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Localization and functional characterization of effector proteins of *Colletotrichum higginsianum*

LINDA JEIMMY RINCÓN RIVERA

Universidad Nacional de Colombia

Facultad de Agronomía

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LINDA JEIMMY RINCÓN RIVERA

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Resumen

El patosistema modelo *Colletotrichum higginsianum- Arabidopsis thaliana* ha permitido avanzar en el entendimiento de las interacciones planta patógeno. Se ha establecido que durante la fase de infección biotrófica hay un conjunto de proteínas efectoras secretadas que le permiten al patógeno evadir el reconocimiento o modular las respuestas de defensa de la planta favoreciendo la infección. La expresión de los proteínas candidatas efectoras de *C. higginsianum* (ChECs) fue inducida por el hospedero y específica a particulares estados de infección. El objetivo de este estudio fue localizar y caracterizar la función de ChEC3, CHEC6, ChEC36 and CHEC89. En el caso de CHEC6 y CHEC36 fueron secretadas de manera focalizada desde el poro de penetración del apresorio, sugeriendo una nueva función para esta estructura de penetración, la secreción localizada de proteínas efectoras. CHEC89 y CHEC3 se acumularon en estructuras formadas en la interface entre las hifas bitróficas y las células del hospedero, implicando a este tipo de hifas en la secreción de efectores. Adicionalmente se evidencio que CHEC3 y CHEC89 incrementaron el crecimiento de bacterias fitopatógenas.

Se estableció que ChEC3, ChEC36, ChEC6 suprimieron la muerte celular causada por las proteínas NLP1 (Necrosis and Ethylene-inducing Peptide1-like proteins), sugiriendo que son capaces de intervenir en la inmunidad modulada por patrones moleculares asociados a patógenos (PTI). Además, estos efectores fueron capaces de bloquear la respuesta hipersensible causada por el reconocimiento de la proteína efectora AvrRps4 de *Pseudomonas syringae* por el gen de resistencia RPS4 de *Arabidopsis*, sugiriendo que estas proteínas efectoras actúan en la supresión de la inmunidad elicitada por efectores (ETI). Es posible que estas proteínas interfieran con componentes corriente abajo en la vía de señalización comunes a las dos vías de defensa de las plantas.

Palabras clave: *Colletotrichum higginsianum*, Proteínas efectoras

Abstract

The model pathosystem *Colletotrichum higginsianum - Arabidopsis thaliana* has allowed advances in the understanding of plant pathogen interactions. It has been established that during the biotrophic phase of infection there are a set of secreted effector proteins that may allow the pathogen to evade host recognition or modulate host defense responses to favor the fungal infection. It was found that the expression of *Colletotrichum higginsianum* Effector Candidates (ChECs) was host-induced and specific to particular infection stages. The aim of this study was the localization and functional characterization of ChEC3, CHEC6, ChEC36 and CHEC89 . It was found that CHEC6 and CHEC36 were focally secreted from appressorial penetration pores, showing a new function for this fungal penetration structure, the local secretion of effector proteins. CHEC89 and CHEC3 were accumulated in structures formed at the interface between biotrophic primary hyphae and living host cells, implicating these hyphae in effector delivery. In addition it was shown that CHEC3 and CHEC89 improved the growth of plant pathogenic bacteria.

It was established that ChEC3, ChEC36, ChEC6 are able to suppress plant cell death caused by Necrosis and Ethylene-inducing Peptide1-like proteins, suggesting that they are able to interfere in Pathogen-associated molecular pattern triggered immunity (PTI). Moreover, these effectors suppressed the hypersensitive response caused by recognition of the AvrRps4 effector protein from *Pseudomonas syringae* by the RPS4 resistance protein from *Arabidopsis*, suggesting these effector proteins are involved in suppressing effector triggered immunity (ETI). It is possible these effectors interfere with downstream signaling pathway components that are common to both plant defense pathways.

Key words: *Colletotrichum higginsianum*, effector proteins

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1. Introduction

1.1 *Colletotrichum higginsianum*

Colletotrichum is a genus of Ascomycete fungi (class Sordariomycetes), which contains 680 species (Crous *et al*., 2004). Members of the genus cause anthracnose leaf spot diseases, blights and post-harvest rots on numerous crop plants in both tropical and temperate regions, including cereals (e.g. maize, sorghum), cassava, yam, grain legumes (e.g. beans, cowpea), vegetables and fruits (Bailey and Jeger, 1992). Yield and economic losses can be very severe, for example *C. graminicola* causes annual losses on maize of approximately 1 billion dollars in the USA alone (Frey *et al*., 2011). According to the strategies used to obtain nutrients from their host, phytopathogenic fungi can be classified as biotrophs, necrotrophs or hemibiotrophs (Mendgen and Hahn, 2002). Biotrophic fungi keep their host plant alive to obtain nutrients, manipulating the host through molecular mechanisms that are still poorly understood. In contrast, necrotrophic fungi take nutrients from dead tissues, killing host cells immediately after infection and eventually decomposing the plant tissue (Van Kan, 2006). Most *Colletotrichum* species are classified as hemibiotrophic pathogens, combining both biotrophic and necrotrophic lifestyles. Initially, intracellular biotrophic hyphae are developed, which invaginate the plasma membrane of living host cells. Later the fungus switches to necrotrophic growth when secondary intercellular hyphae are produced which destroy the infected cells (O´Connell *et al*., 2000, O´Connell *et al*., 2004).

C. higginsianum has a wide host range including members of the Brassicaceae such as *Brassica*, *Raphanus* and *Arabidopsis* (Narusaka *et al*., 2004; O'Connell *et al*., 2004). During the process of infection the conidia of *C. higginsianum* initially adhere to the plant surface, and germinate to produce a germ-tube. Surface cues such as hydrophobicity

induce the germ-tube to differentiate a specialized penetration structure called an appressorium. Mature appressoria have darkly melanized cell walls and generate high turgor pressure required for mechanical penetration of the plant cuticle/cell wall (Deising *et al*., 2000). Studies on *M. oryzae* appressoria have shown that the turgor pressure results from the accumulation of glycerol inside the melanized appressoria, generating turgor pressures as high as 8 MPa (Money and Howard, 1996). *Colletotrichum* appressoria also have a glycerol-dependent mechanism for turgor generation. *C. graminicola* appressoria produce a turgor pressure estimated in 17 μN, comparable to the force excerted by an eight-ton school bus on the palm of a human hand (Money, 1999, Bechinger *et al*., 1999). A needle-like infection peg emerges from a pore in the base of the appressorium and penetrates the plant cuticle and cell wall. After penetration (approximately 24 hours after inoculation), the fungus produces bulbous biotrophic hyphae inside the host epidermal cell, which invaginate the plasma membrane of the penetrated host cell. This initial biotrophic phase is confined to only one epidermal cell. Subsequently (approximately 48 hours after inoculation), the biotrophic hyphae produce narrower, filamentous secondary hyphae, which invade the surrounding epidermal and mesophyll cells. At this necrotrophic stage, all the infected cells, and many uninfected cells, are dead. Eventually the fungus completes its life-cycle by producing sporulating structures (acervuli) on the surface of the dead tissue (O´Connell *et al*., 2004).

1.2 *Arabidopsis thaliana*

Arabidopsis thaliana is a member of the *Brassicaceae* distributed in Asia, Europe, North America, Australia and Japan (Mitchell-Olds and Schmitt, 2006). It is a relatively small plant, with fast growth, high fertility and high seed production and a short generation time. Many well-characterized *Arabidopsis* mutants are available and it is easily genetically transformed. Microarrays are available for genome-wide gene expression profiling. It has also been considered a model plant in plant pathology because it is a host for several pathogens with different taxonomic origins and lifestyles. Obligate biotrophic pathogens, such as *Hyaloperonospora parasitica*, *Albugo* and *Erisyphe*, necrotrophic pathogens, such as *Botrytis*, *Alternaria* and *Rhizoctonia*, and hemibiotrophic pathogens, such as *Phytophthora* and *Colletotrichum*, have all been reported (Adam *et al*., 1999, Holub *et al*.,

1995, Koch and Slusarenko 1990, Roetschi *et al*., 2001, Thomma *et al*., 1999, O´Connell *et al*., 2004).

1.3 The *Colletotrichum higginsianum – Arabidopsis* interaction

The infection of *Arabidopsis thaliana* by *C. higginsianum* was first described by O´Connell and collaborators (2004). *C. higginsianum* causes hemibiotrophic infection on *Arabidopsis* plants, which facilitates the study of both biotrophic and necrotrophic lifestyles. Through the evaluation of 37 *Arabidopsis* ecotypes, it was possible to detect a single dominant allele, called *RCH-1*, which confers resistance against anthracnose caused by *C. higginsianum* in ecotype Eil-0 (Narusaka *et al*., 2004, Birker *et al*., 2009). In addition, two resistance proteins with nucleotide binding site and leucine-rich repeats (NBS-LRR) encoded by *RRS1* and *RPS4* in tandem, confer resistance to *C. higginsianum*. Remarkably, these two genes also confer resistance to two bacterial pathogens; thus *RRS1* confers resistance to a strain of *Ralstonia solanacearum,* while *RPS4* confers resistance to *Pseudomonas syringae* pv. *tomato* carrying the avirulence gene *AvrRps4* (Narusaka *et al*., 2009).

On the pathogen side, it is possible to stably transform *C. higginsianum* with high efficiency by T-DNA transfer mediated by *Agrobacterium tumefaciens*. This allows random insertional mutagenesis (Huser *et al*., 2009), targeted mutagenesis (Ushimaru *et al*., 2010) and expression of transgenes such as fluorescent proteins (O´Connell *et al*., 2004). Also the fungal genome was recently sequenced and annotated (Broad Institute (http://www.broadinstitute.org/annotation/fungi/). It is a significant advantage that in this interaction both host and pathogen can be manipulated genetically and genome sequences are available.

1.4 The Plant-Pathogen Arms Race

Fossil records show that the adaptation of plants to life on land was facilitated by symbiotic fungal interactions, suggesting that coevolution of plants and microbes is very ancient (Gehrig *et al*., 1996). Jones and Dangl (2006) have proposed the so-called ‘zig-zag’ model to describe the evolutionary arms race between plants and pathogens, which

will be discussed in detail below. This model explains how pathogens try to avoid recognition and activation of plant defenses, while plants try to advance in recognition and establish stronger defense responses. Plants are exposed to numerous pathogens, and have developed two major lines of recognition and immune responses, namely pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI).

1.4.1 PAMP-triggered immunity (PTI)

Initially plants evolved the ability to detect highly conserved molecules present in all pathogens. These components are called pathogen-associated molecular patterns (PAMPs). PAMPs are recognizing by cell surface receptor proteins called pattern recognition receptors (PRRs). In addition, plants are able to recognize cuticular or plant cell wall fragments that are released during pathogen infection. Those molecules are called damage-associated molecular patterns (DAMPs).

The classical example of a PAMP is the N-terminal domain of the bacterial flagellin protein called flg22 (Zipfel and Felix 2005, Gomez-Gomez and Boller, 2000). Other examples include lipopolysaccharide (LPS), a major constituent of the outer membrane in Gram-negative bacteria (Newman *et al*., 2007), and peptidoglycan (PGN), the most abundant compound in the cell wall of Gram-positive bacteria (Gust *et al*., 2007). In the case of oomycetes, the cellulose-binding elicitor lectin (CBEL) of *Phytophthora parasitica* var. *nicotianae* was shown to be triggering plant defense responses in *Arabidopsis* and tobacco (Gaulin *et al*., 2006). Very common PAMPs in fungi are the polysaccharide chitin present in the cell wall and ergosterol in the fungal membrane (Nurnberger *et al*., 2004, Boller and Felix *et al*., 2009).

When PAMPs are perceived by PRRs, the first line of defense is activated, which is called PAMP-triggered immunity (PTI). PRRs can be classified as transmembrane receptor kinases (RLK) and transmembrane receptor-like proteins. FLS2 and EFR are two RLKs which recognize flagellin and EF-tu, a bacterial elongation factor, respectively. CERK1, is an RLK containing the lysine motif (LysM), that is necessary to perceive chitin

oligosaccharides in *Arabidopsis*. The extracellular domain of CERK1 mediates recognition on the cell surface but transduction of the signal to the cytoplasm is mediated by its serine/threonine kinase domain (Miya *et al*., 2007). The chitin elicitor-binding protein (CEBiP) is a plasma membrane glycoprotein with LysM motifs which functions as a cell surface receptor for the chitin elicitor in rice. In this case, CEBiP does not have any transmembrane domain, and it needs a co-receptor to interact with chitin, which is called OsCERK1 (Shimizu *et al*., 2010).

After recognition of PAMPs, PTI responses include production of reactive oxygen species (ROS), transcriptional activation of pathogen-responsive genes, ethylene production, deposition of callose and activation of MAPK kinases (MAPK). MAPK kinases are involved in transducing signals from extracellular receptors into cellular responses in eukaryotes (Pitzschke *et al*., 2009). However, treatment of *Arabidopsis* cell cultures with different elicitors such as flagellin and EF-tu independently induces expression of very similar sets of genes (Zipfel *et al*., 2006). This suggests that PAMP recognition involves overlapping signalling pathways (Jones and Dangl, 2006).

Necrotrophic and hemibiotrophic pathogens secrete toxins which favor virulence by provoking host cell death. Necrosis and ethylene-inducing peptide 1-like proteins (NLPs) have been identified as toxins presents in oomycetes, bacteria and fungi (Pemberton and Salmond, 2004). All of them have a necrosis-inducing protein 1 (NPP1) domain, containing a common heptapeptide motif “GHRHDWE” (Fellbrich *et al*., 2002). These proteins are able to enhance virulence and microbial growth through disintegration of the plant plasma membrane and consequent cytolysis (Ottmann *et al*., 2009). These authors proposed that this cell disruption may release host-derived molecules that act as DAMPs. Alternatively plants perceive the physiological changes caused by NLPs to activate defense responses, for example the influx of Ca^{2+} and H^{+}. Similar ion fluxes can be mimicked in plant cells by application of synthetic ionophores, which themselves were shown to trigger plant defense-associated responses in a non-receptor-mediated manner (Jabs *et al*., 1997). A protein homolog to *Phytophthora sojae* NIP (Qutob *et al*., 2002) was identified in *C. higginsianum* and is called ChNLP1 (Kleemann, 2010).

1.4.2 Effector-triggered susceptibility (ETS)

Successful pathogens overcome the first line of plant defense by producing effector proteins that are able to suppress PTI. This is called effector-triggered susceptibility (Jones and Dangl, 2006). Effectors are defined by Kamoun (2006) as: “molecules that manipulate host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defense responses (avirulence factors or elicitors)”. Effector proteins are produced by bacterial, fungal and oomycete pathogens, but the bacterial effectors are the best-characterized. Gram-negative bacteria produce many effectors, which are secreted by the type III secretion system (TTSS), a molecular “syringe” that allows bacteria to deliver effectors inside the plant cell (Chisholm *et al*., 2006).

Suppression of PAMP-triggered transcriptional responses has been demonstrated directly through the use of strains of pathogenic bacteria that are deficient in their TTSS, which is indispensable to inject effector proteins into the plant cytoplasm (Thilmony *et al*., 2006). *Pseudomonas syringae* secretes 20 to 30 effectors, several of which have been shown to suppress host immunity through various mechanisms. The bacterial effectors studied so far are mostly involved in three main processes: protein turnover and secretion, RNA homeostasis and phosphorylation pathways (Block *et al*., 2008). The *P. syringae* effectors AvrRpt2 and AvrRpm1 inhibit PAMP-signaling due to their interaction with RIN4, a negative regulator of PAMP signaling (Kim *et al*., 2005). The largest effector family found in *Xanthomonas* species are the TAL (transcription activator-like) effectors, which includes AvrBs3 (Boch and Bonas, 2010). These effectors contain a nuclear localization signal and a modular DNA-binding domain, which allows them to act as transcriptional activators in the plant nucleus. TAL effectors bind to the promoter sequences of particular plant target genes to activate their expression to enhance bacterial colonization, symptom development, or dissemination (Boch and Bonas, 2010).

Other effectors allow pathogens to overcome physical barriers developed in the pathogen infection site. For example, *P. syringae* AvrPto suppresses formation of callose wall-thickenings called papillae, and both AvrE and Hop PtoM also suppress callose deposition (DebRoy *et al*., 2004). XopJ from *Xanthomonas campestris* pv. *vesicatoria* suppresses callose deposition and affects plant protein secretion (Bartetzko *et al*., 2009),

suggesting that the host secretory pathway can be interfered by the pathogen. Another method used by bacteria to modify the host is through the modulation of plant signaling that regulates plant defense, namely salicylic acid (SA), jasmonic acid (JA) and ethylene. This is the case for coronatine, which is produced during *Pseudomonas syringae* infection. SA and JA are antagonistic plant defense signaling pathways, and the production of coronatine mimics JA, inducing suppression of SA-mediated host responses and thereby contributing to bacterial virulence (He *et al*., 2004, Reymond and Farmer, 1998).

Fungal pathogens such as *Cladosporium*, *Magnaporthe* and *Ustilago* can also modify host gene expression during infection, thereby inducing defense-related genes at lower levels, or delaying their transcription, while host genes related to cell death suppression are induced (Doehlemann *et al*., 2008, van Esse *et al*., 2009, Mosquera *et al*., 2009). The oomycete effector ATR13, from *Hyaloperonospora parasitica*, suppresses PAMP-induced callose deposition and ROS accumulation (Sohn *et al*, 2007). *Cladosporium fulvum* evades PTI by secreting an effector called Ecp6, which acts in the plant apoplast to capture chitin oligosaccharides released from the fungal cell wall that would otherwise activate PTI (de Jonge *et al*., 2010).

