predispose it for invasion, but no experimental data were provided to support this hypothesis. We attempted to abolish the ethylene biosynthetic pathway in *B. cinerea*. Catabolism of L-methionine and other amino acids involves bacterial branched-chain aminotransferases (BCAs) (Engels *et al.*, 2000; Thage *et al.*, 2004), some of which accept a specific amino acid as substrate while others have broad substrate preference. It was proposed that conversion of L-methionine into KMBA in the yeast *Yarrowia lipolytica* is catalyzed by a BCA (Bondar *et al.*, 2005). We considered the possibility that a (possibly methionine-specific) BCA in *B. cinerea* might be essential in production of KMBA, which would subsequently decompose into ethylene. The *B. cinerea* genome contains at least 4 genes that encode predicted proteins with homology to bacterial BCAs. One of the *B. cinerea* genes (designated *Bcat*1) showed 37% identity to a BCA from *Lactococcus lactis* (accession number AF164204). Although we were aware that

the presence of other putative BCA genes in the *B. cinerea* genome might provide functional redundancy, the *Bcat*1 gene was selected for functional analysis. Deletion mutants in this gene were generated in an attempt to abolish ethylene synthesis in *B. cinerea*. Cultures of *Bcat*1-deficient mutants grown in presence of methionine showed reduction in KMBA production but no notable reduction in ethylene production when compared with the parental strain B05.10 (F. Borges & J. van Kan, unpublished). *Bcat*1-deficient mutants were equally virulent as the wild type. A concentration of at least 5 mM methionine is required to achieve detectable levels of ethylene production by *B. cinerea* (A. ten Have & J. van Kan, unpublished). This concentration is well beyond the amount of free methionine available in plant tissue. We consider it unlikely that *B. cinerea* produces ethylene in plant tissue and ethylene produced by *B. cinerea* is thus unlikely to play any role in the infection process.

Besides the ability to produce ethylene, there were reports that *B. cinerea* is able to respond to exogenously applied ethylene. Ethylene treatment stimulated germination of *B. cinerea* conidia and growth of germ tubes (Kepczynska, 1993). Moreover, the rates of conidial germination and germ tube growth on glass, tomato and bean leaf surfaces were enhanced in the presence of ethylene (Elad *et al.*, 2002). Chagué *et al.* (2006), however, reported that ethylene inhibited mycelial growth in cultures. Thus, ethylene may have different effects on the fungus at different developmental stages and in different experimental systems (Sharon *et al.*, 2004). Taken together, these observations suggested that an ethylene receptor and signalling cascade may be present in *B. cinerea*. In order to search for a possible ethylene receptor in *B. cinerea*, we first verified that ethylene indeed affects mycelium growth either *in vitro* or during plant colonization (Chapter 2). The fungus was cultured in different media and exposed to a range of ethylene concentrations. In sharp contrast to previous reports of others, no differences were observed between cultures in the morphology or growth rates in the presence or absence of ethylene. It should be noted that Kepczynska (1993) used ethephon as ethylene releasing agent, instead of pure ethylene. Ethephon decomposes into ethylene, phosphonic acid and hydrochloric acid (Lawton *et al.*, 1994; van Kan *et al.*, 1995). Effects of ethephon on *B. cinerea* may not necessarily be attributed only to a response to ethylene. Our experiments were performed several times using the same strain and conditions as reported by Chagué *et al*. (2006), however, effects of ethylene on fungal growth were never observed. A concentration of 200 µl/l is very high considering that the amount of ethylene released in red tomato fruit is 5 $nl.h^{-1}.gFW^{-1}$ (E.J. Woltering, *pers. comm.*) and the ethylene released by *B. cinerea*-infected tomato fruit ranges from 28 to 60 $nl.h^{-1}.gFW^{-1}$ (Cristescu *et al.*, 2002).

Experiments were conducted *in planta* to study whether ethylene production by the host plant affects disease development. Detached leaves from tomato mutant plants with altered ethylene production were inoculated with *B. cinerea*. The results showed that even if *B. cinerea* were able to sense ethylene produced by the host, it did not affect disease development. It should be noted that experiments were conducted in leaves and not in fruits, since it was difficult to get uniformity in the ripening stage of fruits. Processes that facilitate infections in fruit may differ from those in vegetative organs. Cantu *et al*., (2008) demonstrated that simultaneous suppression of expansin and polygalacturonase in ripening tomato fruit reduced wall disassembly, and slowed fruit softening *in vivo*, which decreased susceptibility to *B. cinerea*.

Infection of a range of plant species and tissues by *B. cinerea*, as well as other pathogens, results in a large increase in ethylene production in the infected tissue (Elad, 1988a, 1990). Cantu *et al.* (2009) observed that the transcriptional changes that occur following *B. cinerea* infection of mature green tomato fruit resemble, in qualitative terms, the changes that were observed during normal ripening in uninfected fruit. In mature green fruit, *B. cinerea* infection caused 5- and 7-fold up-regulation of *ACS2* and *ACS4*, respectively, key enzymes in the ripening-associated ethylene biosynthetic pathway. Expression of *ACS2* and *ACS4* increased 31- and 139- fold, respectively, in healthy red ripe fruits compared to healthy mature green fruit. *B. cinerea* appears to exploit endogenous developmental programs and actively induce, in unripe tomato fruit, some of the processes that normally occur during fruit ripening, possibly to predispose the host tissue to fungal colonization (Cantu *et al.*, 2009).