1.4.3 Effector-triggered immunity (ETI)

The plant's counter-defense against pathogen effectors is based on the evolution of protein receptors called resistance (R) proteins that are able to detect effectors directly, or indirectly by recognizing the activity of effectors. Recognition by R proteins provokes plant defense responses with a higher strength and durability than PTI, and this is called effector-triggered immunity (ETI). The R protein receptors are usually intracellular proteins with nucleotide binding site-leucine rich repeat (NBS-LRR) domains, the latter providing recognition specificity (Martin *et al*., 2003). A hallmark of ETI is the localized programmed cell death of infected cells, called the hypersensitive response (HR). This is typically associated with an increase in cytosolic calcium, depolarization of the plasma membrane, nitric oxide production and MAPK cascade activation (Dangl and Jones, 2001). Antimicrobial molecules such as phytoalexins, chitinases and glucanases are produced by the surrounding tissue, ultimately leading to the restriction of pathogen growth.

ETI provides the molecular basis for the so-called 'gene-for-gene' resistance (Flor, 1942), leading to either compatible or incompatible plant-pathogen interactions. In gene-for-gene resistance, a plant *R* gene matches with an effector-encoding avirulence (*Avr*) gene in the pathogen. To evade ETI, pathogens continuously evolve to lose, modify or create new effectors. Thus, the mutation or absence of an *Avr* gene prevents recognition by the corresponding R gene, leading to disease (i.e. gain of virulence, loss of avirulence) in a previously incompatible interaction. In turn, the host plant is then under selection pressure to evolve new *R* gene genotypes that favor ETI.

Evidence for the direct recognition of effectors is not frequent. However, direct interaction between AvrPita, an effector from the rice blast fungus *Magnaporthe oryzae*, and Pi-ta, an R protein from rice, has been proven by yeast two-hybrid and *in vitro*-binding assays (Jia *et al*, 2000). PopP2 from *Ralstonia solanacearum* is recognized by the RRS1 resistance protein in *Arabidopsis* (Deslandes *et al*., 2003), which also confers resistance to *C. higginsianum* (Narusaka *et al*., 2009). One example of *R* gene and effector gene coevolution due to their direct interaction is the *R* gene *RPP13* from *Arabidopsis* and the *ATR13* effector gene from *Hyalonospora parasitica*. There is high diversity in *ATR13* alleles and also in *RRP13*. For the latter, sequencing 24 *Arabidopsis* accessions showed that the *RRP13* locus has high levels of polymorphism (Rose *et al*., 2004). In the case of indirect recognition, the 'guard model' has been described, where the resistance gene perceives a change produced in another host protein by the activity of the effector protein, thereby eliciting plant defenses (van der Hoorn and Kamoun, 2008). An example is the resistance protein Cf-2 from tomato, which is activated when *Avr2* from *C. fulvum* binds to the tomato protease *Rcr3* (Rooney *et al*., 2005).

1.4.4 Effector delivery by filamentous pathogens

Effector proteins produced by filamentous pathogens (oomycetes and true fungi) typically have an N-terminal signal peptide for secretion via the endoplasmic reticulum and Golgi (exocytosis). According to their localization during infection, effectors can be classified as apoplastic, which are secreted into plant extracellular space and interact with extracellular targets or surface receptors, and cytoplasmic effectors, which are secreted into the plant cytoplasm, involving translocation across the plant plasma membrane (Kamoun *et al*., 2006).

Many biotrophic and hemibiotrophic oomycetes and true fungi share a similar infection process, inserting into living host cells a special structure to obtain nutrients, known as the haustorium. This structure invaginates the plant plasma membrane, producing a highly modified extrahaustorial membrane (Koh *et al*., 2005) and induces reorganization of the plant cytoskeleton and endomembrane system (Kobayashi *et a*l., 1994, Heath, 1997). The haustorium provides a site for intimate interaction, including uptake of nutrients and water into the pathogen and delivery of effectors into the plant cell (Voegle and Mendgen, 2011; Catanzariti *et al*., 2006).

Most fungal effectors are considered to act in the plant cytoplasm but in the majority of cases there is no experimental evidence to prove their translocation into plant cells, and often their intracellular localization has only been inferred from the cytoplasmic location of their corresponding host R proteins (Stergiopoulos and Wit, 2009). In a few cases, effector secretion into host cells has been proven. For example, in the case of *RTP1*, rust transferred protein 1 from *Uromyces fabae*, immunogold labeling was used to localize the effector in the extrahaustorial matrix and in the plant nucleus (Kemen *et al*., 2005). Using immunofluorescence, it was also possible to detect the presence of AvrM from *M. lini* inside flax cells during late infection stages (Rafiqui *et al*., 2010).

The translocation of cytoplasmic effectors has also been proven or inferred for other pathogens which do not produce haustoria. For example, AVR-Pita from *Magnaporthe oryzae* is a secreted protein, which is recognized inside rice host cells by the resistance protein Pi-ta (Jia *et al*., 2000). In *M. oryzae*, effector secretion has been related to their accumulation in the biotrophic interfacial complex (BICs), which is a structure formed on the surface of intracellular biotrophic hyphae into which fluorescently labeled effectors appear to be locally secreted (Mosquera *et al*., 2009, Khang *et al*., 2010). During biotrophic infection of rice cells by *M. oryzae*, fungal genes encoding several secreted proteins, including effector candidates and known Avr proteins, are up-regulated, and these biotrophy-associated secreted (BAS) proteins show different localization patterns. For example, PWL2 and BAS1 proteins accumulate in BICs and are translocated into the rice cytoplasm (Mosquera *et al*., 2009, Khang *et al*., 2010). In contrast, BAS4 was

localized uniformly over the surface of the intracellular hyphae but remained in the plant apoplast and did not enter into the plant cytoplasm (Mosquera *et al*., 2009).

Many oomycete effector proteins contain the conserved amino acid domain RxLR, located 60 amino acids downstream from the signal peptide. This domain is required for effector translocation into host cells (Whisson *et al*., 2007, Morgan and Kamoun 2007). In the case of *Phytophthora*, another motif dEER located 5-21 amino acids further downstream from the RxLR, is also implicated in effector translocation. The RxLR motif is similar in sequence to the PEXEL motif found in *Plasmodium falciparum*, the human malaria parasit that is required for targeting effectors into host red blood cells (Bhattacharjee *et al*., 2006). The PEXEL motif was interchangeable with the RxLR motif from the *P. infestans* effector Avr3a (Grouffaud *et al*., 2008). Recent studies have shown controversial results, which explain a possible association to the AVR1b RxLR from *P. sojae* motif with phosphatidyl inositol phosphates (PIPs) mediating its entrance into the host plant. It was explained by RXLR binding to the PIPs located on the plant plasma membrane surface, favouring the effector endocytosis into the plant cells (Kale *et al*., 2010). However, Yaneoa *et al*., 2011, working with AVR3a found that it contains a conserved patch, which is necessary to binding PIPs rather than the RXLR domain, besides it mutations affecting PIP binding do not abolish AVR3a recognition by the resistance protein R3a. Then is still unclear which is the mechanism mediating oomycetes effector translocation.

Cladosporium fulvum is a fungal pathogen that colonizes plant intercellular spaces, where it delivers many apoplastic effectors. They are typically rich in cysteine residues, which form disulfide bridges, thereby generating structural stability to protect against attack by the plant proteases present in the apoplast (Stergiopoulos and Wit, 2009). For the apoplastic effectors Avr4 and Avr9 it was demonstrated that disulfide bridges formed between cysteine residues provide protein stability and are required for effector activity (Van den Burg *et al*., 2003, Van den Hooven *et al*., 2001). Some apoplastic effectors are able to inhibit the activity of plant proteases, chitinases and glucanases. Thus, Avr2 from *C. fulvum* is able to bind and inactivate the tomato protease Rcr3 (Rooney *et al*., 2005). Similarly, GIP1 and GIP2 are secreted effector proteins of *P. sojae* that inhibit the soybean endo-β-1,3 glucanase EGaseA (Rose *et al*., 2002).

Few effectors have been reported for *Colletotrichum* species. CgDN3 from *C. gloeosporioides* was described as a biotrophy-expressed effector involved in the suppression of plant defenses. CgDN3 knock-out mutants elicited a localized host cell death resembling the hypersensitive response in *Stylosanthes guianensis* (Stephenson *et al*, 2000). In *C. lindemuthianum* and *C. higginsianum*, an effector protein called CIH1 has been identified which is expressed exclusively during the biotrophic phase of infection in bean and Arabidospsis, respectively. CIH1 may prevent plant recognition by chitin camouflage during biotrophic infection (Perfect *et al*., 1998, Takahara *et al*., 2009), similar to *Cladosporium* Ecp6 (de Jonge *et al*., 2010).

2. Objectives

C. higginsianum is an intracellular hemibiotrophic fungus that initially penetrates into, and grows inside, living host cells (biotrophy) before later switching to destructive necrotrophic growth, when it feeds on dead tissues. We assume that during early infection phases it secretes effector proteins that allow the fungus to evade defense responses, keep the host cell alive, and reorganize the plant cytoplasm to accommodate invasion by fungal hyphae. To identify effector proteins expressed before or during biotrophic invasion, stage-specific cDNA libraries were generated previously. These included appressoria formed *in vitro*, biotrophic hyphae purified from infected plants using fluorescence-activated cell sorting, epidermal strips containing plant-penetrating appressoria and the late necrotrophic stage (Kleemann *et al*., 2008; Takahara *et al*., 2009; Kleemann *et al*., 2012). The resulting ESTs were then screened for genes encoding soluble secreted proteins using bioinformatic prediction tools, based on the presence of an N-terminal signal peptide and absence of transmembrane domains or predicted sites for glycophosphatidylinositol (GPI)-mediated anchoring to the fungal membrane/cell wall. *C. higginsianum* effector candidates (ChECs) were defined as secreted proteins without sequence similarity to known proteins, or resembling putative effectors from other fungi. By analyzing the EST composition of the contigs, a set of 102 ChECs appeared to be preferentially expressed at stages that are relevant to the establishment of biotrophy (appressoria) or maintenance of biotrophy (biotropic hyphae). These candidates were previously identified in the O'Connell laboratory at Max Planck Institute for Plant Breeding Research (Kleemann *et al*., 2010).

From this repertoire, four effectors were selected for further characterization in the present study. ChEC3 and ChEC36 were selected due to their similarity to putative effectors identified from other fungi, namely CgDN3 from *C. gloeosporioides* and SIX6 from *Fusarium oxysporum* f.sp. *lycopersici*, respectively (Stephenson *et al*, 2000; Chakrabarti *et al*., 2011). ChEC6 and ChEC89 were chosen due to their very high

expression level during plant infection (abundant ESTs from appressoria and biotrophic hyphae but not necrotrophic hyphae). To better understand the functions of these proteins in fungal pathogenicity, my aim was to localize them during infection and experimentally test their ability to promote pathogen virulence and suppress plant cell death.

Specific objectives were as follows:

- To profile the expression patterns of genes encoding ChEC3, ChEC6, ChEC36 and ChEC89 during different stages of plant infection and *in vitro*. Are the genes only expressed during *in-planta infection*? Are they only induced at particular stages or expressed constitutively?
- To determine the destination of effector proteins ChEC3, ChEC36 and ChEC89 after secretion into infected *A. thaliana* plants. Do they stay in the plant apoplast or enter into the plant cytoplasm? Are they uniformly distributed at the plant-fungal interface? Are they associated with particular fungal cell types/infection structures?
- To evaluate the capacity of ChEC3, ChEC6, ChEC36 and ChEC89 to enhance the virulence of bacterial pathogens and non-adapted fungal pathogens. This would provide indirect evidence that they suppress or evade plant immunity in some way.
- To evaluate the capacity of ChEC3, ChEC6, ChEC36 and ChEC89 to suppress plant cell death. The ability to keep host cells alive is likely to be important for biotrophic pathogens, and in the biotrophic phase of hemibiotrophs such as *C. higginsianum*.

3. Materials and Methods

3.1 Localization of *C. higginsianum* effector candidates (ChECs)

3.1.1 Plant and fungal material and growth conditions

A. thaliana accessions *Landsberg erecta* (Ler-0) and Columbia (Col-0) which are susceptible ecotypes to *C. higginsianum* were used for plant infection assays. Plants were grown in a peat-based compost. *Arabidopsis* seeds were stratified for two days at 4 °C in darkness to allow for synchronous germination. Germination was induced by transfer of the plants to controlled environment chambers under a regime of a 10-h light period at 150 to 200 mE $m^{-2}s^{-1}$, 65% relative humidity, with 22 °C during the day and 20°C during the night.

C. higginsianum isolate IMI 349063A was used as background strain to obtain DNA templates for cloning ChECs, to generate transformants expressing fluorescent-tagged proteins, and for plant infection as a wild-type control. Fungal cultures were grown and brought to sporulation as described by O'Connell *et al*. (2004). Conidial suspensions were obtained by irrigation of 8- to 12-day-old cultures and the spore concentration was adjusted using a haemocytometer. Fungal transformants were grown on potato dextrose agar (PDA, Difco), supplemented with hygromycin (100 µg/mL), cefotaxime and spectinomycin (both 50 µg/mL) (Sigma-Aldrich).

3.1.2 Cloning of fungal sequences

All primers used in this study were designed with Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Annex 1). All preparative PCRs for fungal sequences cloning were carried out with the High-Fidelity DNA Polymerase Phusion Finnzymes (Biolabs). Final concentrations of dNTP, primer and

enzyme in 50 µL reactions were 0.3 mM, 0.2 µM and 0.02 U/µL, respectively. The thermal cycling conditions were 98 °C for 10 sec and 35 cycles of 98 ° for 10 sec, X °C for 30 sec, 72°C for 15-30 sec/kb and final extension at 72 °C for 3 minutes with X being the predicted primer melting temperature. Preparative PCR reactions were purified with NucleoSpin extract II Kit (Macherery&Nagel). Chemically-competent *Escherichia coli* DH5α cells (Top10, Invitrogen) were used for plasmid propagation containing cloned inserts. Bacterial clones were checked by colony PCR with insert and vector-specific primers before plasmid isolation (NucleoSpin Plasmid Kit, Macherery&Nagel). Insert sequences were verified by in-house sequencing (Max Planck Genome Centre Cologne). For fungal transformation, verified constructs were introduced into *A. tumefaciens* C58C1 strain transformed -competent cells, carrying a genomic rifampicin resistance (50 µg/mL).

3.1.3 Cloning into expression vectors for fluorescent protein tagging

To localize ChECs by fluorescent protein-tagging, ChEC genes including at least 1.5 kb or the entire upstream intergenic region and lacking stop codon, were amplified from cDNA derived from epidermal peels infested with penetrating appressoria or biotrophic hyphae. After TOPO cloning (Invitrogen) and sequence verification, cloned sequences were shuttled *via* Gateway recombination into pFPL-R, and pNLS binary destination vectors providing C-terminal translational fusions to monomeric red fluorescent protein (mRFP). *A. tumefaciens* strain C58C1 was used as recipient strain for fungal transformation. These binary destination vectors were created and kindly provided by Dr. Mark Farman (University of Kentucky, Lexington, KU). The plasmid pNLS was used to express ChECs with a nuclear localization signal and to determine whether ChECs were translocated into plant cells, in which case they should become concentrated in the plant nucleus (Khang *et al*., 2010).

3.1.4 Fungal transformation and screening fungal transformants

Transformation was done as described by Huser and co-workers (2009). To identify the positive transformants, it was necessary to screen at least 24 independent colonies. For this, mycelium of each transformant was obtained by growing it on PDA medium supplemented with hygromycin (50 µg/ml). The transformants were incubated at least eight days at 25 ºC to obtain enough mycelium to inoculate Erlenmeyer flasks containing

Mathur's agar medium (Tu, 1985). Sterile water (1.5 ml) was added to each flask and shaken to disperse conidia over the agar surface. Inoculated flasks were then incubated for 10 days at 25ºC to produce spores.

3.1.5 Plant infection

For ChEC localization, twelve-day-old seedlings (cotyledons and hypocotyls) of susceptible *Arabidopsis* accession Ler-0 were spray-inoculated with spore suspension (1×10^5 conidia/mL) from the positive fungal transformants using an atomizer. Inoculated plants were placed in sealed propagator boxes to maintain 100% humidity and incubated in a controlled environment chamber at 25 ºC (16-h light period) for 24-48 hai. To evaluate if ChECs were translocated into plant cells and mobilized to the nucleus, transgenic Col-0 plants expressing green fluorescent protein (GFP) targeted to the nucleus (Chytilova et al., 1999) were used for inoculations with fungal transformants expressing ChECs carrying nuclear localization signals.

To evaluate the presence of mRFP-tagged effector proteins underneath appressoria, inoculated leaves of the *Arabidopsis* Col glabrous mutant (Herman and Marks, 1989) were used to obtain plant penetrating appressoria. The underside (abaxial surface) of excised leaves was inoculated with spore suspension (5×10^5 conidia ml^{-1}), which was applied over nylon mesh (50 µm pore size) to disperse the spore suspension as a thin liquid film and to ensure uniform coverage of the hydrophobic leaf surface. At 22 h after inoculation (hai) the leaves were brushed with a solution of 50% (w/v) cellulose acetate in acetone and when dry the cellulose acetate film containing the embedded appressoria was removed using fine forceps.

To check effector expression *in vitro*, cellophane membrane pieces were placed on microscope slides and inoculated with spore suspension (5×10^5 conidia ml^{-1}). The suspension was dispersed uniformly over the cellophane using nylon mesh as described. The microscope slides were incubated for 40h in a humid chamber at 25°C.