Reports that the transcript levels of certain *B. cinerea* genes are induced by exposure to ethylene (Chagué *et al.*, 2006), led us to characterize the role of the fungal histidine kinase BcHHK5, which structurally resembles plant ethylene receptors. Gene replacement mutants in the *Bchhk5* gene were neither affected in growth *in vitro* nor in virulence. The expression of the two genes, *Bchsp*30 and *Bcspl*1, reported by Chagué *et al*. (2006) to be ethylene-responsive were not significantly and consistently altered upon exposure to ethylene in the *B. cinerea* wild type strain nor in the mutant. In conclusion, our experiments did not provide any evidence that *B. cinerea* senses ethylene, nor that the BcHHK5 protein acts as an ethylene receptor. We propose that the stimulation of disease development by ethylene is exclusively the result of the ethylene-induced senescence and ripening processes in the host. These processes provoke softening and disintegration of tissues that facilitate the entry and proliferation of the pathogen. The effects of ethylene are not a direct consequence of an ethylene response in the pathogen.

Functional analysis of *B. cinerea* NLPs

NLPs constitute a protein family that is produced by a wide range of microbes, including both prokaryotic and eukaryotic organisms (Bailey, 1995; Fellbrich *et al.*, 2002; Gijzen and Nürnberger, 2006). Most of the plant pathogenic microbes that produce NLPs exhibit a hemibiotrophic or necrotrophic life style. NLPs are considered proteinaceous toxins that trigger necrosis and ethylene production in plants. Besides their stimulation of immune-associated defenses specifically in dicotyledonous plants, little is known about their mode of action. Ottmann *et al.* (2009) demonstrated that an NLP from *Pythium aphanidermatum* is able to disintegrate plant plasma membranes and provoke subsequent cell lysis.

Many fungi and oomycetes posses several copies of NLPs in their genomes. Fungi can posses from one to four copies, whereas oomycetes like *Phytophthora infestans* possess up to 60 copies (Gijzen and Nürnberger, 2006; Garcia *et al.*, 2007; Motteram *et al.*, 2009). *B. cinerea* possesses two NLP proteins, named BcNEP1 and BcNEP2 and these have orthologs in all other *Botrytis* species (Staats *et al.*, 2007). BcNEP1 is the ortholog of all other NLPs identified thus far. No ortholog of BcNEP2 was detected in other species, except the closely related pathogen *Sclerotinia sclerotiorum* (Schouten *et al.*, 2008). The two proteins have low sequence similarity (39 %) and possess different post-transcriptional modification motifs which, however, do not contribute to the necrosis-inducing activity of the proteins. Amino acid substitutions in the conserved hepta-peptide GHRHWDE abolish the necrosis-inducing activity on *N. benthamiana* and *N. tabacum* (Chapter 4). This region is part of a negatively charged cavity exposed at the protein surface and is implicated in coordination of a divalent cation within this cavity (Ottmann *et al.*, 2009).

Although both BcNEP1 and BcNEP2 are able to induce necrosis and ethylene production in a dose-dependent manner, BcNEP1 is more phytotoxic than BcNEP2. BcNEP1 is able to cause necrosis more rapidly and the protein concentration needed to induce ethylene production is lower. Other features of these proteins suggested that they may have different functions or have similar functions at different stages of the infection. *Bcnep*1 is transiently expressed during the formation of primary lesions in early stages of infection, while *Bcnep*2 expression is detected when the infection is established and the lesions are expanding (Chapter 3). Activity of BcNEP1 was independent of light, whereas the necrosis-inducing activity of BcNEP2 was compromised when the protein was infiltrated in leaves of dark-adapted plants and the infiltrated plants were kept in darkness (Chapter 5). The mechanism underlying light-dependent activity of BcNEP2 remains to be resolved.

Gene replacement of *Bcnep*1 or *Bcnep*2 showed that these genes are dispensable for virulence (Chapter 3). Disease development by the mutant strains on tomato and *N. benthamiana* was similar to the wild type strain B05.10. The lack of BcNEP1 or BcNEP2 in single mutants was not compensated by the overexpression of the other gene since the genes are differentially expressed during infection. The fact that these genes are not essential in the infection process is in agreement with several reports. Single deletion of the two *NLP* genes from *B. elliptica* had no effect on virulence on lily (Staats *et al.*, 2007). Likewise, deletion of an *NLP* gene in *Mycosphaerella graminicola*, present in a single copy in the genome, had no effect on virulence of this fungus on wheat, and the MgNLP protein, produced in *Pichia pastoris*, failed to induce necrosis on wheat leaves (Motteram *et al.*, 2009). Both *B. elliptica* and *M. graminicola* are pathogens of monocots, and it is well established that NLPs are not able to cause necrosis in monocots (Gijzen and Nürnberger, 2006; Staats *et al.*, 2007; Schouten *et al.*, 2008; Motteram *et al.*, 2009). So why would pathogens that attack monocot plants contain NLP genes? Also non-pathogenic microbes like *Neurospora crassa, Bacillus halodurans, Streptomyces coelicolor* posses NLP genes (Pemberton and Salmond, 2004). It is tempting to speculate that NLPs have a structural role or a developmental role in microbes instead of a role in pathogenesis. Their phytotoxic activity may be an inadvertent side effect of a different function.

We attempted to determine which cellular processes and pathways in plants are required for the necrosis-inducing activity of BcNEP proteins. *N. benthamiana* leaves were pre-treated with several inhibitors of cellular processes and then infiltrated with BcNEP1 protein (Chapter 5). Only dynasore was able to reduce the necrosis-inducing activity of BcNEP1 but not of BcNEP2. Dynasore blocks coated vesicle formation and thereby inhibits endocytotic pathways known to depend on dynein (Macia *et al.*, 2006). Does this suggest that BcNEP1 enters plant cells through endocytosis whereas BcNEP2 uses a different strategy for entry? Previous reports established that BcNEP1 and BcNEP2 are associated with cell and nuclear membranes and may act as membrane-altering toxins that can penetrate deeply into the cell (Schouten *et al.*, 2008). Schouten *et al.* (2008) proposed that NLPs might bind to plant lectins. Interestingly, there is a NLP in *Bacillus thuringiensis* serovar *israelensis*, annotated as having insecticidal activity but of which further details have remained unpublished. This protein contains a NLP domain fused to a lectin domain homologous to ricin B. The ricin B domain binds to galactose-containing receptors in (mammalian) cell membranes (Fu *et al.*, 1996). Bacteria produce several toxins, whose actions often depend on glycan-binding subunits that

allow the toxin to interact with membrane glyco-conjugates and deliver the active toxic subunit across the plasma membrane. Binding of a toxin or bacterium to a glycolipid also may increase the likelihood of further interactions with membranes (Esko and Sharon, 2009).

Ottmann *et al.* (2009) and Küfner *et al*. (2009) proposed that NLP_{Pya}, from the plant pathogenic oomycete *Pythium aphanidermatum*, targets specific components of membrane bilayers via a surface-exposed cavity and that it is distantly related to actinoporins and lectins. Actinoporins are cytolytic toxins that form transmembrane pores via their flexible N-terminal regions (Mancheño *et al.*, 2003). The N-terminal region of NLP_{Pya} is required for NLP-induced necrosis and plant defense activation and it was proposed that NLPs and actinoporins share a cytolytic, membrane-disintegrating mode of action (Ottmann *et al.*, 2009).

Pathogen-derived compounds like toxins trigger a complex spectrum of plant defence responses requiring activation of distinct signalling pathways. The phytohormones ethylene, jasmonic acid (JA) and salicylic acid (SA) have been implicated in plant defence signalling (Thomma *et al.*, 1998; van Loon *et al.*, 1998; Petersen *et al.*, 2000). Arabidopsis mutants altered in ethylene, JA and SA production or signalling, as well as tomato mutants altered in ethylene production or signalling, were infiltrated with BcNEP proteins, and showed necrotic lesion development indistinguishable from the wild type progenitor (Chapter 5). Similar results were found for NLP_{PP}, which induced lesions in SA-deficient *nah*G-expressing Arabidopsis plants, suggesting that SA is not required for this response (Qutob *et al.*, 2006). Various experiments using genetic and pharmacological approaches (Chapter 5) have not identifed any target or cellular process that may be essential for the mode of action of *B. cinerea* NLPs. BcNEP1 and BcNEP2, as well as other NLPs, may indeed act as pore-forming toxins and permeate membranes without any active participation by the plant cell, as proposed by Küfner *et al*. (2009). Their role as virulence factors for *B. cinerea* appears to be marginal and may be overruled by two phytotoxic metabolites (botrydial and botcinic acid) that induce chlorosis and cell collapse. These toxic metabolites have very distinct chemical structures, yet seem to display functional overlap. The production of at least one of these two phytotoxic metabolites is essential for virulence of *B. cinerea* (Siewers *et al*., 2005; Pinedo *et al*., 2008; Paul and Bettina Tudzynski, *pers. comm.*). *B. cinerea* field isolates that do not produce these toxins are significantly less virulent as compared to isolates that produce either one or both of these toxins (Reino *et al*.,2004). The lack of any detectable role of BcNEP1 and BcNEP2 in virulence of *B. cinerea* and the difficulty in unravelling their mode of action will lead to discontinuation of research on these proteins.

REFERENCE LIST

Bailey, B. A. (1995). "Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*." Phytopathology **85**(10): 1250-1255.

Bondar, D. C., J. M. Beckerich and P. Bonnarme (2005). "Involvement of a branched-chain aminotransferase in production of volatile sulfur compounds in *Yarrowia lipolytica*." Applied and Environmental Microbiology **71**(8): 4585-4591.

Cantu, D., B. Blanco-Ulate, L. Yang, J. M. Labavitch, A. B. Bennett and A. L. T. Powell (2009). "Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires NOR but not RIN or Ethylene." Plant Physiology **150**(3): 1434-1449.

Chagué, V., L. V. Danit, V. Siewers, C. S. Gronover, P. Tudzynski, B. Tudzynski and A. Sharon (2006). "Ethylene sensing and gene activation in *Botrytis cinerea*: A missing link in ethylene regulation of fungus-plant interactions?" Molecular Plant-Microbe Interactions **19**(1): 33-42.

Chague, V., Y. Elad, R. Barakat, P. Tudzynski and A. Sharon (2002). "Ethylene biosynthesis in *Botrytis cinerea*." FEMS Microbiology Ecology **40**(2): 143-149.

Cristescu, S. M., D. De Martinis, S. Te Lintel Hekkert, D. H. Parker and F. J. Harren (2002). "Ethylene production by *Botrytis cinerea in vitro* and in tomatoes." Applied and Environmental Microbiology **68**(11): 5342-5350.

Elad, Y., Z. Lapsker, I. Kolesnik and B. Kirshner (2002). Involvement of ethylene in plant *Botrytis cinerea* interaction. 7th International Mycological Congress, Oslo.

Engels, W. J. M., A. C. Alting, M. M. T. G. Arntz, H. Gruppen, A. G. J. Voragen, G. Smit and S. Visser (2000). "Partial purification and characterization of two aminotransferases from *Lactococcus lactis* subsp. cremoris B78 involved in the catabolism of methionine and branched-chain amino acids." International Dairy Journal **10**(7): 443-452.

Esko, J. D. and N. Sharon (2009). Microbial Lectins: Hemagglutinins, Adhesins, and Toxins. Essentials of Glycobiology. A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart and M. E. Etzler. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press: 784.

Fellbrich, G., A. Romanski, A. Varet, B. Blume, F. Brunner, S. Engelhardt, G. Felix, B. Kemmerling, M. Krzymowska and T. Nürnberger (2002). "NPP1, a Phytophthora-associated trigger of plant defense in parsley and Arabidopsis." Plant Journal **32**(3): 375-390.