3.1.6 Microscopy evaluations

For examination by confocal microscopy, infected cotyledons were detached and mounted with the upper (adaxial) side facing upwards on microscope slides in water under a coverslip. Excised hypocotyl segments (3 mm long) were mounted on slides in

the same way. To determine whether mRFP-tagged proteins were located in the plant apoplast or inside the plant cytoplasm, infected hypocotyl samples were plasmolyzed using 0.85M KNO_3 before examination. Light and confocal microscopy was done using a Zeiss LSM 700 confocal laser scanning microscope equipped with a 63X oil immersion objective. For imaging mRFP, excitation was at 555 nm and emission was detected at 557-600 nm. To discriminate mRFP emission from autofluorescence, we used spectral imaging in the lambda mode of the Zeiss LSM 510 microscope. Using the Meta detector and 545 nm excitation line, image stacks with 558–648 nm emission were recorded. To separate mixed fluorescent signals and resolve the spatial distribution of mRFP fluorescence, linear unmixing was employed using the mRFP emission spectrum and several autofluorescence spectra as references.

3.1.7 Electron microscopy

For transmission electron microscopy immunogold labelling, infected plant material was fixed in 4% *p*-formaldehyde and 0.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.9, for 2 h. After progressive low-temperature dehydration in a graded water-ethanol series, samples were embedded in LR White resin (Plano GmbH, Wetzlar, Germany). Immunogold labelling of mRFP was described by Micali and co-workers (2011), except that anti-RFP primary antibodies (Invitrogen) and goat anti-rabbit IgGs conjugated with 10 nm colloidal gold (British Biocell International) were used.

3.2 Profiling ChEC gene expression

3.2.1 Quantitative real-time PCR

RNA samples representing different stages of *C. higginsianum* development *in vitro* and *in planta* were collected as follows. Ungerminated spores were harvested from cultures on Mathur's medium (Tu, 1985), after eight days of incubation at 25°C. Saprophytic mycelium was harvested from potato dextrose broth culture which was in constant agitation for 2 days at 25°C. *In vitro* appressoria were obtained by germinating spores on polystyrene (Kleemann *et al*., 2008). Inoculated leaves of the *Arabidopsis* Col glabrous mutant (see Section 3.1.5) were used to obtain plant-penetrating appressoria and biotrophic hyphae by collecting epidermal strips at 22 and 40 hai, respectively. The correct stage of the samples was verified by microscopic evaluations before collecting the

epidermal strips, which were obtained using fine forceps to remove the infected epidermal cells. Samples representing the switch from biotrophy to necrotrophy were obtained by cutting pieces of leaf tissue showing the first water-soaked lesions at 60 hai. Samples representing the late necrotrophic stage were obtained from heavily infected, macerated leaves at 4 days after inoculation.

Three biological replicates were obtained for each sampled fungal stage. cDNA was obtained from 1 µg total RNA using the iScript cDNA synthesis kit (Bio-Rad) in a volume of 20 µL. Two µL of cDNA (5 ng/µL) were amplified in 1X iQ SYBR Green Supermix (Bio-Rad) with 1.6 µM primers using the iQ5 Real-time PCR detection system (Bio-Rad). Specific primers (Annex 2.) amplified fragments ranging from 106 to 329 bp with efficiencies ranging from 97 and 123%. GeNorm (http://medgen.ugent.be/wjvdesomp/genorm/) was used to assess expression stability of five commonly used reference genes of which α-tubulin and actin were most stable (stability value 0.047 and 0.051, respectively) and used to normalize gene expression values.

3.3 Functional assays

3.3.1 Enhancement of bacterial virulence assay

- **3.3.1.1 Cloning effectors for delivery to plant cells through the bacterial type III secretion system**

To clone versions of ChEC3, ChEC6, ChEC36 and ChEC89 without their signal peptides, codons following the predicted signal peptide cleavage site were fused to an artificial start codon and included the stop codon (Annex 1). Following TOPO cloning (Invitrogen) and sequence verification, cloned sequences were shuttled *via* Gateway recombination into the 'Effector Detector Vector' plasmid pEDV6 (Sohn *et al*., 2007) using *E. coli* competent cells (TOP10, Invitrogen) as recipient. The positive transformants were selected by resistance to gentamycin (25 µg/ml) and detection by colony PCR using insert and vector-specific primers. Insert sequences were verified by in-house sequencing (Max Planck Genome Centre Cologne).

▪ 3.3.1.2 Triparental mating for assaying enhanced bacterial virulence

Triparental mating utilized *Pseudomonas syringae* pv. *tomato* DC3000, *E. coli* HB101 pRK2013 (helper strain) (Sohn *et al*., 2007) and one of the *E. coli* strains obtained above. *E. coli* strains carrying pEDV6 with the *H. parasitica* ATR13 effector or the yellow fluorescent protein (YFP). During bacterial conjugation, *Pst* DC3000 becomes transformed with pEDV. Upon plant inoculation or infiltration, pEDV uses the bacterial TTSS to release proteins inside host cells. This is possible because the fungal effector is fused to the N-terminal part of the *Pst* AvrRPS4 effector, which naturally is cleaved inside plant cells to release the fungal effector protein (Sohn *et al*., 2009). ATR13 effector from *H. parasitica* was shown to increase growth of *Pst* DC3000 (Sohn *et al*., 2007) and was used here as a positive control, while YFP was included as a negative control. The mating was done by mixing small portions of fresh bacterial cultures from *Pst* (Rifampicin 100 μg/ml, Kanamycin 25 μg/ml), Helper strain (Kanamycin 50 μg/ml), *E. coli* carrying pEDV (Gentamycin 25 μg/ml) in the proportions 2:1:1. The mix was resuspended in water, plated on LB 1% agar plates (without antibiotics) and incubated overnight at 28 ºC. Arising bacterial colonies were streaked onto nutrient yeast glycerol agar (NYGA) solidified medium (5 g/L bactopeptone, 3 g/L yeast extract, and 20 ml/L glycerol, with 15 g/L agar, containing antibiotics (Rifampicin 100 μg/ml, Kanamycin 25 μg/ml, Gentamycin 25 μg/ml) to obtain single *Pst* colonies, which were then analyzed by colony PCR to verify that the effector gene was present.

▪ 3.3.1.3 Selection of positive transformants by colony PCR

A small quantity of bacteria from the selection plates was taken using a pipet tip and transferred to a PCR tube by touching the bottom. PCR reactions were carried out using Taq DNA polymerase. Final concentrations of dNTP, primer and enzyme in 20 µL reactions were 0.5 mM, 0.2 µM and 0.4 U/µL, respectively. The thermal cycling conditions were 95 °C for 2 min with 35 cycles of 95°C for 10 sec, X °C for 30 sec, 72°C for 1 min/kb and final extension at 72 °C for 3 min with X being the predicted primer melting temperature. Positive transformants were selected using a plasmid-specific primer combined with an effector-specific primer.

▪ 3.3.1.4 Inoculation of plants with bacteria

Four week-old Col-0 plants, which are susceptible to *Pst* DC3000, were spray-inoculated with bacterial suspension adjusted to OD=0.2 (measured at 600 nm) in 10 mM $MgCl_2$ with 0.04% (v/v) Silwet detergent. In addition, four week-old plants of Col-0 were spray-inoculated with *P. syringae* carrying YFP as a control. For each treatment, at least 18 plants were inoculated (two pots, each containing nine plants).

▪ 3.3.1.5 Sampling for bacterial colony counting

Leaf samples were collected at two time points: day zero (i.e. the same day, 3 hours after inoculation) and day 3 after inoculation. Samples for day zero comprised three replicates per treatment. Each replicate had nine leaf discs (6mm diameter) which were disinfected with 70% ethanol for 10 seconds and then collected in 1.5 ml 10 mM $MgCl_2$ containing 0.01% Silwet, and agitated at 650 rpm for 1h at 28ºC. After agitation, 20 μl aliquots were plated onto NYGA medium supplemented with antibiotics (rifampicin 100 μg/ml kanamycin 25 μg/ml, gentamycin 25 μg/ml), and incubated over night at 28ºC before counting bacterial colonies. After taking the first sample, plants were incubated in a climatic chamber at 20ºC for three days. Samples from day three comprised four replicates per treatment. Each replicate had nine leaf discs (6mm diameter), which were collected in 0.5 ml 10mM $MgCl_2$ containing 0.01% Silwet, and agitated as described above. After agitation, serial dilutions 10^{-1}- 10^{-5} were prepared, plated onto NYGA medium supplemented with antibiotics, prior to colony counting (cfu/cm^2).

3.3.2 ETI suppression assay

▪ 3.3.2.1 Triparental mating for assaying ETI suppression

Pseudomonas fluorescens is a soil bacterium which has no effectors of it own and as a consequence it is not able to cause HR in *Arabidopsis* accession Ws-0. For this assay, a strain of *P. fluorescens* engineered to contain the TTSS was used (Thomas *et al*., 2009). Triparental mating utilized *P. fluorescens* (chloramphenicol 50 μg/ml and tetracycline 20 μg/ml), *E. coli* HB101 pRK2013 (helper strain) (kanamycin 50 μg/ml) and *E. coli* containing plasmid pEDV6 carrying inserts with individual ChECs (gentamycin 25 μg/ml), or yellow fluorescent protein (YFP) (chloramphenicol 50 μg/ml, tetracycline 20 μg/ml,

gentamycin 25 μg/ml). During mating, *P. fluorescens* is transformed by the pEDV system, as described in Section 3.3.1.1. After mating, single bacterial colonies were obtained on selective medium supplemented with chloramphenicol 50 μg/ml, tetracycline 20 μg/ml and gentamycin 25 μg/ml. *P. fluorescens* harbouring *AvrRps4* was used as a positive control. This *Pst* avirulence protein is recognized by resistance gene *Rps4* in *Arabidopsis* accession Ws-0, provoking hypersensitive cell death (Narusaka *et al*., 2009).

- **3.3.2.2 Inoculation of plants with bacteria**

Four week-old plants of *Arabidopsis* ecotype Ws-0 were infiltrated with a 1:1 mixture of *P. fluorescens* harbouring individual ChECs (final OD_{600}=0.3) and *P. fluorescens* carrying AvrRps4 (final OD=0.3). The mixture of *P. fluorescens* carrying AvrRps4 (final OD_{600}=0.3) and *P.fluorescens* secreting YFP (final OD_{600}=0.3) was used as a control to induce cell death. *P. fluorescens* secreting only YFP at OD_{600}=0.6 was used to verify not cell death induction. For each treatment at least 18 leaves were pressure-infiltrated until the entire leaf area was covered. Infiltration was done manually using a 1 ml needle-less syringe.

- **3.3.2.3 Sampling for ETI suppression assay**

After infiltration, 18 leaf discs were taken from each treatment, and washed in sterile water with constant agitation for 1h at room temperature. Leaf discs from the same treatment were distributed in a multi-well plate, three-leaf discs per well. Immediately afterwards, ion leakage was measured using a conductivity meter (Horiba Twin). The background conductivity of the water used in these experiments was 2-3 μS/cm. The measurements were obtained by adding to the conductivity meter 30 μl from the water in which the leaf discs were submerged. The conductivity meter was cleaned between treatments, using distillated water, until obtaining background conductivity. Conductivity measurements were taken during three days. During this time the multi-well plates containing the samples were kept at room temperature and continuous light. Increased conductivity indicates electrolyte leakage, which is a marker for plant cell death (Rizhsky *et al*., 2004).

3.4 Cloning fungal coding sequences for transient expression assays

To evaluate the capacity of ChECs to suppress plant cell death, effector proteins were cloned without signal peptide, adding an artificial start codon and omitting a stop codon, into the pENTR vector (Invitrogen). A *C. higginsianum* homolog of ethylene-inducing Peptide1-Like Protein (ChNLP1) and YFP were similarly cloned. Following sequence verification, cloned sequences were shuttled *via* Gateway recombination into the binary plant expression vector pB7WG2, providing expression from the CaMV 35S promotor (VIB Gent, Gent university). The *A. tumefaciens* strain C58C1 pGV2260 was used as recipient strain (selection on rifampicin, carbenicillin, spectinomycin and streptomycin, all at 50 μl/mg) for transient expression in *N. benthamiana* leaves.

3.5 Transient expression in *N. benthamiana*

Bacterial cells were collected in the stationary phase to maximize the transformation efficiency (Marion *et al*., 2008). pelleted and resuspended in infiltration buffer (10 mM $MgCl_2$, 5 mM MES (pH 5.6) supplemented with 200 μM acetosyringone) before infiltration into the abaxial side of *N. benthamiana* leaves using a needle-less syringe. *N. benthamiana* was grown under long day conditions in a greenhouse with an ambient temperature of 22-25°C and high light intensity. Agroinfiltration experiments were conducted with 4-6-week-old plants. To evaluate possible suppression of cell death caused by ChNLP1, infiltration mixtures containing bacterial strains harbouring constructs for co-expression of cell death inducer (ChNLP1) together with ChECs or YFP as control were prepared. In these bacterial mixtures, ChECs were used at OD_{600}=1, ChNLP1 was used at OD_{600}=0.1 and p19 at OD_{600}=0.5. p19 is a protein of *Tomato bushy stunt virus* that suppresses gene silencing and was used to prevent the onset of post-transcriptional gene silencing in the infiltrated tissues, allowing high levels of transient expression (Voinnet *et al*., 2003). Infiltration mixtures were kept at room temperature for 2 h before infiltration into full-expanded leaves of *N. benthamiana* plants (4 weeks old). To allow pair-wise comparisons, infiltration mixtures containing ChEC/cell death inducer constructs and YFP/cell death inducer constructs were infiltrated side-by-side into the same leaf. Plants were incubated in a controlled environment chamber (19°C/21°C day/night temperature cycles and 16-h-light/8-h-dark cycles) to which they were adapted at least 24 h before infiltration. Six to eight days after infiltration, infiltration site pairs were inspected in a

blinded manner to determine whether the site co-expressing ChECs with cell death inducer showed reduced necrosis compared to corresponding control site on the same leaf expressing YFP with cell death inducer.

4. Results

4.1 Sequence analysis of the selected effector candidates and their homology to other proteins

ChEC36 encodes a protein of 224 amino acids (predicted size 24.8 kDa) that is cysteine-rich (9 cysteines) and includes an N-terminal signal peptide for secretion. This protein does not have homology to any protein from other *Colletotrichum* species but resembles SECRETED IN XYLEM6 (SIX6), an effector of unknown function that is secreted into the tomato xylem by *Fusarium oxysporum* f. sp. *lycopersici* (Chakrabarti *et al*., 2011). ChEC36 and SIX6 share 40% amino acid identity, and 8 cysteines are conserved between the two proteins (Figure 1).

After sequencing ChEC36 PCR products amplified from cDNA generated from infected leaves 20 hours post inoculation (hai) with *C. higginsianum*, three splice variants were found: ChEC36, ChEC36-1 and ChEC36-2. Fungal introns have been described as being short introns, and the majority (98%) correspond to the canonical splice site 5´GT----AG 3´ in cDNA (Kupfer *et al*, 2004). We found that ChEC36-1 has a canonical splice site but not ChEC36-2 (5'GA----TG3', Figure 2). The original ChEC36 does not have any introns. The corresponding protein sequences are identical except at the C terminus, where the splice variants lacked an amino acid motif that was later shown to be required for suppression of ChNLP1-induced cell death (Figure 3).

ChEC3 encodes a protein of 70 amino acids (predicted size 7.7 kDa), with 3 cysteines. This protein has a paralog in *C. higginsianum*, called ChEC3a (51% amino acid identity), and an ortholog in *Colletotrichum gloeosporioides*, called CgDN3 (39% identity). The three versions have 24 amino acids in common (Figure 4). In *C. gloeosporioides* this gene is required for virulence: *CgDN3* mutants were unable to infect and reproduce on intact host leaves, because they elicited a localized host cell death resembling the hypersensitive-like response (Stephenson *et al*, 2000). This result suggests that CgDN3

may function to suppress plant cell death or defense responses. Thus, ChEC3 represents a good candidate for a cell death suppressor and was selected for further characterization.

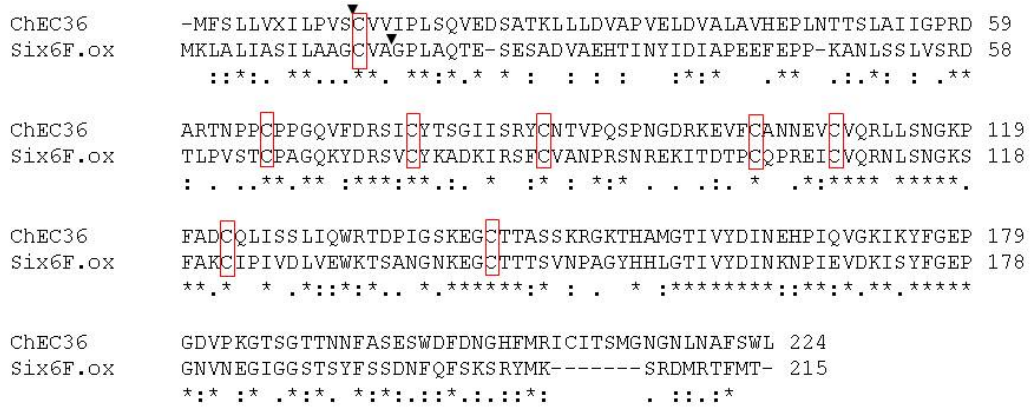

Figure 1. Amino acid alignment of ChEC36 and the homologous SIX6 effector of *Fusarium oxysporum* f.sp. *lycopersici.* The two effector proteins share 40% identical amino acids. The predicted signal peptide cleavage site is marked with a triangle. Identical amino acids are labelled (*). (:) indicates conservation between groups of amino acids with strongly similar chemical properties (.) indicates conservation between groups of amino acids with weakly similar chemical properties. Conserved cysteine residues are highlighted in red.