Fu, T., C. Burbage, E. Tagge, J. Chandler, M. Willingham and A. Frankel (1996). "Double-lectin site ricin B chain mutants expressed in insect cells have residual galactose binding: evidence for more than two lectin sites on the ricin toxin B chain." Bioconjugate Chemistry **7**(6): 651-658.

Garcia, O., J. A. N. Macedo, R., Tibúrcio, G., Zaparoli, J., Rincones, L. M. C., Bittencourt, G. O., Ceita, F., Micheli, A., Gesteira, A. C., Mariano, M. A., Schiavinato, F. J. Medrano, L. W. Meinhardt, G. A. G. Pereira and J. C. M. Cascardo (2007). "Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete *Moniliophthora perniciosa*, the causal agent of witches' broom in *Theobroma cacao*." Mycological Research **111**(4): 443-455.

Gijzen, M. and T. Nürnberger (2006). "Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa." Phytochemistry 67(16): 1800-1807.

Jarvis, W. R. (1977). Botryotinia and Botrytis species: taxonomy, physiology, and pathogenicity. . Otawa, Research Branch Canada Department of Agriculture.

Kepczynska, E. (1993). "Involvement of ethylene in the regulation of growth and development of the fungus Botrytis cinerea Pers. ex. Fr." Plant Growth Regulation **13**: 65-69.

Klee, H. J., M. B. Hayford, K. A. Kretzmer, G. F. Barry and G. M. Kishore (1991). "Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants." Plant Cell **3**(11): 1187-1193.

Küfner, I., Ottmann, C., Oecking, C. and Nürnberger, T. (2009) "Cytolytic toxins as triggers of plant immune response." Plant Signalling and Behaviour **4**: 977-979.

Lawton, K. A., S. L. Potter, S. Uknes and J. Ryals (1994). "Acquired Resistance Signal Transduction in Arabidopsis Is Ethylene Independent." Plant Cell **6**(5): 581-588.

Macia, E., M. Ehrlich, R. Massol, E. Boucrot, C. Brunner and T. Kirchhausen (2006). "Dynasore, a Cell-Permeable Inhibitor of Dynamin." Developmental Cell **10**(6): 839-850.

Mancheño, J. M., J. Martín-Benito, M. Martínez-Ripoll, J. G. Gavilanes and J. A. Hermoso (2003). "Crystal and electron microscopy structures of sticholysin II actinoporin reveal insights into the mechanism of membrane pore formation." Structure **11**(11): 1319-1328.

Motteram, J., I. Kufner, S. Deller, F. Brunner, K. E. Hammond-Kosack, T. Nürnberger and J. J. Rudd (2009). "Molecular characterization and functional analysis of MgNLP, the sole NPP1 domain-containing protein, from the fungal wheat leaf pathogen Mycosphaerella graminicola." Molecular Plant Microbe Interactions **22**(7): 790-799.

Ottmann, C., B. Luberacki, I. Küfner, W. Koch, F. Brunner, M. Weyand, L. Mattinen, M. Pirhonen, G. Anderluh, H. U. Seitz, T. Nürnberger and C. Oecking (2009). "A common toxin fold mediates microbial attack and plant defense." Proceedings of the National Academy of Sciences of the United States of America **106**(25): 10359-10364.

Pemberton, C. L. and G. P. C. Salmond (2004). "The Nep1-like proteins - A growing family of microbial elicitors of plant necrosis." Molecular Plant Pathology **5**(4): 353-359.

Petersen, M., P. Brodersen, H. Naested, E. Andreasson, U. Lindhart, B. Johansen, H. B. Nielsen, M. Lacy, M. J. Austin, J. E. Parker, S. B. Sharma, D. F. Klessig, R. Martienssen, O. Mattsson, A. B. Jensen and J. Mundy (2000). "Arabidopsis map kinase 4 negatively regulates systemic acquired resistance." Cell **103**(7): 1111-1120.

Pinedo, C., C. M. Wang, J. M. Pradier, B. Dalmais, M. Choquer, P. Le Pecheur, G. Morgant, I. G. Collado, D. E. Cane and M. Viaud (2008). "Sesquiterpene synthase from the botrydial biosynthetic gene cluster of the phytopathogen Botrytis cinerea." ACS Chemical Biology **3**(12): 791-801.

Qadir, A., E. W. Hewett and P. G. Long (1997). "Ethylene production by *Botrytis cinerea*." Postharvest Biology and Technology **11**(2): 85-91.

Qutob, D., B. Kemmerling, F. Brunner, I. Kufner, S. Engelhardt, A. A. Gust, B. Luberacki, H. U. Seitz, D. Stahl, T. Rauhut, E. Glawischnig, G. Schween, B. Lacombe, N. Watanabe, E. Lam, R. Schlichting, D. Scheel, K. Nau, G. Dodt, D. Hubert, M. Gijzen and T. Nürnberger (2006). "Phytotoxicity and innate immune responses induced by Nep1-like proteins." Plant Cell **18**(12): 3721-3744.

Reino, J. L., Hernández-Galán, R., Durán-Patrón, R. and Collado, I. G. (2004). "Virulence–toxin production relationship in isolates of the plant pathogenic fungus *Botrytis cinerea*." Phytopathology **152**: 563-566.

Schouten, A., P. Van Baarlen and J. A. L. Van Kan (2008). "Phytotoxic Nep1-like proteins from the necrotrophic fungus Botrytis cinerea associate with membranes and the nucleus of plant cells." New Phytologist 177(2): 493-505.

Sharon, A., Y. Elad, R. Barakat and P. Tudzynski (2004). Phytohormones and *Botrytis*. *Botrytis*: Biology, Pathology and Control. Y. Elad, B. Williamson, P. Tudzynski and N. Delen. Dordrecht, the Netherlands, Kluwer Academic Publishers: 163-179.

Staats, M., P. Van Baarlen, A. Schouten and J. A. L. Van Kan (2007). "Functional analysis of NLP genes from Botrytis elliptica: Short communication." Molecular Plant Pathology **8**(2): 209-214.

Thage, B. V., F. P. Rattray, M. W. Laustsen, Y. Ardo, V. Barkholt and U. Houlberg (2004). "Purification and characterization of a branched-chain amino acid aminotransferase from *Lactobacillus paracasei* subsp. paracasei CHCC 2115." Journal of Applied Microbiology **96**(3): 593-602.

Thomma, B. P., K. Eggermont, I. A. Penninckx, B. Mauch-Mani, R. Vogelsang, B. P. Cammue and W. F. Broekaert (1998). "Separate jasmonate-dependent and salicylate-dependent defense-response pathways in arabidopsis are essential for resistance to distinct microbial pathogens." Proceedings of Natural Academy of Science of the United States of America **95**(25): 15107-15111.

van Kan, J. A., T. Cozijnsen, N. Danhash and P. J. De Wit (1995). "Induction of tomato stress protein mRNAs by ethephon, 2,6-dichloroisonicotinic acid and salicylate." Plant Molecular Biology **27**(6): 1205-1213.

van Loon, L. C., P. A. Bakker and C. M. Pieterse (1998). "Systemic resistance induced by rhizosphere bacteria." Annual Review of Phytopathology **36**: 453-483.

Summary

Botrytis cinerea can infect more than 200 plant species, including a wide range of economically important crops. During pathogen infection, plants release ethylene and it has been hypothesized that ethylene may predispose host tissue for infection by inducing senescence and ripening. This thesis focused on the roles that ethylene production and perception, both by the pathogen and the plant, play in the interaction between *B. cinerea* and crops, using tomato as a model. Furthermore, functional analysis was performed of *B. cinerea* Nep1-Like Proteins (NLPs), called BcNEP1 and BcNEP2, with emphasis on their role in virulence and mode of action.

Ethylene regulates several developmental processes in plants and plays an important role in plant-pathogen interactions. We investigated possible effects of ethylene on *B. cinerea* during infection of tomato *Solanum lycopersicum* (Chapter 2). There were previous reports that ethylene released by the plant could stimulate germination of *B. cinerea* conidia and affect germ tube growth and infection structure differentiation. Based on growth experiments *in vitro* in the presence of ethylene, we conclude that ethylene does not affect hyphal development of the fungus. Also the virulence of *B. cinerea* on tomato genotypes with a reduced or an enhanced ethylene production level was unaltered. Neither did ethylene induce fungal gene expression as was previously reported. We studied a *B. cinerea* gene encoding a histidine kinase (BcHHK5) with strong structural similarity to plant ethylene receptors. Mutants in which the *Bchhk*5 was deleted were neither affected in growth *in vitro* nor in virulence. We propose that the effects of ethylene on *B. cinerea* disease development are not a direct consequence of an ethylene response in the pathogen, but rather a consequence of induced senescence and ripening processes in the host. These processes provoke softening and disintegration of tissues that facilitate the entry and proliferation of the pathogen.

Functional analysis was performed of two *B. cinerea* NLPs, named BcNEP1 and BcNEP2, produced in *Pichia pastoris* (Chapter 3). Infiltration of purified proteins into *N. benthamiana* leads to induction of ethylene in a dose-dependent manner. BcNEP1 was able to induce ethylene and necrosis at lower concentrations as compared to BcNEP2. Transcriptional studies (Chapter 3) showed that *Bcnep*1 is transiently expressed during early stages of infection when primary lesions develop, while *Bcnep*2 is expressed when the infection is established and lesions are expanding. Altogether these results suggested that BcNEP1 and BcNEP2 may have different functions or they have a similar function at different stages of the

infection process. Single knock-out mutants of either *Bcnep*1 or *Bcnep*2 gene showed no reduction of virulence on tomato or *N. benthamiana.* Ethylene emitted by leaves inoculated with *Bcnep* mutants was not significantly different from leaves inoculated with the parental wild type strain B05.10. These results demonstrate that BcNEP proteins are not essential in the infection process of *B. cinerea* and that ethylene produced in *B. cinerea*-infected tissue does not result from a response to BcNEP proteins (Chapter 3).

By transiently expressing site-directed mutant BcNEP proteins in *N. benthamiana* and *N. tabacum* through *Agrobacterium tumefaciens,* we could study structure-function relationships (Chapter 4). The conserved hepta-peptide GHRHWDE, in the central part of the protein sequence, was shown to be essential for the necrosis-inducing activity. Also the first two cysteine residues, C68 and C94, which are predicted to form a disulfide bridge, are important for necrosis-inducing activity. The two proteins contain different post-transcriptional modification motifs, however, none of these motifs is essential for necrosis-inducing activity.

Necrosis-inducing activity of BcNEP1 was independent of light, whereas the activity of BcNEP2 was compromised when the protein was infiltrated in leaves of dark-adapted plants and the infiltrated plants were kept in darkness (Chapter 5). We studied the role of the plant in the mode of action of BcNEP proteins using genetic and pharmacological approaches (Chapter 5). In spite of several efforts, we were not able to identify any cellular process or signaling pathway in plants that is required for the necrosis-inducing activity of BcNEP proteins. The target(s) and mode(s) of action of BcNEP proteins remain unresolved.