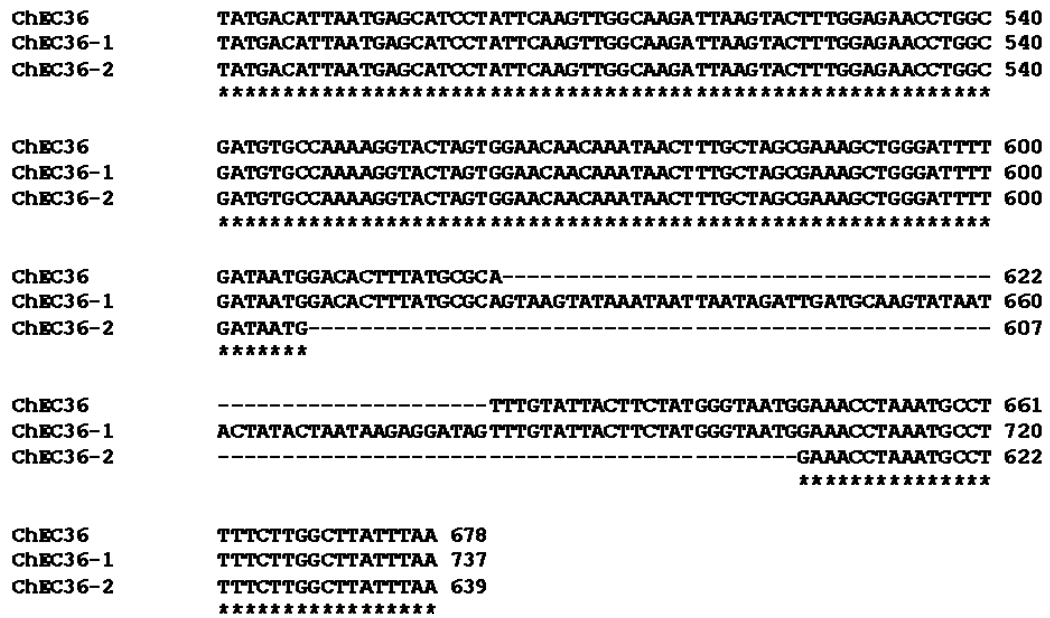

Figure 2. Nucelotide sequences of ChEC36 splice variants. Three different splice variants of ChEC36 were found, called ChEC36, ChEC36-1 and ChEC36-2. ChEC36 has no splice site, ChEC36-1 has a canonical splice site, and ChEC36-2 has a non-canonical splice site. Identical nucleotides are labelled (*).

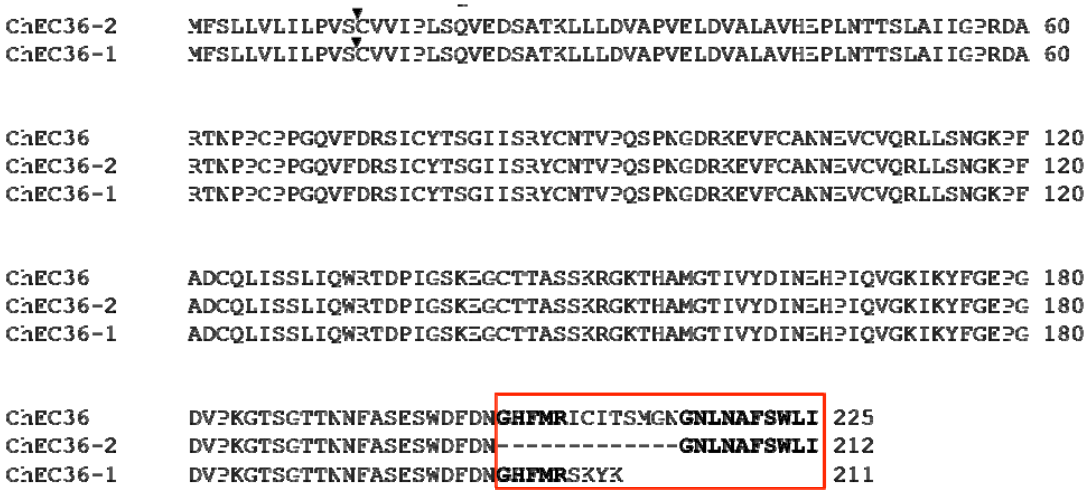

Figure 3. Alignment of protein sequences encoded by ChEC36 splice variants. The three different predicted proteins differ at their C-termini (highlighted in red). Amino acids conserved between variants appear in bold. The predicted signal peptide cleavage site is marked with a triangle.

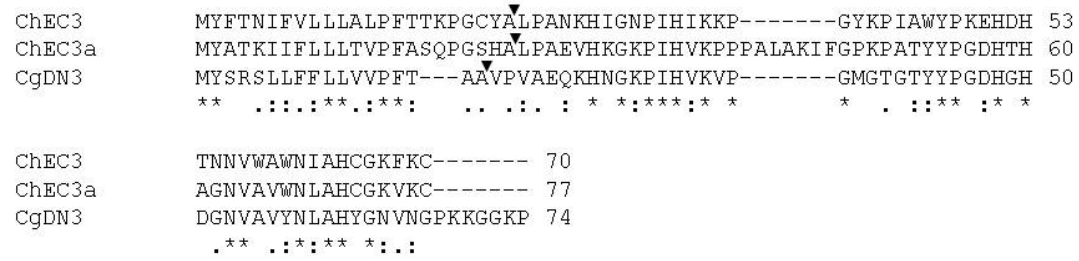

Figure 4. ChEC3 paralogs and orthologs. Alignment of ChEC3, ChEC3a and *C. gloeosporioides* CgDN3 protein sequences. Identical amino acids are labelled (*). The predicted signal peptide cleavage site is marked with a triangle. (:) indicates conservation between groups of amino acids with strongly similar chemical properties (.) indicates conservation between groups of amino acids with weakly similar properties.

ChEC89 encodes a protein of 83 amino acids (predicted size 9.2 kDa), with 4 cysteines. This protein showed 38% amino acid identity to a hypothetical protein of *Magnaporthe oryzae* (NCBI accession number XB001409354.1), which includes 3 conserved cysteine residues. Three further paralogs were also found in the *C. higginsianum* genome with one cysteine conserved in all of them (Figure 5).

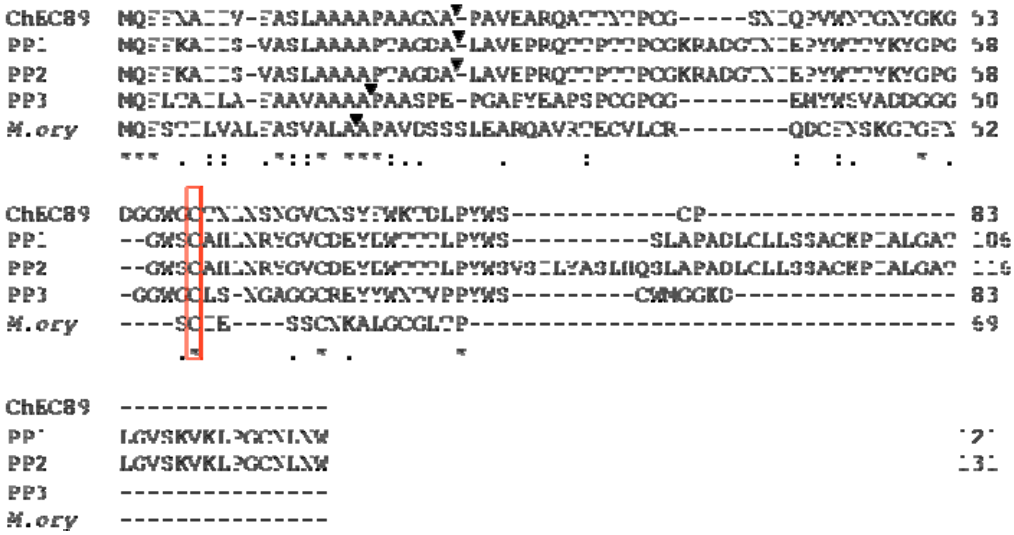

Figure 5. Alignment of ChEC89 homologs and paralogs. ChEC89 shares a conserved cysteine with three paralogous proteins in *C. higginsianum* (PP1, PP2, PP3) and a homolog in *Magnaporthe oryzae*. Identical amino acids are labelled (*). The predicted signal peptide cleavage site is marked with a triangle. (:) indicates conservation between groups of amino acids with strongly similar chemical properties (.) indicates conservation between groups of amino acids with weakly similar properties.

ChEC6 encodes a protein of 89 amino acids (predicted size 9.7 kDa) and does not have homology to any known proteins in the NCBI non-redundant protein database but it has a homolog in *Glomerella graminicol*a, the sexual stage of *C. graminicola* (NCBI accession number EFQ24895.1), with which it shares 51% amino acid identity (Figure 6).

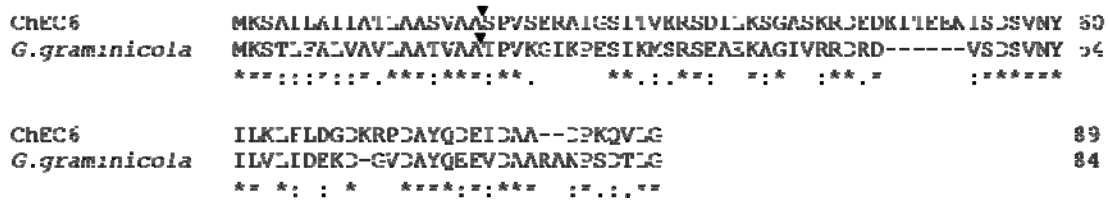

Figure 6. Alignment of ChEC6 and a homologous protein from *G. graminicola*. Identical amino acids are labelled (*). The predicted signal peptide cleavage site is marked with a triangle. (:) indicates conservation between groups of amino acids with strongly similar chemical properties. (.) indicates conservation between groups of amino acids with weakly similar properties.

4.2 Expression profiles of genes encoding effector candidates

Although the four selected ChECs were identified from infection stage-specific EST libraries, expression profiling by quantitative real-time PCR (qPCR) was necessary to determine more precisely their expression *in vitro* and *in planta* and to confirm the EST predictions. In this way, it could be determined whether ChECs are constitutively expressed in all stages and cell types or only at specific infection stages, e.g. biotrophy-relevant stages (penetrating appressoria, biotrophic hyphae) or during the necrotrophic stage. A further aim was to establish whether ChECs are exclusively expressed *in planta* or if they are expressed also under *in vitro* conditions. The following RNA samples were collected for analysis: ungerminated spores (SP), saprophytic mycelium (MY), appressoria formed *in vitro* (VA), unpenetrated appressoria formed *in planta* (PA), penetrating appressoria with young biotrophic hyphae (BH), the switch from biotrophy to necrotrophy (SW) and the late necrotrophic stage (LN). Internal control genes (*C. higginsianum* α -tubulin and actin) were used to normalize the data and to compensate for variation in fungal biomass between the different stages.

The qPCR analysis confirmed the unique expression of the four ChECs in unpenetrated appressoria formed *in planta* and during the biotrophic stage. Transcripts were not detectable during necrotrophic stages or under any *in vitro* conditions, confirming that expression of the four ChECs only occurs *in planta*. However, different levels of ChEC gene expression were found, relative to the control genes. The ChEC36 expression profile showed that this gene was most highly expressed in unpenetrated appressoria *in planta*. ChEC89 was most highly expressed in the biotrophic hyphae stage, while ChEC3 was highly expressed in both penetrating appressoria and biotrophic hyphae. ChEC6 showed a similar expression profile to ChEC3 and had the highest level of expression of all the effector genes, relative to the two controls, indicating very strong gene induction *in planta* (Figure 7).

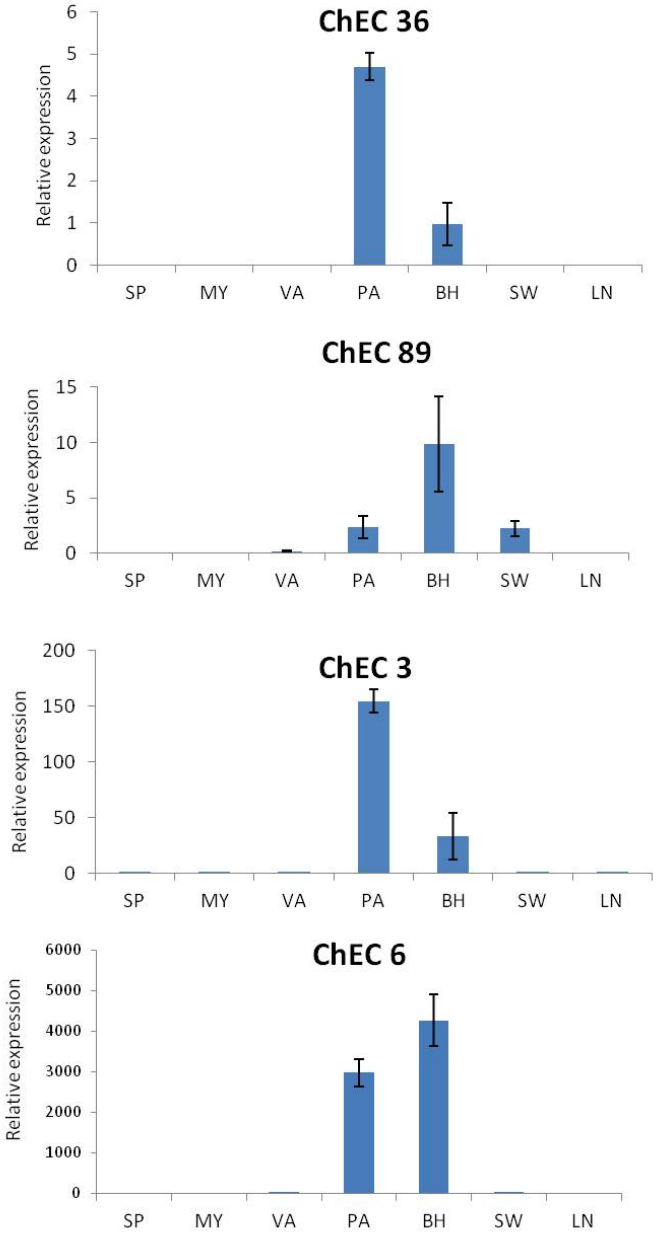

Figure 7. Expression profiling of ChEC genes during fungal development in planta and in vitro. qPCR expression profiles of effector candidates ChEC3, ChEC6, ChEC36 and ChEC89. Data show ChEC gene expression relative to the control genes α-tubulin and actin, which were used to normalize the data and compensate for the variation in fungal biomass between the different stages. The samples correspond to: SP: ungerminated spores, MY: saprophytic mycelium, VA: unpenetrated appressoria formed *in vitro*, PA: unpenetrated appressoria formed *in planta*, BH: penetrating appressoria with biotrophic hyphae, SW: switch from biotrophy to necrotrophy, LN: Late necrotrophic stage.

4.3 Localization of effector candidates *in planta*

In order to characterize ChECs, their localization during plant infection was one of the major aims in my study. To localize ChECs, fungal transformants were generated that expressed effector proteins as C-terminal fusions with the monomeric Red Fluorescent Protein (mRFP) under the native promoter (~1.5 kb upstream sequence). Fusion proteins were localized during infection of *Arabidopsis* seedlings (cotyledons or hypocotyls) by confocal laser scanning microscopy. At least three independent transformants were analysed per gene.

4.3.1 Localization of effector candidate ChEC36

Confocal microscopy of transformants expressing ChEC36:RFP revealed that only unpenetrated appressoria on the plant surface showed fluorescent labelling. The fluorescent signal was localized at the base of the appressoria, in the penetration pore (Figure 8 A-F). This suggests ChEC36 accumulates in the pore before host penetration. A side-view projection computed from the recorded image stack of a penetrating appressorium showed that the fluorescence signal was also present in the penetration peg emerging from the base of penetrating appressorium (Figure 8, G). In addition to the penetration pore and peg, a weak fluorescence signal was also detected as a spotty labeling pattern in the appressorial cytoplasm (Figure 9 A-C). These structures may represent fungal vacuoles (see below). Mature, expanded biotrophic hyphae developing from appressoria did not show mRFP fluorescence (Figure 10 A, B).

Gene expression profiling by qPCR (Section 3.2) indicated that ChEC36 is specifically induced *in planta* and not in appressoria formed *in vitro*. To test whether expression may be linked to morphogenesis of the appressorial penetration peg, transformants expressing ChEC36:RFP were germinated on cellophane membranes, where they form appressoria which penetrate and form pseudo-biotrophic hyphae inside the membrane. Neither appressoria penetrating cellophane nor pseudo-biotrophic hyphae formed inside the membrane showed any detectable mRFP labeling (Figure 10 C, D), indicating that ChEC36 protein expression is specifically induced during plant penetration only.