Samenvatting

De schimmel *Botrytis cinerea* kan meer dan 200 plantensoorten infecteren, waaronder een groot aantal gewassen van grote economische betekenis. Tijdens infectie door ziekteverwekkers produceren planten ethyleen. Er is gepostuleerd dat ethyleen in een waardplant de rijping en veroudering van weefsels induceert, waardoor de plant vatbaarder wordt voor ziekteverwekkers. Dit proefschrift richtte zich op het bestuderen van de rol van ethyleenproductie en -perceptie, zowel door de ziekteverwekker als door de plant, in de interactie tussen *B. cinerea* en planten, waarbij tomaat als modelgewas werd gebruikt. Bovendien werd een functionele analyse uitgevoerd aan twee fytotoxische 'Nep1-Like Proteins' van *B. cinerea*, genaamd BcNEP1 and BcNEP2, met bijzondere aandacht voor de rol van deze eiwitten in virulentie en voor hun werkingsmechanisme.

Ethyleen reguleert in planten verschillende ontwikkelingsprocessen en het speelt een belangrijke rol in plant-pathogeen interacties. We bestudeerden de mogelijke effecten van ethyleen op *B. cinerea* tijdens de infectie van tomaat, *Solanum lycopersicum* (Hoofdstuk 2). Er waren eerdere publikaties die beschreven dat ethyleen, geproduceerd door een plant, in staat was om de kieming van *B. cinerea* conidia te stimuleren, en ook de kiembuisgroei en differentiatie van infectie structuren van de schimmel te beïnvloeden. Op basis van *in vitro* groeiproeven in aanwezigheid van ethyleen, konden wij concluderen dat ethyleen de ontwikkeling van hyfen van de schimmel niet beïnvloedt. Ook de ziekteontwikkeling van *B. cinerea* op tomaat genotypes met verminderde of verhoogde ethyleen productie was onveranderd. Ethyleen kon evenmin de expressie induceren van twee schimmelgenen, zoals door anderen werd gerapporteerd. We bestudeerden een *B. cinerea* gen dat codeert voor een histidine kinase (BcHHK5), dat structurele gelijkenis vertoont met ethyleenreceptoren van planten. *B. cinerea* mutanten waarin het *Bchhk*5 gen was uitgeschakeld vertoonden geen veranderde groei *in vitro* en ook geen verminderde virulentie. We concluderen dat de effecten van ethyleen op de ziekteontwikkeling van *B. cinerea* geen direct gevolg kunnen zijn van een ethyleenrespons in de schimmel, maar resulteren van de inductie van veroudering en rijping in de waardplant. Deze processen veroorzaken plantenweefselafbraak en vergemakkelijken de toegang en proliferatie van *B. cinerea* (Hoofdstuk 2).

Eveneens werd een functionele analyse uitgevoerd aan twee fytotoxische eiwitten van *B. cinerea*, genaamd BcNEP1 en BcNEP2 die werden geproduceerd in de gist *Pichia pastoris* (Hoofdstuk 3). Infiltratie van de eiwitten in *Nicotiana benthamiana* leidde op een

concentratie-afhankelijke wijze tot inductie van ethyleen productie. BcNEP1 induceerde ethyleen en necrose bij lagere concentraties dan BcNEP2. Transcript analyse (Hoofdstuk 3) toonde aan dat *Bcnep*1 tot expressie komt in de vroege infectiestadia, terwijl *Bcnep*2 tot expressie komt als de *B. cinerea* lesies uitgroeien. Alles overziend suggereren deze resultaten dat BcNEP1 en BcNEP2 verschillende functies hebben, of ze hebben dezelfde functie op verschillende tijdstippen van de infectie door *B. cinerea*. Deletiemutanten in het *Bcnep*1 of *Bcnep*2 gen vertoonden geen verminderde virulentie op tomaat of *N. benthamiana*. Ethyleen productie in bladeren die waren geïnoculeerd met *Bcnep* mutanten was niet lager dan in bladeren die waren geïnoculeerd met de wild type *B. cinerea* stam B05.10. BcNEP eiwitten zijn dus niet essentieel voor virulentie van *B. cinerea* en ethyleen productie in *B. cinerea*-geïnfecteerd weefsel is niet het gevolg van respons op BcNEP eiwitten (Hoofdstuk 3).

Door transiënte expressie van gemuteerde BcNEP eiwitten in *N. benthamiana* en *N. tabacum* door middel van *Agrobacterium tumefaciens*, konden we structuur-functie relaties bestuderen (Hoofdstuk 4). Het geconserveerde hepta-peptide domein, GHRHWDE, in het centrale deel van de eiwitsequentie, was essentieel voor de necrose-inducerende activiteit. Ook de eerste twee cysteine residuen, C68 en C94, die volgens structuurvoorspellingen een zwavelbrug vormen, zijn belangrijk voor necrose-inducerende activiteit. De twee eiwitten bevatten geheel verschillende motieven voor post-translationele modificaties, maar deze motieven zijn niet essentieel voor necrose-inducerende activiteit (Hoofdstuk 4).

Necrose-inducerende activiteit van BcNEP1 was niet afhankelijk van licht. Daarentegen nam de necrotische reactie op BcNEP2 af als het eiwit werd geïnfiltreerd in bladeren van in het donker geplaatste planten, en de geïnfiltreerde planten vervolgens in het donker bleven (Hoofdstuk 5). We bestudeerden de rol van de plant in het werkingsmechanisme van BcNEP eiwitten door genetische en farmacologische benaderingen (Hoofdstuk 5). In verschillende typen experimenten werden geen processen geïdentificeerd die van belang zijn voor necrose-inducerende activiteit van BcNEP eiwitten. Het werkingsmechanisme en de moleculaire targets van BcNEP eiwitten blijven onopgehelderd.

Acknowledgements

The completion of this project has been possible thanks to the support and contribution of several people.