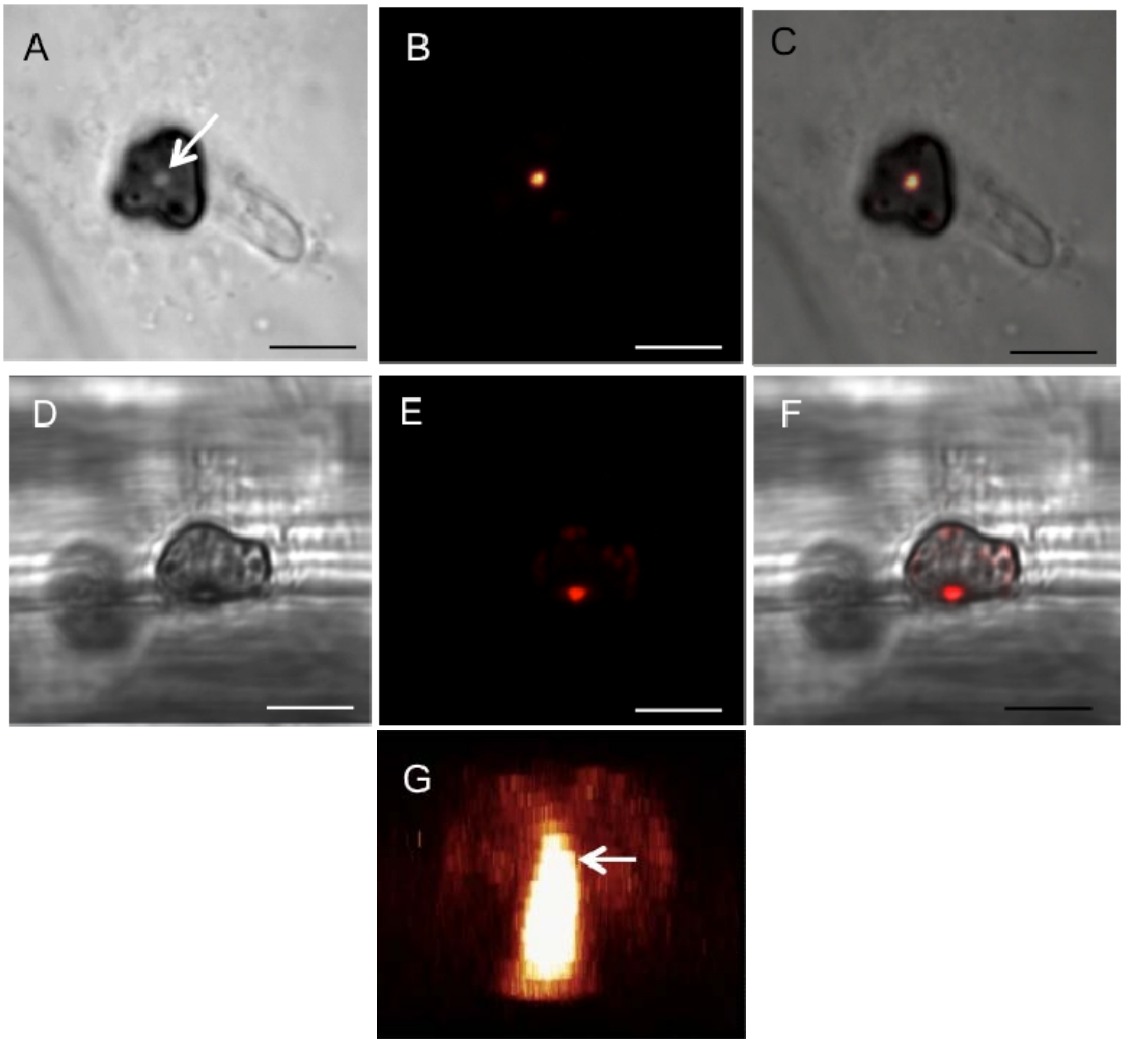

Figure 8. ChEC36 co-localizes with the appressorial penetration pore. Confocal microscopy images of appressoria of *C. higginsianum* expressing RFP-tagged ChEC36. A. Bright field image, showing the penetration pore (arrow) in the base of the darkly melanized, unpenetrated appressorium, viewed from above B. Red fluorescence channel (RFP). C. Overlay of bright field and fluorescence channel. D, E, F side-view of an unpenetrated appressorium showing the fluorescence signal in the basal penetration pore. G. Side-view 3D projection of a penetrating appressorium, showing fluorescence signal localized in the infection peg (arrow) emerging from base of the appressorium. Bars = 5 µm. Images were recorded at 24 hpi.

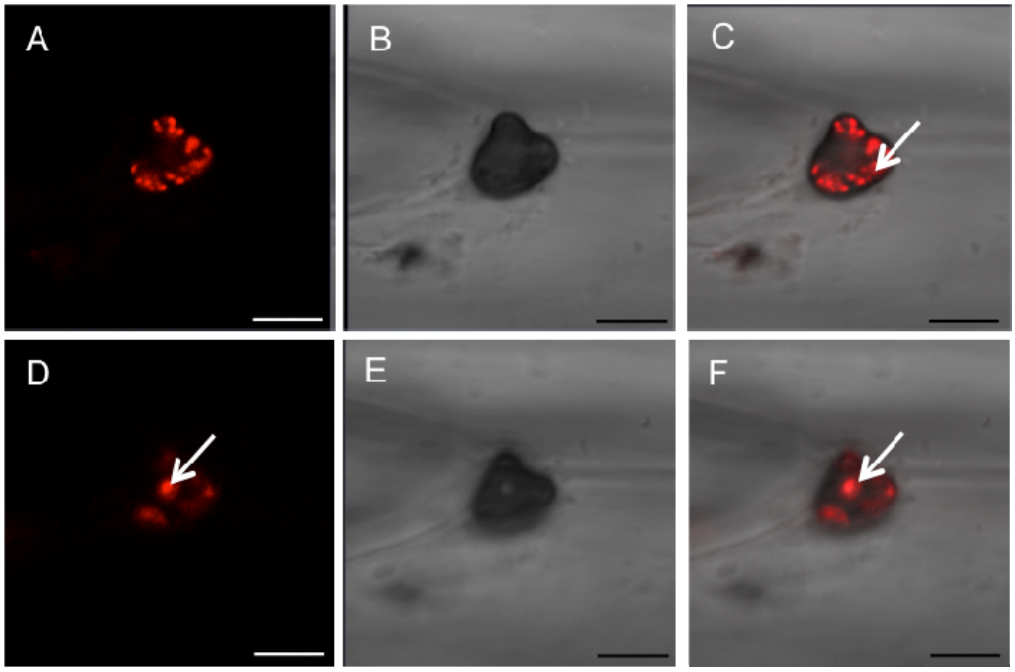

Figure 9. ChEC36 localizes to the appressorial penetration pore and fungal vacuoles. Confocal microscopy images of *C. higginsianum* appressoria expressing RFP-tagged ChEC36. (A-F) An appressorium viewed in two different focal planes. A) First focal plane showing the presence of RFP fluorescence in vacuoles (arrow) in the appressorial cytoplasm. B) Bright field signal. C) Overlay of bright field and fluorescence channels. D-F) second focal plane at the base of the same appressorium showing D) brightly fluorescing spot in the center (arrow), corresponding to the penetration pore, E) Bright field image and F) Overlay of bright field and fluorescence channels. Bars = 5 µm. Images were recorded at 24 hpi.

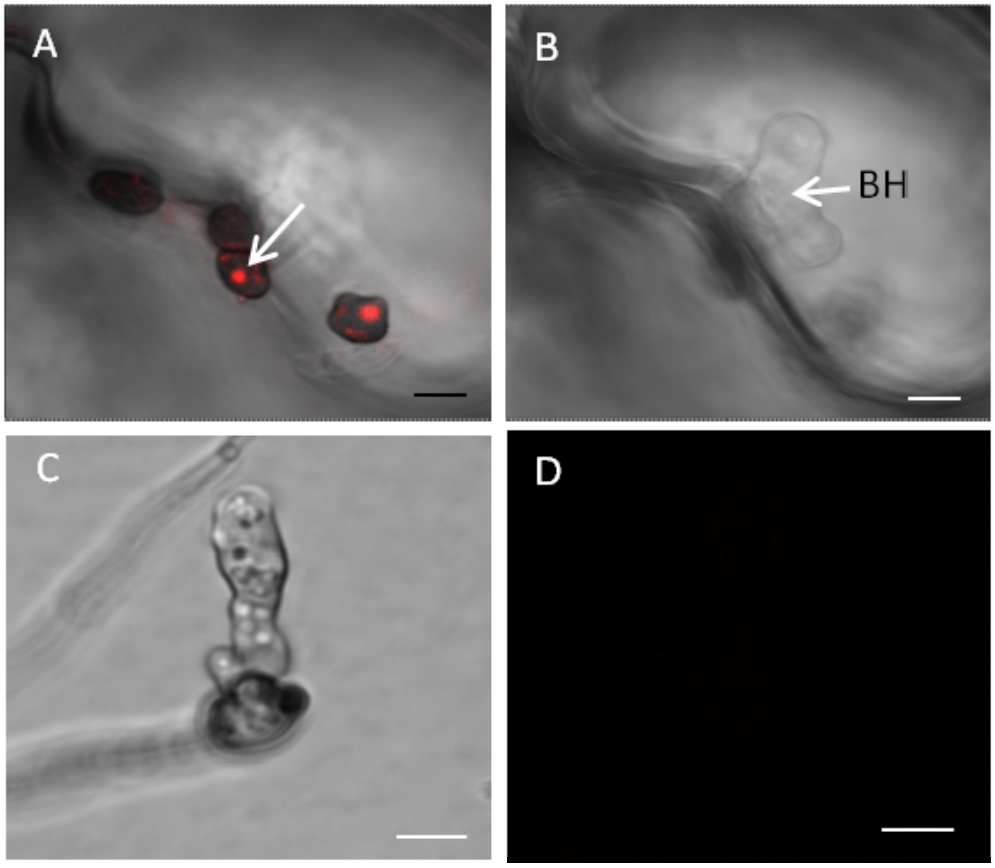

Figure 10 . ChEC36 is not expressed in biotrophic hyphae and during penetration of cellophane. A, B: Individual optical sections from two different focal planes of appressoria expressing ChEC36:RFP, of which one has penetrated the host cell to form a biotrophic hypha (arrow in B). A) Overlay of bright field and fluorescence channels. Note the brightly fluorescing spots, corresponding to the penetration pore (arrow) of unpenetrated appressoria. B) Same region as in A) in a different focal plane showing a biotrophic hypha (BH) that is not expressing ChEC36:RFP. C, D: Appressorium penetrating cellophane membrane to form pseudo-biotrophic hyphae inside the membrane. C, Bright field image, D Fluorescence channel. ChEC36:RFP was not detected in appressoria or pseudo-biotrophic hyphae formed *in vitro* on cellophane membranes. Bars = 5 µm. Images were recorded at 42 hai.

To determine whether ChEC36 was secreted out of the appressorium into the plant apoplast, the leaf area immediately underneath appressoria was examined for the presence of RFP fluorescence signals. For this, appressoria were removed from the surface of inoculated leaves as follows. Leaves were brushed with a solution of cellulose acetate in acetone, and after drying, the cellulose acetate was stripped off using fine

forceps. Both, cellulose acetate strips containing the embedded appressoria as well as the stripped leaf surface were evaluated using confocal microscopy to detect the RFP fluorescence signal. When the removed appressoria were evaluated, it was possible to find RFP fluorescent labelling inside appressoria, forming a ring around the basal penetration pore (Figure 11 A-C). On the corresponding stripped leaf surface, the RFP fluorescence was detected in the plant cell wall as a central bright spot, representing the penetration peg, surrounded by a small halo of diffuse fluorescence, providing evidence for the secretion of ChEC36-RFP into the plant apoplast (Figure 11 D-G). The signal coming from the halo was verified through spectral scanning, confirming that the fluorescence signal had the characteristic spectrum of mRFP and that it was not autofluorescence.

It is known that some pathogen effector proteins can be translocated across the plant plasma membrane and enter into the host cell cytoplasm (Catanzariti *et al*., 2006, Dodds *et al*., 2004). However, the ChEC36:RFP fluorescence signal was not detected in the plant cytoplasm. This could be because the concentration of ChEC36:RFP was below the detection limit of confocal microscopy. As a more sensitive approach to demonstrate translocation into the plant cytoplasm, ChEC36 was expressed as a translational fusion to mCherry coupled with an artificial nuclear localization signal (NLS). Assuming translocation into the host cell, the NLS should result in the fluorescent fusion protein being concentrated in a small compartment (i.e. the plant nucleus), allowing more sensitive detection of small amounts of translocated effector protein (Khang *et al*., 2010). Figure 11 (H-J) shows transformant appressoria expressing ChEC36:NLS:mCherry inoculated onto transgenic *Arabidopsis* plants expressing NLS:GFP as a nuclear marker (Chytilova *et al*., 1999). However, it was not possible to detect any RFP signal in the nucleus of infected plant cells, suggesting that the fusion protein was not translocated across the plant plasma membrane.

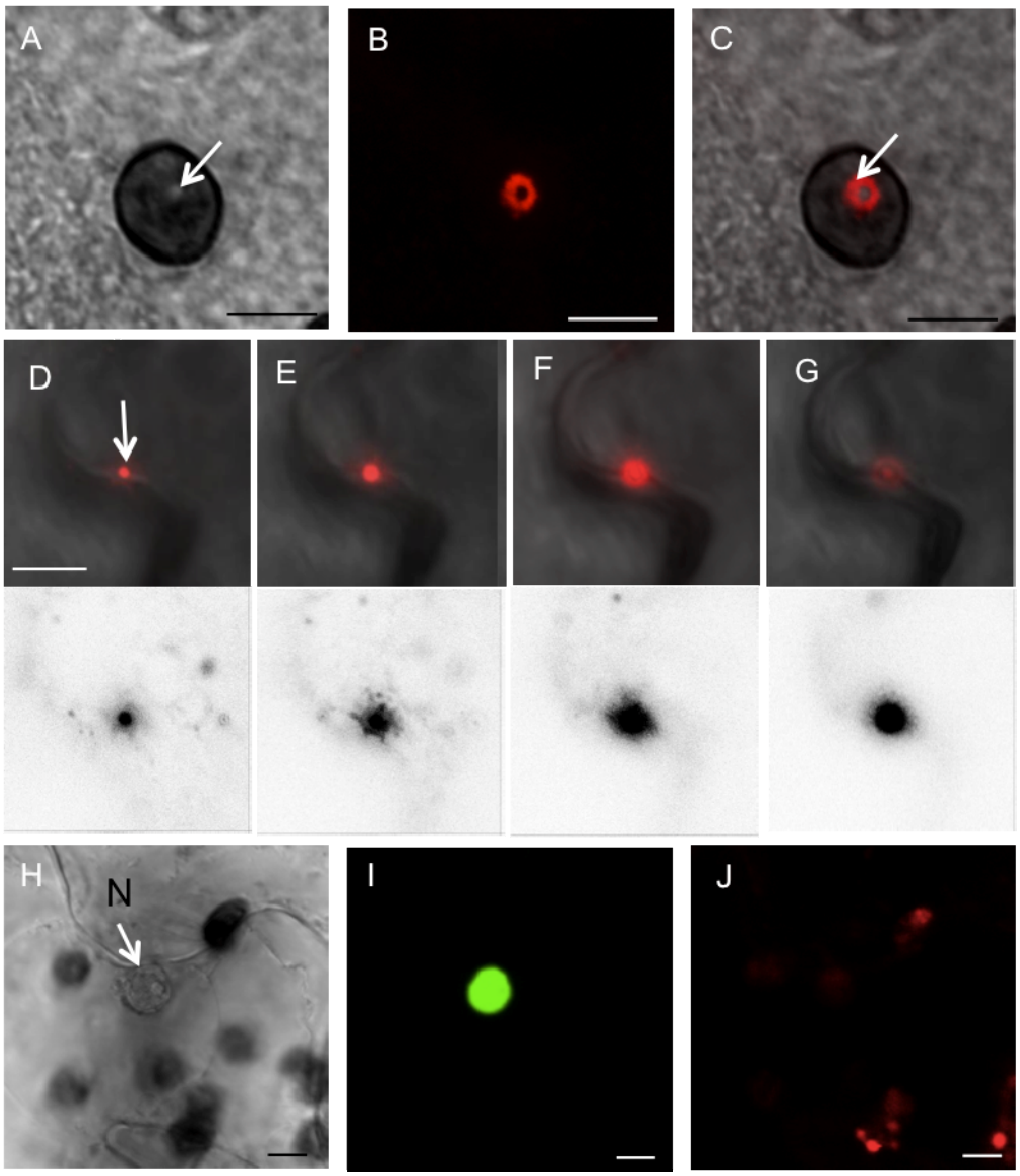

Figure 11. ChEC36 is secreted into the plant cell wall but not into the plant cytoplasm. A,B,C: Base of an appressorium removed from the leaf surface by cellulose acetate-stripping. A. Bright field image showing the appressorial penetration pore (white arrow). B. Fluorescence image of the same appressorium. C. Overlay of bright field and fluorescence channels. White arrow indicates RFP-labelled ring around the penetration pore. D,-G Top: Leaf surface after removing appressoria by cellulose acetate-stripping. Series of confocal microscope optical sections representing different focal planes and shown as overlays of bright field and red fluorescence channels (top panels), and RFP fluorescence converted to black-and white to improve image contrast (lower panels). The ChEC36-RFP signal diffuses a short distance laterally and downwards from the penetration site (arrow). H-J: Confocal micrographs showing a fungal transformant expressing ChEC36:RFP:NLS inoculated onto transgenic *Arabidopsis* plants expressing GFP targeted to the plant nucleus. Images were recorded 30 hai.

Confocal microscopy suggested that effector protein ChEC36 is focally secreted to appressorial pores. Transmission electron microscopy (TEM) combined with immunogold labeling with antibodies specific to RFP was used to verify this localization pattern at higher resolution. Rabbit polyclonal anti-mRFP antibody was applied to ultrathin resin sections at a dilution of 1 in 500. Goat anti-rabbit IgG antibodies conjugated with 10 nm colloidal gold particles were used as secondary antibodies. TEM-immunogold labeling confirmed that ChEC36:RFP is localized in appressorial pores (Figure 12 C), but not in mature biotrophic hyphae (Figure 12 B). The ChEC36:RFP fusion protein was localized outside the appressorial plasma membrane (Figure 12 D, E), indicating that ChEC36:RFP is secreted. In addition, the pore ring which forms a cell wall layer continuous with the penetration peg cell wall, was intensely labelled in some appressoria (Figure 12 E). The punctate labelling in the appressorial cytoplasm visible with confocal microscopy appeared to correspond to protein accumulation inside vacuoles (Figure 12 A). There was no detectable labelling in cells of the wild-type fungus (Figure 12 F), indicating that the antibody used was specific for RFP.

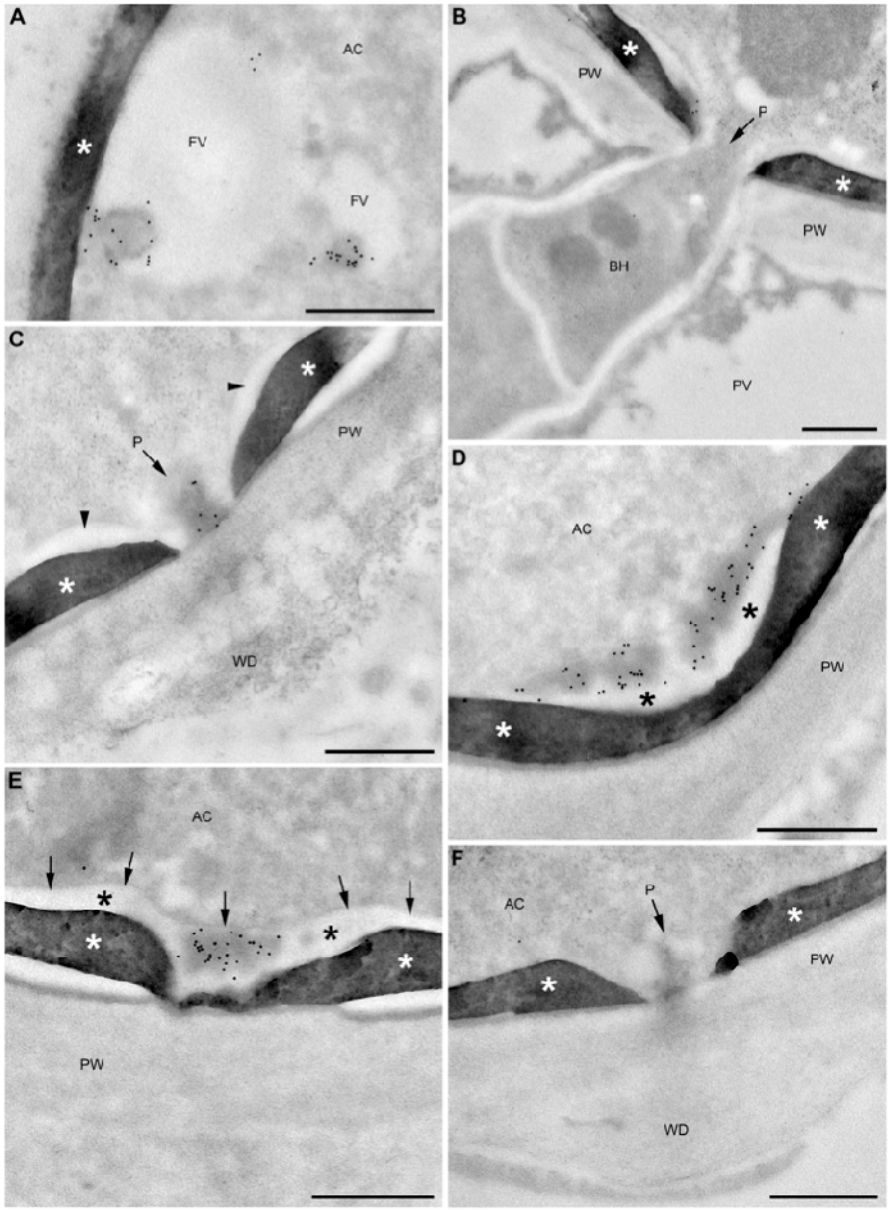

Figure 12 . TEM-Immunogold localization of ChEC36:mRFP using antibodies recognizing RFP. A, Labelled protein inclusion bodies inside fungal vacuoles (FV). B, Unlabelled mature biotrophic hyphae (BH). C, Labelled appressorial pore (P) surrounded by an unlabelled pore ring (arrowheads). D, Tangential section through a penetration pore ring (black asterisks) labelled on the inner surface of the pore ring. E, Pore labelling is external to the appressorial plasma membrane (arrows). F, Wild-type appressorium showing absence of any labelling. White asterisks: melanized appressorial cell wall, AC, appressorial cytoplasm, PV, plant vacuole, P, penetration pore. PW, plant cell wall. WD, plant cell wall deposits. Scale bars, 500 nm.

4.3.2 Localization of effector candidate ChEC89

Confocal microscopy of transformants expressing ChEC89:RFP revealed that the fusion protein was localized to the surface of the biotrophic hyphae but was not detectable in appressoria (Figure 13 A-F). Similar results were obtained using transformants expressing ChEC89 as a C-terminal fusion with the green fluorescent protein (GFP) (Figure 13 G-I). On the surface of many biotrophic hyphae (~50%) small, brightly fluorescent foci were observed (Figure 13 A-F), suggesting that effector proteins accumulate after secretion into small regions at the plant fungal interface, hereafter called interfacial bodies. At later infection stages, at the switch from biotrophy to necrotrophy, it was possible to visualize the protein on the surface of the biotrophic primary hyphae, but not on the surface of the necrotrophic secondary hyphae, confirming the expression profile of this gene (Figure 14 A-F). The expression of ChEC89:RFP was plant-induced and specific to pathogenesis because there was no detectable RFP fluorescence on pseudo-biotrophic hyphae formed after appressorial penetration into cellophane membranes *in vitro* (Figure 14 G, H).

After plasmolysis of the infected tissue, it was possible to detect the RFP fluorescence signal in the expanded apoplast of the infected plant cell, between the plant plasma membrane and fungal cell wall, confirming that this effector is solubly secreted (Figure 15). However, a portion of ChEC89:RFP also appeared cell wall-associated because the signal was visible in septa (Figure 14 E, F) and was retained on the surface of hyphae in dead host cells, when the plant plasma membrane was probably destroyed (Figure 14 D-F).

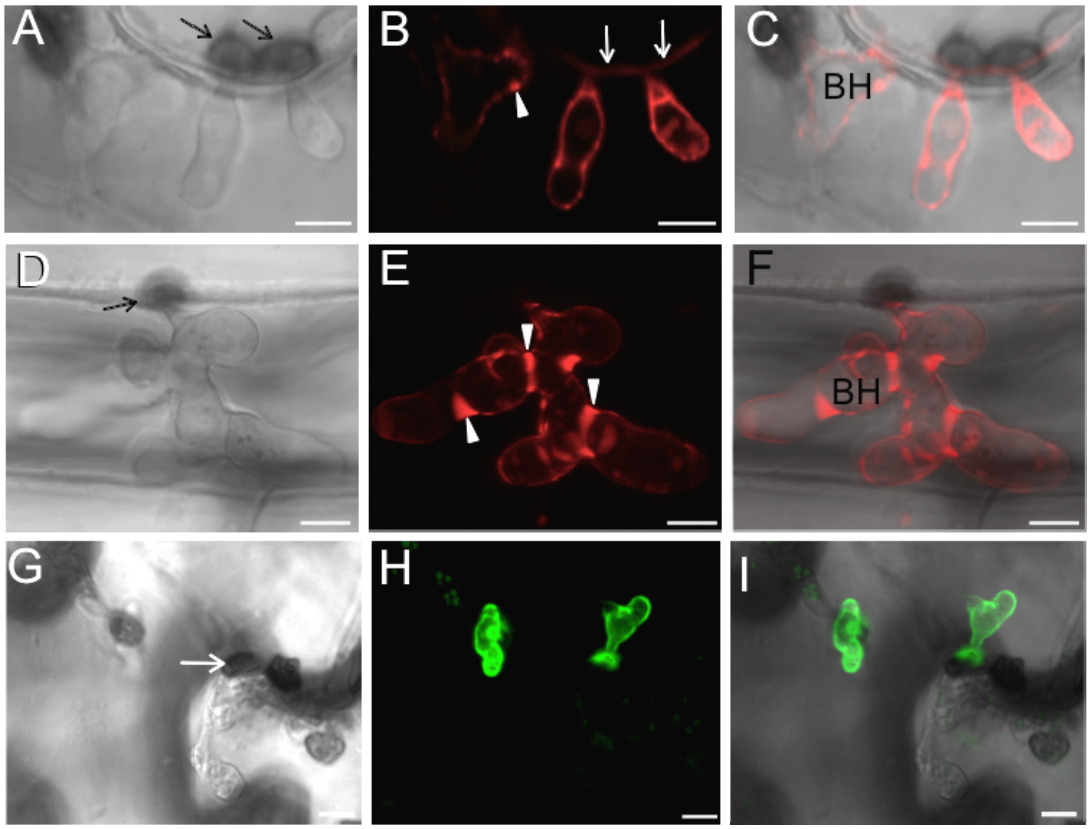

Figure 13. ChEC89 localization. Confocal microscopy of *C. higginsianum* expressing RFP-tagged ChEC89. A, D, G Bright field image. B, E, H fluorescence image. C, F, I. Overlay of bright field and fluorescence channels. A. Appressoria (arrows) and biotropic hyphae. B,C biotrophic hyphae showing fluorescent foci (arrowhead) on the hyphal surface and labelling of the plant cell wall (arrows). D Mature biotrophic hyphae, (E and F), showing fluorescence accumulation in hyphal concavities (arrowheads). Arrow: appressorium. G. Bright field image showing appressoria (white arrow) H) Fluorescence image showing GFP-tagged ChEC89 on the surface of young biotrophic hyphae. Images were recorded 42 hai.

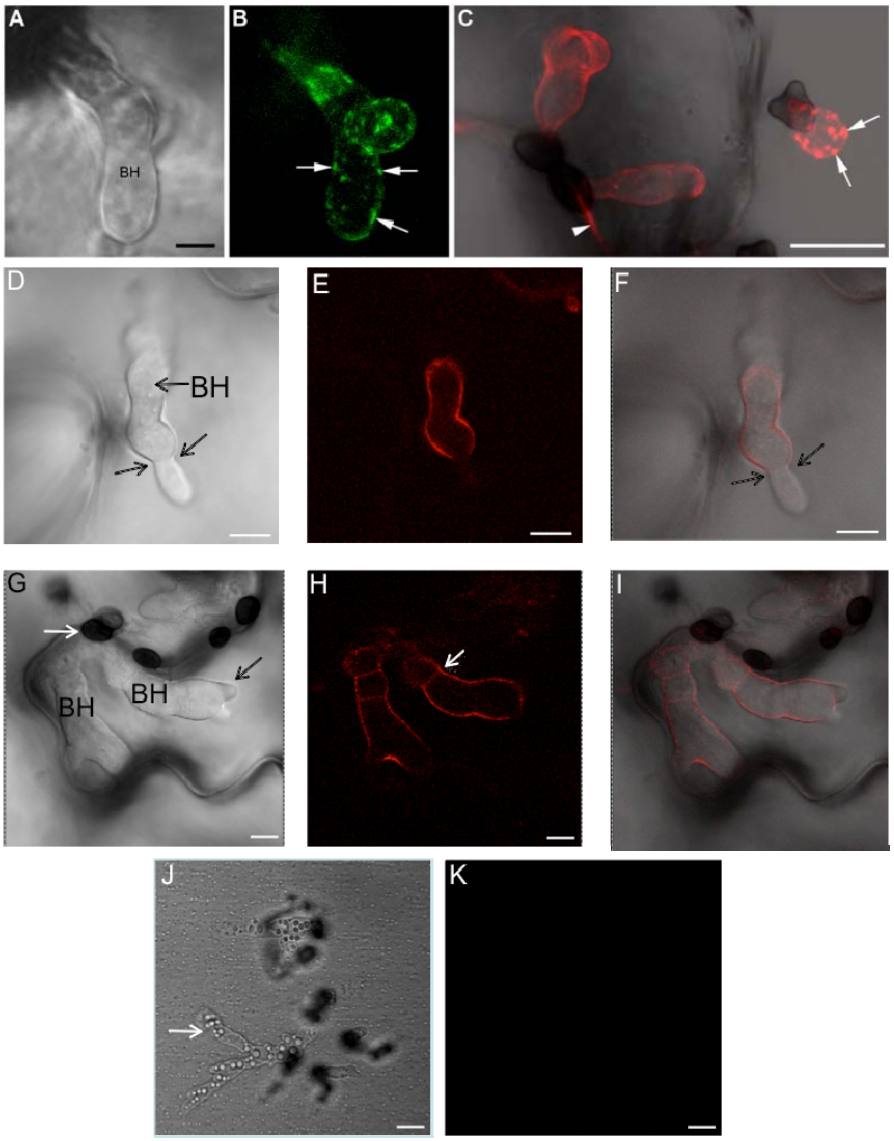

Figure 14. ChEC89 localization. Confocal microscopy of *C. higginsianum* expressing RFP-tagged ChEC89. A, D, G J Bright field image. B, C. E, H.K fluorescence image. C, F, I. Overlay of bright field and fluorescence channels. A, B Transformant biotrophic hypha expressing effector ChEC89:GFP. C Maximum fluorescence intensity overlay of biotrophic hypha expressing fluorescent foci (arrowheads) on the hyphal surface. Note the plant cell wall is labelled (arrowhead). White arrows in B and C indicate fluorescent foci. D. Biotrophic primary hyphae (BH) switching to the necrotrophic stage, producing a thin secondary hypha. Arrowheads indicate point of transition. F. RFP fluorescence is detectable on the surface of the biotrophic hypha but not the necrotrophic hypha. G. Appressorium (white arrow) and biotrophic hypha (BH) starting to switch to the necrotrophic stage, producing a thin secondary hypha at the tip (black arrow). H. RFP fluorescence is on the surface of biotrophic hyphae and in the septum (white arrow) I. RFP fluorescence is present on the biotrophic primary hypha but not the emerging secondary hyphae. J,K) Pseudo-biotrophic hyphae (white arrow) growing inside a cellophane membrane. J) Bright field image; K) Fluorescence image, showing there is no detectable RFP signal on the hyphae. Bars = 5μm. Images were recorded at 55 hpi.

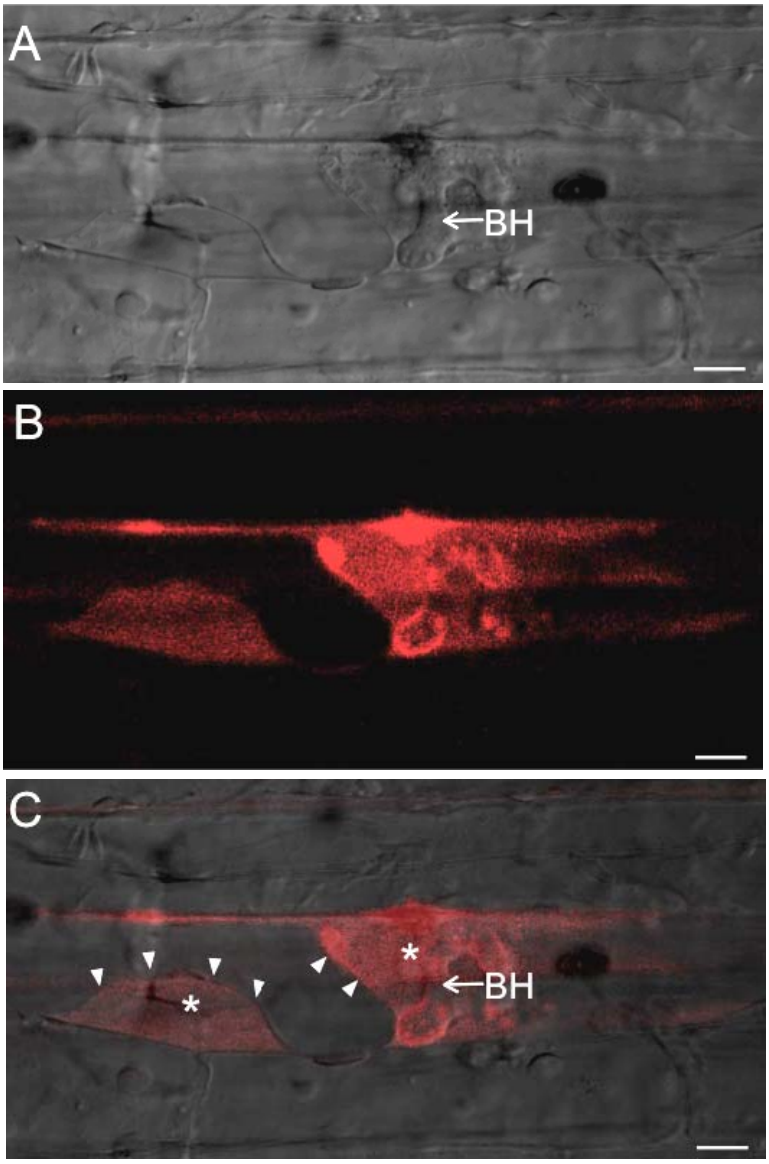

Figure 15. ChEC89 is solubly secreted into the plant cell apoplast. Confocal microscopy images of *Arabidopsis* hypocotyls infected with *C. higginsianum* and plasmolyzed in 0.85M KNO_3. A. Bright field image. B. RFP Fluorescence image. C. Overlay of bright field and fluorescence channels. A. Biotrophic hyphae (BH) infecting a plant epidermal cell with contracted cytoplasm. B, C. RFP fluorescence signal is present in the enlarged plant apoplast (*). White arrowheads show the contracted plant plasma membrane. Bars = 5 µm. Images were recorded at 55 hpi.

4.3.3 Localization of effector candidate ChEC3

Using confocal microscopy of *Arabidopsis* plants inoculated with transformants expressing RFP-tagged ChEC3, the fusion protein was localized exclusively on the surface of biotrophic hyphae but not in appressoria or in necrotrophic hyphae (Figure 16), consistent with the qPCR expression profile of this gene. After plasmolysis of the infected tissue, it was possible to detect the RFP fluorescence signal in the expanded apoplast of the infected plant cell, similar to ChEC89 (Figure 17). In addition, it was verified that the protein expression was only induced during plant infection and not under *in vitro* conditions by pseudo-biotrophic hyphae growing inside cellophane membranes (Figure 16 G-I).