Jan, my appreciation and esteem for your guidance and the commitment to bring me to the completion of this thesis. Without you I would not have been able to come to these final pages. I don't have words to thank you for the efforts you made and for encouraging me. When you and Sander gave me the possibility to start this project, you rescued me from a difficult situation, I am also thankful for that. Since then, you and Sander have taught me almost everything that I know in Molecular Biology and helped me to grow as a scientist.

In 2001, I arrived in the Laboratory of Phytopathology to do my MSc research with Sander and Jos. Since then, it has been a long journey. Sander, you have been my daily supervisor for the MSc and PhD research; you were very patient and kind with me. Thank you for all your teaching and guidance. You also brought a lot of enthusiasm to the Botrytis group. I would like to thank also Martijn, Ilona, Lia and Peter for the time that we spent together in and out of the lab as members of the Botrytis group

The help of Ester at the end of the project was decisive. You are an excellent technician and I learnt a lot from you including keeping the lab in order ☺. I would like to thank as well Eric for his input in the research and being a funny roommate.

Beatrice, Filipe, Miriam O., Mirjam D., and Peter V., all of you as students contributed to experiments that are described in this thesis. I cannot forget José, who made me visit his home country with his nice stories during lab work. I enjoyed a lot the company of all you guys.

I would like to express my gratitude to Paul and Bettina Tudzynski for their advices during the Dutch-German Botrytis meetings and more importantly letting me stay in their labs for a while. Thanks also to Verena for helping me out with the experiments.

I was very lucky to be member of Phytopathology. My friends were always complaining that Dutch people are very tough, direct… In my case it was not true, as my colleagues were very kind and always ready to help even when you don't ask. I appreciate a lot the friendly environment and of course the phyto-excursions without biking. I enjoyed during all those years having nice chats and sometimes very funny ones with John, Harold, Rob, Ioannis, Marco, Peter or Koste. Harold, I was delighted to be your co-pilot while benefiting from your advice for writing this thesis and to be a good mother. Thanks again for being such a gentleman. Every time I got trapped in technical matters, I always looked for Klaas who was

always ready to help me with troubleshooting. Thank you. Emilie: you were my special colleague, I will never forget your inestimable friendship and you named my first child☺.

I will take as nice memories our girls dinner that allowed me to enjoy the sympathy and the delicious food that we shared during very long evenings. Thanks to all phyto girls.

I would like to thank also the ones that were involved in my research indirectly as their help has been crucial. My gratitude to Bert, André and Henk for taking care of the plants and their good advices. Ali, you were always ready to help me with administrative stuff even when I was not longer part of the staff, I appreciate your efficiency a lot. Diane, I enjoyed a lot our times measuring ethylene and talking about Dutch and Cuban culture. Ernst, thanks you for the supervision of the ethylene experiments and the advices during STW user committee meetings. I would also like to acknowledge STW for financing this project and the rest of the user STW committee members as well as Productschap Tuinbouw

Wageningen is a small city where you can make great friends.

I would like to thank to the French connection: Stephanie Pringent, Stephanie Guillotin Laurice and Natalie, for the very good moments that we spent together. Maelle, thanks to you I came back to Holland to pursue the PhD title. You also make me easy the communication with your compatriots, thanks for the French lessons!! I appreciate you a lot. Thierry and Hoa, thank you for everything that you have done for me and my little family. Merci à tous

Douglas, Berna, Lourdes, Sjaak, Rosita y Norka a todos muchas gracias por su incondicional amistad, les agradeceré eternamente haber formado parte de esta etapa de mi vida.

Arantza, ni volviendo a nacer podría agradecerte todo lo que has hecho por mi durante todo este tiempo. Tu has sido mas que mi amiga y tu entusiasmo y determinación han sido muy importante para ponerle punto final a este proyecto.

Me gustaría agradecer en especial a Ester y sus “amigos” por sus predicciones y consejos.

Me gustaría agradecer a mi extensa familia, que desde la distancia me ha estimulado y motivado. A mi abuela, por su amor incondicional que me ha ayudado en los momentos mas difíciles sin mirar atrás y gracias a ti pude terminar mis estudios universitarios. En especial mi tia Sara, que es la loca mas cuerda que conozco y me ha apoyado y ayudado en esta aventura científica. Mi profunda gratutid a mi madre que ha venido desde tan lejos para cuidar de mis hijos para poder redactar estas últimas líneas.

William, tu amor| y sacrificios han sido un pilar en donde guarecerme durante todo este trayecto, te doy mil gracias.

...ah se me olvidaba... GRACIAS TIO ☻

Curriculum vitae

Yaite Cuesta Arenas was born on September 20, 1974 in Havana City, Cuba. In 1992, she received her high school diploma at Pre- university Institute of Exact Sciences "Heroes of Humboldt 7" with specialization in Mathematics. Then, she pursued studies at Havana University during 5 years. After graduating as BSc in Microbiology in 1997, she began to work as junior scientist at the laboratory of microorganism collection in the Institute for Fundamental Research on Tropical Agriculture "Alejandro de Humboldt" (INIFAT) in Havana City. In 2000, she started an MSc in Biotechnology at Wageningen University thanks to a fellowship granted by this university. During her MSc project she worked on resistance mechanisms in *Botrytis cinerea* against antibiotics produced by the biocontrol agent *Pseudomonas fluorescens*, under the supervision of Dr. Alexander Schouten and Dr. Jos Raaijmakers at the Laboratory of Phytopathology. In 2002, she returned to Cuba to work in an international project to obtain secondary metabolites from fungi to use in agriculture and medicine at INIFAT. From 2004 to 2008, she conducted her PhD thesis research entitled "The role of ethylene production and perception, by both pathogen and plant, in the interaction of *Botrytis cinerea* and tomato" under the supervision of Dr. Jan van Kan at the Laboratory of Phytopathology, Wageningen University.