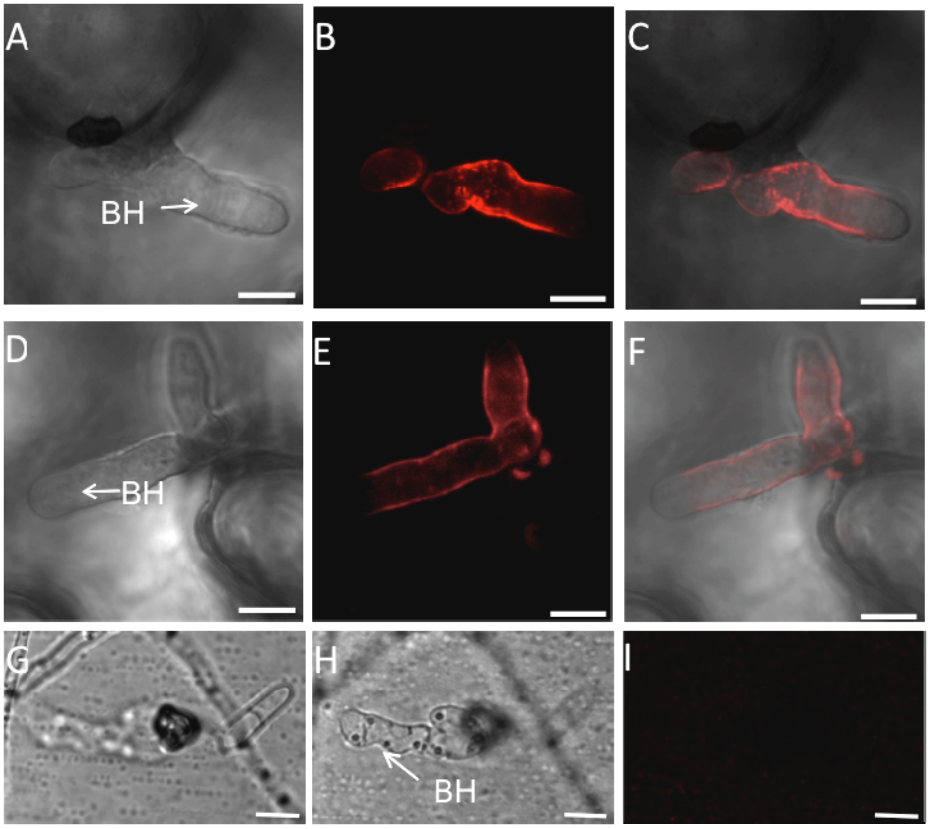

Figure 16. Localizaton of RFP-tagged ChEC3. Confocal microscopy images of biotrophic hyphae of *C. higginsianum* expressing RFP-tagged ChEC3. A, D. Bright field image. B, E. RFP fluorescence image showing labeling on the surface of biotrophic hyphae (BH). C, F. Overlay of bright field and fluorescence channels. G, H, I. Pseudo-biotrophic hypha (BH) that penetrated a cellophane membrane *in vitro*. G. Bright field image; H. RFP fluorescence signal. I. Overlay of bright field and fluorescence channels. Note the appressorium and hypha show no detectable RFP fluorescent. Bars = 5 µm. Images were recorded 42 hai.

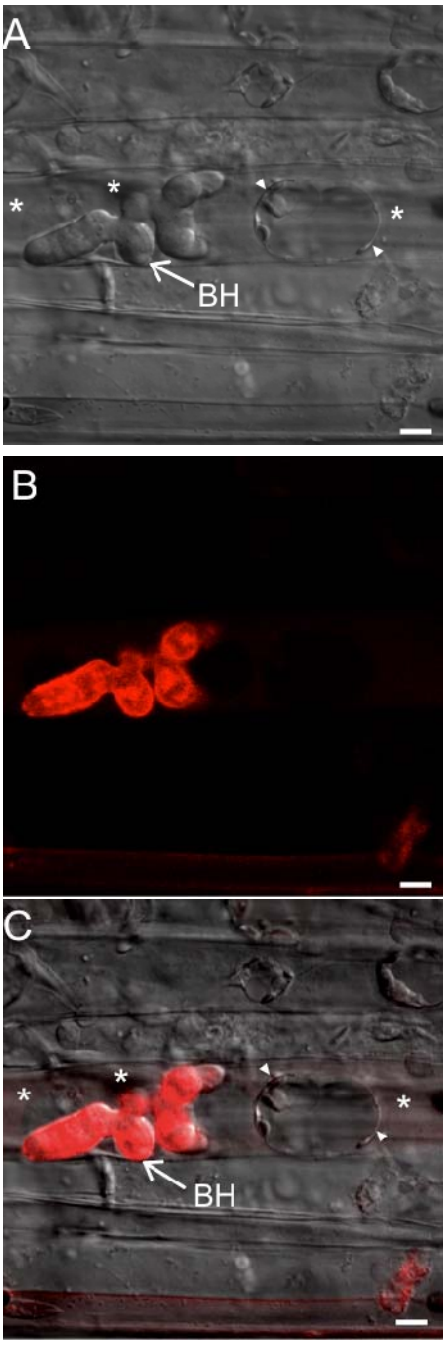

Figure 17. ChEC3 is secreted into the plant apoplast. Localization of RFP-tagged ChEC3 by confocal microscopy. A. Bright field image. B. RFP fluorescence image. C. Overlay of bright field and fluorescence channels. A. Biotrophic hyphae (BH) infecting plant cell after plasmolysis. B, C. RFP fluorescence is detectable in the enlarged plant apoplast (*). White arrowheads show the contracted plasma membrane. Bars = 5 µm. Images were recorded 42 hai

4.3.4 Localization of effector candidate ChEC6

Confocal microscopy of transformants expressing ChEC6:RFP had a similar localization pattern to ChEC36:RFP. The fluoresence signal was visible exclusively in appressoria, in the penetration pore, but there was not detection of fluorescence signal in the appresorial cytoplasm. This results were obtained previously by Dr Kleemann (Kleemann *et al*., 2012) (Figure 18 A-C). The *ChEC6* expression is induced only during plant infection, there was no fluorescence signal during *in vitro* conditions. (Figure 18 D-G).

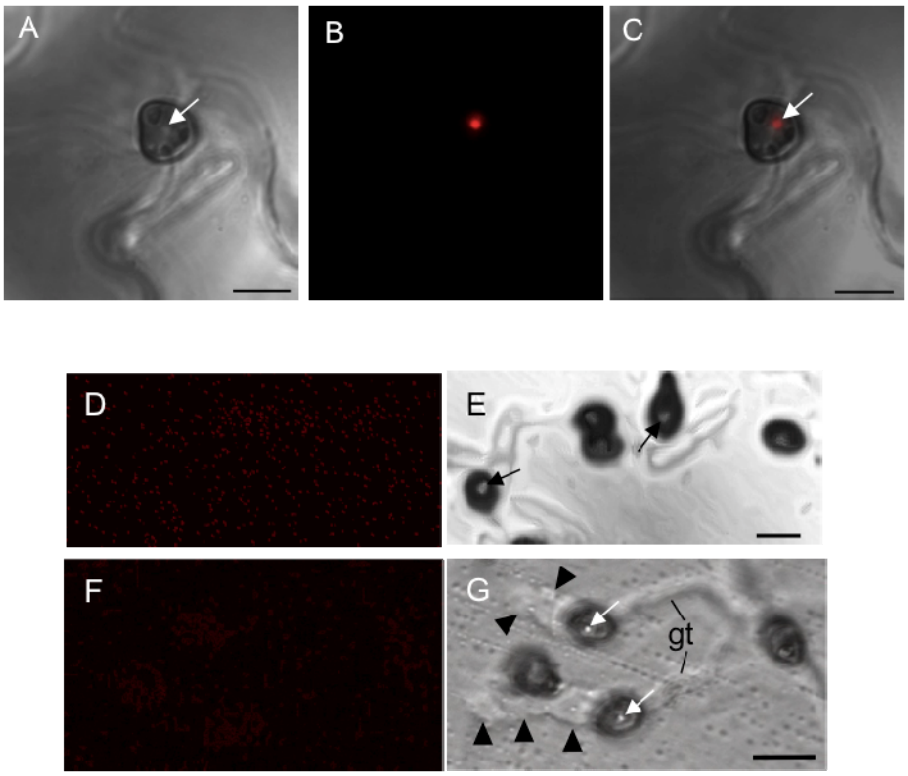

Figure 18. ChEC6 co-localizes with the appressorial penetration pore. Confocal microscopy images *C. higginsianum* appressoria expressing RFP-tagged ChEC6. A-C. *In planta*, D-G. *in vitro* (cellophane). A. Bright field image, showing the penetration pore (arrow) in the base of the darkly melanized, unpenetrated appressorium, viewed from above B. Red fluorescence channel (RFP). C. Overlay of bright field and fluorescence channel. D, F. RFP fluoresence signal. E,G. Bright field image. D-E. There is no ChEC6:RFP fluorescence signal in unpenetrated appresoria (black arrows). F-G. There is no ChEC6:RFP fluorescence signal after penetration (white arrows). gt, germ tubes. Arrowheads, penetration hyphae within the cellophane. Bars = 5 µm. Images were recorded at 24 hpi.

4.4 Putative functions of effector candidates

One advantage of the *C. higginsianum-Arabidopsis* interaction is that fungal genes of interest can be knocked out by targeted mutagenesis to test their contribution to pathogenicity. However, in previous studies the deletion of ChEC genes did not produce measurable infection phenotypes, possibly due to functional redundancy (Kleemann, 2010). As an alternative approach, the direct expression of ChECs in plant cells can be used to evaluate their possible functions, e.g. repressing or inducing plant cell death, or suppressing plant defense responses.

4.4.1 Effector proteins are able to suppress plant cell death

During the biotrophic phase of infection, *C. higginsianum* must avoid damaging or killing host cells. To evaluate whether the effector candidates have the ability to suppress plant cell death, they were transiently co-expressed in *Nicotiana benthamiana* leaves together with a necrosis-eliciting protein (NLP1) from *C. higginsianum*. Necrosis- and Ethylene-inducing Peptide1-Like Proteins (NLPs) belong to a family of proteins which are able to disrupt the plasma membrane, thereby causing cell death and promoting the virulence of fungi, oomycetes and bacteria (Gjizen and Nürnberger, 2006, Ottmann *et al*., 2009). All of them contain the heptapeptide GHRHDWE, which has been defined as an essential domain for full biological activity (Ottmann *et al.,* 2009). Recently, six NLP proteins have been described in *C. higginsianum* (ChNLP1-6) (Kleemann *et al*., 2010) which were identified through comparison with the NLP from *Phytophthora sojae* (Kanneganti *et al*., 2006, Gijzen and Nürnberger 2006). The most similar homolog (*ChNLP1*) was specifically expressed at the switch from biotrophy to necrotrophy (Kleemann *et al*., 2012). ChNLP1 was selected as a suitable elicitor of cell death for the assay described below.

Effectors were transiently expressed in *N. benthamiana* leaves by infiltrating a mixture of *Agrobacterium tumefaciens* strains carrying constructs for expression of ChECs (without their predicted signal peptide for secretion) or NLP1 protein expression in one half of the leaf. Into the other half of the leaf, a control mixture was infiltrated comprising *A. tumefaciens* strains carrying constructs for the expression of NLP1 and the yellow fluorescent protein (YFP). This experimental design (Fig 19 A) allowed pair-wise comparisons of necrosis intensity in the same leaf to take account of the leaf-to-leaf

variation in necrosis development. All effectors were strongly over-expressed under the control of the *Cauliflower mosaic virus* 35S promoter. The cell death reduction was quantified by determining the proportion of infiltrated sites that showed necrosis reduction (exemplified in Fig 19 B) or showed no necrosis reduction (exemplified in Fig 19 C). As a negative control, a fungal secreted chitinase was used. This was previously found to be strongly up-regulated during biotrophy (Takahara *et al*, 2009). This experiment was repeated at least three times for each effector protein, and at least 30 leaves per repetition were evaluated, with reproducible results for experimental repetitions.

Co-expression of ChEC3 and ChEC6 significantly impaired ChNLP1-induced cell death in 93% and 88% of the evaluated infiltration sites (P<0.02 and <0.005 in Student´s test) compared to the chitinase negative control (Figure 19 D). In contrast, co-infiltration of ChEC36, ChE36-1 and ChEC89 resulted in no significant reduction of NLP1 induced necrosis.

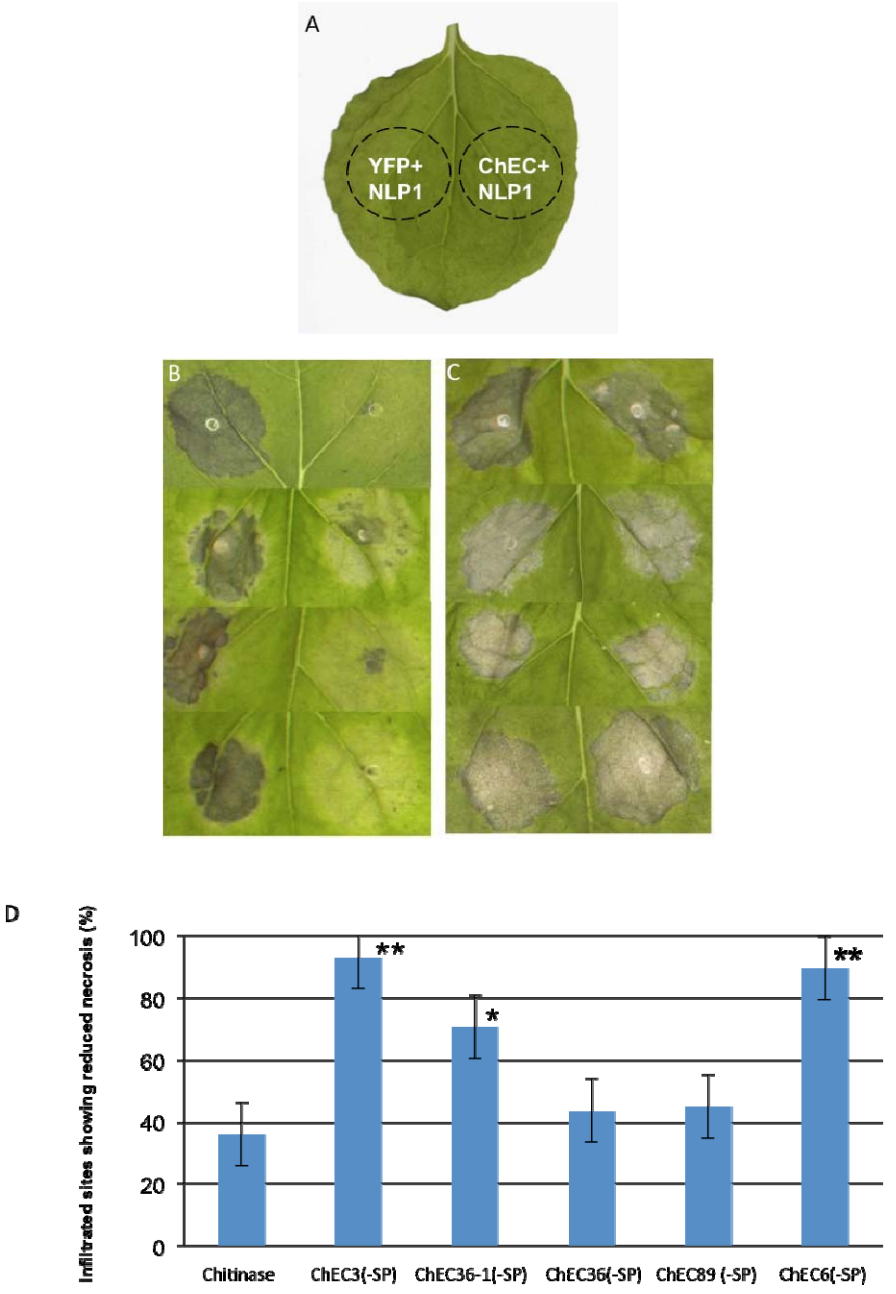

Figure 19. Cell death suppressing activity of the *C. higginsianum* Effector Candidates (ChECs). A, Scheme showing the infiltrated combinations used for the transient co-expression assay in *Nicotiana benthamiana* leaves. Agrobacteria containing constructs for ChEC or YFP expression were mixed with those for expression of the cell death-inducer NLP1. (B), Examples of infiltration site pairs scored as showing necrosis reduction six days after infiltration. (C), Examples of infiltration site pairs scored as showing no reduction in necrosis. (D), Quantification of cell death suppressing activity of ChEC3, ChEC6, ChEC36-1, ChEC36-2 and ChEC89, all expressed without their signal peptide for secretion. Asterisks * and ** indicate significant differences from the chitinase control at P<0.02and <0.005, respectively (Student's t-test). Data represent the means from at least three independent experiments, with at least 15 leaves/experiment/co-expression combination (± standard error).

4.4.2 Effector proteins are able to increase virulence of plant pathogenic bacteria

To test whether ChECs are able to make host cells more susceptible to other *Colletotrichum* species, preliminary experiments were done in which agroinfiltrated sites in *N. benthamiana* leaves expressing ChECs were challenged with *Colletotrichum* spp. that are adapted to infect *Nicotiana*, namely *C. orbiculare* and *C. destructivum*. However, no enhancement of pathogen growth was detectable (data not shown), possibly as a result of plant immune responses triggered by the infiltration of agrobacteria. Therefore, I used the well-described 'effector detector vector' system to deliver ChECs *via* the type III secretion system of transgenic *Pseudomonas syringae* pv. *tomato* (*Pst*) into *Arabidopsis* leaves to determine whether ChECs can manipulate the plant immune system in such way that they enhance bacterial multiplication and virulence (Sohn *et al*., 2007). Four ChECs (ChEC3, ChEC6, ChEC36 and ChEC89) were evaluated. The *Hyaloperonospora parasitica* effector ATR13^{Emco5} is known to enhance the virulence of *Pst* (Sohn *et al*., 2007) and was therefore used as a positive control in this assay. YFP served as a negative control. This experiment was repeated at least three times for each effector protein, sampling per treatment 63 leaf discs, showing similar results among treatments (Figure 20).