PUBLICATIONS

Alexander Schouten, Olesya Maksimova**, Yaite Cuesta-Arenas,** Grardy van den Berg and Jos M. Raaijmakers (2008)."Involvement of the ABC transporter BcAtrB and the laccase BcLCC2 in defence of *Botrytis cinerea* against the broad-spectrum antibiotic 2,4-diacetylphloroglucinol" Enviromental Microbiology. **10** (5); 1145-1157

Yaite Cuesta, Ester Dekkers and Jan A.L. van Kan (2010) Ethylene perception by *Botrytis cinerea* does not affect pathogenesis on tomato. Molecular Plant Pathology. *Submitted*

Yaite Cuesta Arenas, Ester Dekkers, Alexander Schouten, Eric Kalkman, Miriam Oses Ruiz, Beatrice Uwumukiza, Mirjam Dieho, Peter Vredenbregt, Jan A.L. van Kan "Functional analysis and mode of action of *Botrytis cinerea* NEP-like proteins." *In preparation*

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Yaite Cuesta Arenas
Date: 17 May 2010
Group: Laboratory of Phytopathology, Wageningen University

1) Start-up phase		*date*
►	**First presentation of your project**	
	The role of ethylene production and perception, by both pathogen and plant, in the interaction of Botrytis cinerea and tomato; perspectives for	Dec 2004
►	**Writing or rewriting a project proposal**	
►	**Writing a review or book chapter**	
►	**MSc courses**	
►	**Laboratory use of isotopes**	
	Safehandling radioisotopes, level 5B	2004
	Subtotal Start-up Phase	*3.0 credits**
2) Scientific Exposure		*date*
►	**EPS PhD Student Days**	
	EPS PhD Student Day, Amsterdam	Jun 03, 2004
	EPS PhD Student Day, Wageningen	Sep 19, 2006
	EPS PhD Student Day, Wageningen	Sep 13, 2007
►	**EPS Theme Symposia**	
	EPS Theme 2 Symposium: Interaction between plants and biotic agents, Utrecht	Sep 17, 2004
	EPS Theme 2 Symposium: Interaction between plants and biotic agents, Leiden	Jun 23, 2005
►	**NWO Lunteren days and other National Platforms**	
	The ALW-NWO meeting in Lunteren	Apr 05-06, 2004
	The ALW-NWO meeting in Lunteren	Apr 04-05, 2005
	National Botrytis workgroup meeting, Wageningen	Apr 12, 2005
►	**Seminars (series), workshops and symposia**	
	Seminar Nick Talbot	May 03, 2003
	Genomic Momentum, Rotterdam	Aug 30-Sep 01, 2004
	Dutch-German Workshop on molecular aspects of pathogenicity of *Botrytis cinerea*	2004, 2005
	CBS/Wageningnen Phytopathology Symposium, Wageningen	2004
	Seminar Sophien Kamoun	Okt 05, 2005
	CBS/Wageningnen Phytopathology Symposium, Wageningen	Jun 2007
	Bio Career Event	May 31, 2008
►	**Seminar plus**	
►	**International symposia and congresses**	
	13th International Botrytis Symposium, Antalya, Turkey	Oct '21-25, 2004
	8th European Conference on Fungal Genetics Vienna, Austria	Apr 08-11, 2006
	2nd Botrytis Genome Workshop, Versailles, France	Oct 05-07, 2006
	14th International Botrytis Symposium, Cape Town, South Africa	Oct 21-25, 2007
	Workshop Botrytis-tomato interaction, University of California, Davis, USA	Mar 19, 2007
	24th Fungal Genetics Conference, Asilomar USA	Mar 20-25, 2007
►	**Presentations**	
	13th International Botrytis Symposium, Antalya, Turkey (poster)	Oct 21-25, 2004
	Dutch-German Workshop on molecular aspects of pathogenicity of *Botrytis cinerea* (two oral presentations)	2004, 2005
	8th European Conference on Fungal Genetics Vienna, Austria (poster)	Apr 08, 2006
	24th Fungal Genetics Conference, Asilomar USA (poster)	Mar 20-25, 2007
	14th International Botrytis Symposium, Cape Town, South Africa (oral presentation)	Oct 21-25, 2007
	Workshop Botrytis-tomato interaction, University of California, Davis, USA (oral presentation)	Mar 19, 2007
►	**IAB interview**	Sep 07, 2006
►	**Excursions**	
	Laboratoy Molecular Biology and Biotechnology of Fungi, Institute of Botany, Munster, Germany	Aug 26-30, 2005
	Subtotal Scientific Exposure	*19.6 credits**
3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	EPS-Summerschool: Environmental Signaling : Arabidopsis as a model	Aug 22-24, 2005
	EPS-Summerschool: Signaling in Plant Development and Defense towards System Biology	Jun 19-21, 2006
►	**Journal club**	
	Phytopathology group	2004- 2008
►	**Individual research training**	
	Subtotal In-Depth Studies	*4.8 credits**
4) Personal development		*date*
►	**Skill training courses**	
	Techniques for writing and presenting a scientific paper	Jun 29-Jul 02, 2004
	Academic Writing	Jan 15-Apr 02, 2007
	Working with Endnote 7	Feb 01,2004
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3.9 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**31.3**

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

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

This research was performed at the Laboratory of Phytopathology, Binnenhaven 5, 6709 PD Wageningen, the Netherlands, and within the graduate school for Experimental Plant Sciences (EPS).

This research was funded by the Dutch Technology Foundation STW (project WEB6084) with financial support by the Horticultural Product Board (Productschap Tuinbouw).

Cover: a tomato leaflet inoculated with *Botrytis cinerea*
Printed at GVO B.V. /Ponsen & Looijen, Ede