Two ChECs tested, ChEC3 and ChEC89 significantly ($P<0.0001$; $P< 0.02$; Student´s test) enhanced the virulence of *Pst*, resulting in bacterial titers even higher than the positive control gene ATR13^{Emco5} (Figure 20). In contrast, ChEC6 and ChEC36 lacked activity in this assay, similar to the negative control YFP. These results suggest that ChEC3 and ChEC89 can increase the virulence of plant pathogenic bacteria, presumably by suppressing host defense responses (Sohn *et al*., 2007).

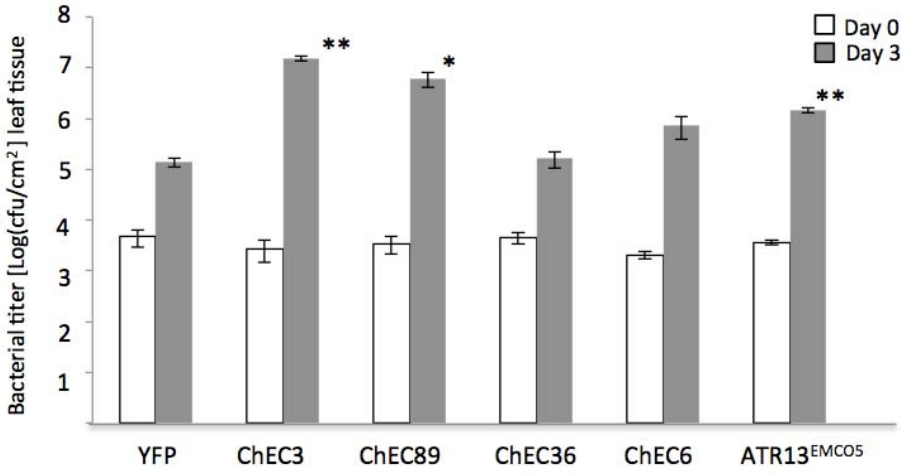

Figure 20. ChECs supporting multiplication of plant pathogenic bacteria. Bacterial titers in Arabidopsis Col-0 leaves infected with *Pseudomonas syringae* pv. *tomato* expressing the indicated ChECs as fusions with a bacterial effector mediating delivery via the type III secretion system. $ATR13^{Emco5}$ and YFP were included as positive and negative controls, respectively. Colony forming units (cfu) were counted 0 and 3 days after spray inoculation. * and ** indicate significant difference from the YFP control at P<0.03 and P<0.0005, respectively. Bars represent means of 4 replicates (each replicate correspond to nine leaves per tratament) ± standard error.

4.4.3 ChECs can suppress the hypersensitive cell death response (ETI)

P. fluorescens (*Pfo*) carrying the *Pst* effector AvrPS4 is recognized by the corresponding R protein, RPS4, in the *Arabidopsis* accession Ws-0, thereby triggering a hypersensitive cell death response (HR). Cell death can be quantified by measuring electrolyte (ion) leakage from the dead cells using a conductivity meter (Rizhsky *et al*., 2004). To test whether ChECs are able to suppress the HR induced by this *Avr-R* gene interaction (effector-triggered immunity), leaves of Arabidopsis Ws-0 were pressure-infiltrated with a 1:1 mixture of *Pfo* cells carrying *AvrRps4* to induce HR and *Pfo* cells carrying individual fungal effectors (ChEC3, ChEC6, ChEC36 and ChEC89). After infiltration, leaf discs were taken from each treatment, washed in sterile water and incubated in destilled water in a multi-well plate, three leaf discs per well. Ion leakage was then measured at intervals using a conductivity meter (Figure 21). In each experiment, 18 leaves were sampled and the experiment was repeated four times. The ion leakage measurements showed

considerable variability between replicate leaf discs but the overall trend of the data was consistent and a representative set of measurements are shown in Figure 21.

The control treatments with *Pfo* cells carrying *AvrRPS4* alone and a mixture of *AvrPS4* and *YFP* provoked high levels of conductivity (77.5 µS/cm and 101 µS/cm), in contrast to the negative control *Pfo* strain harbouring YFP alone, which showed the lowest level of conductivity (12.8 µS/cm). It was found that mixtures of *Pfo* strains carrying *AvrRPS4* and *ChEC36, AvrRPS4* and *ChEC3* or *AvrRPS4* and *ChEC6* all showed significantly lower levels of conductivity ($P<0.05$) compared with the positive control mixture of *AvrPS4* and *YFP*. This suggests that these three fungal effector proteins interfere in some way with expression of the HR cell death response triggered by this *AVR-R* gene interaction. However, the *Pfo* strain mixture carrying *AvrRPS4* and *ChEC89* was not able to block HR, as shown by the high levels of ion leakage. It was clear that there was no activation of HR in the negative control with YFP alone. In contrast, AvrRPS4 provoked high conductivity levels, demonstrating the successful recognition of AvrRPS4 by RPS4 in this assay.

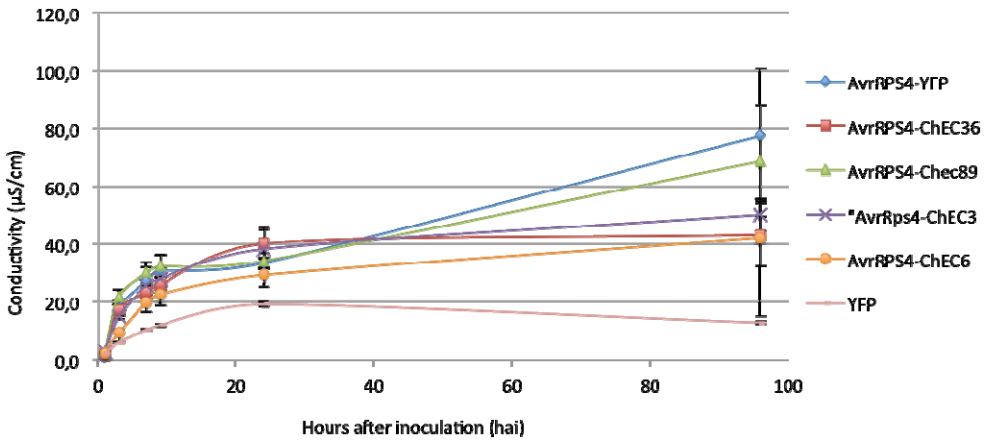

Figure 21. ChECs are able to suppress the hypersensitive cell death response triggered by the recognition of AvrRPS4 by RPS4 (effector-triggered immunity). Ion leakage measurements were made at the indicated time points in leaf discs of 4-week-old Ws plants carrying RPS4, after inoculation of ChEC-AvrRPS4. Bars represent means of 3 replicates (each replicate correspond to eighteen leaves per treament) ± standard error.

5. Discussion

Through this work it was possible to characterize the expression patterns, localization and possible functions of four *C. higginsianum* effector candidates (ChECs). It was shown that ChEC3, ChEC6, ChEC36 and ChEC89 were all specifically upregulated during plant infection, in appressoria and/or during the biotrophic phase, suggesting that these proteins may play important roles during host infection. A novel localization pattern was described for ChEC36, which was focally secreted to, and out of, the appressorial penetration pore. Appressoria have long been recognized as fungal structures enabling adhesion and mechanical penetration of host surface barriers. This study describes a new function for appressoria, namely the local delivery of effector proteins into the plant through the nanoscale interface formed by the penetration pore. In contrast, ChEC89 was found to accumulate in small compartments on the surface of biotrophic hyphae called interfacial bodies, similar to the biotrophic interfacial complex (BIC) of *M. oryzae*, where fluorescent protein-tagged effectors also accumulate. ChEC3 and ChEC6 were shown to suppress plant cell death caused by the *C. higginsianum* Necrosis and Ethylene inducing peptide 1-like protein ChNLP1. In addition, ChEC3 and ChEC89 were found to enhance the growth of *Pseudomonas syringae* in susceptible *Arabidopsis* plants, suggesting they interfere with plant basal defense (PTI responses). Finally, ChEC3, ChEC6, and ChEC36 suppressed the HR death triggered by the interaction of AvrRPS4 and RPS4, suggesting that these effectors are able to interfere with effector triggered immunity (ETI).

5.1 Homology of *C. higginsianum* effectors to proteins from other fungi

A survey of the sequence similarity of the ChECs selected for this study with other known proteins in public databases revealed that in general they have few homologs in the

genus *Colletotrichum* or in other fungi. ChEC3 has a paralog called ChEC3a in *C. higginsianum,* and a homolog called CgDN3 in *C. gloeosporioides*. ChEC6 has a homolog in *C. graminicola* but resembles no other proteins outside the genus. In contrast, ChEC36 and ChEC89 have homology to known secreted effectors of *F. oxysporum* and *M. oryzae*, respectively, but no homologs were found in *C. graminicola*. This is remarkable because although *Fusarium* and *Magnaporthe* belong to the Sordariomycetes, they are not closely related phylogenetically to *Colletotrichum* Possibly these effector genes were present in a common ancestral fungus but were subsequently lost from *C. graminicola* during the course of evolution. The SIX6 homolog of ChEC36 in *Fusarium oxysporum* has been described as a protein effector that contributes to fungal virulence (Takken and Rep, 2010). In the present study, ChEC36 and its splice variants were not able to increase bacterial growth or suppress cell death induced by ChNLP1 proteins. However, the splice variants differed in their ability to suppress plant cell death. Thus, transient expression of ChEC36-1 (with the canonical intron) reduced ChNLP1 induced necrosis in a higher percentage of leaf infiltration sites than ChEC36. This suggests that there may be functional diversification among the splice variants of this gene. These splice forms encode identical proteins with exception of the C terminus. It therefore appears that this protein region contains the functional portion of this protein an amino acid motif required for suppression of ChNLP1-induced cell death.

5.2 Localization of *C. higginsianum* effectors during plant infection

Previously the appressorium has been described as a specialized infection structure capable of providing turgor-driven fungal penetration of host surface barriers (Deising *et al*., 2000, Latunde-Dada, 2001). Penetration is a complex process that involves the release of adhesive extracellular matrix, the formation of a basal penetration pore, the deposition of extra cell wall layers and deposition of the phenolic polymer melanin in the appressorial cell wall. Melanization is a prerequisite for the generation of high turgor pressure through the accumulation of glycerol, providing the mechanical force to allow *Colletotrichum* species to penetrate the host cuticle/cell wall (Bechinger *et al*. 1999).

However, according with my results, it has been possible to define another function for the appressorium, namely the delivery of effector proteins into the host plant. The delivery of these effector proteins is extremely localized to a nanoscale interface formed between host and pathogen which is defined by the 200-nm diameter penetration pore. For plant-pathogenic fungi it has been described that effectors localize to the surface of intracellular hyphae, e.g. *Ustilago maydis* Pep-1 (Doehlemann, 2009), or in special structures such as the *M. oryzae* biotrophic interfacial complex (BIC) (Mosquera *et al*., 2009), from where they may be translocated into host cells (Khang *et al.*, 2010). Also in the case of oomycetes such as *Phytophthora* (Kamoun, 2006, Whisson *et al*., 2007) and true fungi such as *Blumeria graminis* (Ridout *et al*., 2006) and *Melampsora lini* (Catanzariti *et al*., 2006), the extrahaustorial matrix has been described as a structure that plays a critical role in the accumulation and delivery of effector proteins. However, the delivery of effectors by fungal appressoria has not been previously reported.

ChEC36 was focally secreted to and from the appressorial penetration pore and was specifically expressed during plant infection, but not under *in vitro* conditions (including appressoria formed *in vitro*). These findings suggest that expression of this gene is activated by signals derived from the host plant. It is known that several components associated with plant surfaces, for example cutin monomers, epicuticular waxes, as well as the phytohormone ethylene, can induce the germinating spores and germ-tubes of *Colletotrichum* to differentiate into appressoria (Flaishman and Kolattukudy 1994; Podila *et al*. 1993; Dickman *et al*. 2003). However, the ability of fully-developed appressoria to perceive and respond to plant signals was not previously reported for any fungi.

Little is known about the mechanisms by which fungi perceive plant signal molecules. In *M. oryzae*, two putative sensor genes, called *MoMSB2* and *MoSHO1*, have been described. These genes are expressed during plant infection and required for appressoria development. Sensors encoded by these genes are able to detect chemical and physical signals on the rice surface, activating the protein kinase gene PMK1 involved in the development of appressoria and host penetration. *MoMSB2* is able to sense leaf surface hydrophobicity and cutin monomer precursors. *Momsb2* mutants rarely form appressoria

on artificial hydrophobic surfaces. MoSHO1 is important in the perception of leaf waxes, and *Mosho1* mutants are affected in appressoria formation (Liu *et al*., 2011). In contrast, appressorial development on polystyrene by *C. higginsianum* was identical to that found on *A. thaliana* leaf tissue (O´Connell *et al*., 2004, Kleemann *et al*., 2008), suggesting that in this species plant signals are not essential for the induction of appressorium formation *per se*.

It is well known that many genes, not only those encoding effectors, are induced exclusively during plant infection. For example, during *Uromyces fabae* bean infection 31 in-planta induced genes (PIGs) were identified, from a purified haustorial cDNA library. Some of them were involve in nutrient transport and metabolism of carbohydrates (Hahn and Mendgen 1997). Similarly, 21 *in planta*-induced fungal genes were found during infection of susceptible wheat leaves by the rust fungus *Puccinia tritici*. Some of them have homology to pathogenicity-related genes expressed by other fungi during infection (Thara *et al*., 2003). For example, TR24 has homology with a cyclophilin, which is a pathogenicity determinant for the rice blast fungus, *M. oryzae* (Viaud *et al*., 2002).

Previous ultrastructural studies have demonstrated that plant cell wall material is deposited beneath *C. higginsianum* appressoria even before any penetration into the host cell has occurred, when the plant cuticle and cell wall are still intact (Kleemann *et al*., 2012). This suggests that the plant is able to detect and respond to the presence of the fungus very early, before host cells are penetrated. Similarly, the early expression of plant defense response genes also occurs during infection of beans by *C. lindemuthianum*, where appressorium maturation, without penetration, is sufficient to induce plant defense responses (Veneault-Fourrey *et al*., 2005). Therefore, one possibility is that ChEC36 is an effector protein that is delivered at an early stage of infection in order to counteract the pre-penetration host defenses and to prepare the host cell for subsequent invasion.

In contrast to ChEC36:RFP, ChEC89:RFP and ChEC3:RFP both localized to the surface of the biotrophic hyphae, but interestingly, ChEC89:RFP accumulated in small interfacial bodies on the surface of the biotrophic hyphae. These interfacial bodies resemble the

biotrophy interfacial complex (BIC) formed by the *Magnaporthe oryzae*, biotrophic hyphae where effector proteins accumulate and from where they are believed to be translocated into the rice cells (Mosquera *et al*., 2009; Khang *et al*., 2010). However, unlike *M. oryzae* biotrophic hyphae, which produce only a single BIC in each infected cell, *C. higginsianum* hyphae are surrounded by numerous interfacial bodies. Further work is needed to determine if these structures are associated with effector proteins transfer into the plant cell.

In fungi, the mechanisms involved in effector translocation into host cells and the amino acids sequences required for targeting proteins to host cells remain mostly unknown. In the case of oomycetes, effector transfer into host cells is mediated by the RxLR motif, which is similar to a motif present in *Plasmodium* the malaria parasite effectors,which allows translocation of secreted effector proteins into cells of the animal host (Morgan and Kamoun, 2007, Whisson *et al*., 2007). In the case of fungal effectors, transfer across the host plasma membrane into the cytoplasm has been described in several cases, although the mechanism is not clear. For example, the Avr2 (Six3) effector of *F. oxysporum* f. sp. *lycopersici* is secreted to the plant xylem but it is recognized by the intracellular resistance protein I2, providing indirect evidence of effector movement to the plant cytoplasm (Houterman *et al*., 2009). Rust transferred protein 1 (Uf-RTP1p) from *Uromyces fabae* and a homolog protein (Us-RTP1p) of *U. striatus* were found not only in the extrahaustorial matrix, but also in the cytoplasm and nucleus of infected host cells. These proteins were localized through immunocytochemistry (Kemen *et al*., 2005). Another case where translocation of fungal effector proteins has been inferred from their recognition by intracellular resistance proteins, is AvrM from *Melampsora lini*. This effector is secreted to the haustorial cell wall initially and five days after infection the effector was detected inside the host cytoplasm (Rafiqui *et al*., 2010).

Using C-terminal RFP fusions with the ChEC3, ChEC36 and ChEC89 effector proteins, I did not detect their translocation into the plant cytoplasm. This could be explained by the low concentration of the protein in the cytoplasm, making detection by confocal microscopy and immunogold labelling impossible. Also an interference with effector

translocation due the relatively large size of the mRFP tag (28 kDa) could be considered. However, tags of 50 kDa have been used successfully to trace the movement of *M. oryzae* effector proteins into invaded rice cells and their neighbors (Khang *et al*., 2010). In an attempt to increase the detection sensitivity of RFP-tagged effectors by confocal microscopy, I tried to concentrate fusion proteins in the plant cytoplasm through plasmolysis of infected plant tissue, which causes the plant protoplasts to contract (Doehleman *et al*., 2009, Khang *et al*., 2010). However, none of the three fluorescent fusion proteins were detectable in the plant cytoplasm after plasmolysis.

As an alternative approach to obtain a more intense fluorescence signal, and thus more sensitive detection of translocation, I tried a nuclear enrichment strategy by adding an NLS signal to the RFP-effector fusion protein (Khang *et al*., 2010). However, it was not possible to detect any RFP signal in nuclei of plant cells infected by transformants expressing ChEC36:RFP:NLS. For this effector I could only obtain evidence for highly localized movement of ChEC36:RFP into the plant apoplast directly beneath appressoria. Taken together, these findings suggest that ChEC36 should be classified as an apoplastic effector, which may act in the plant extracellular space (Stergiopoulos and deWit, 2009). Consistent with this, ChEC36 contains a relatively large number of cysteine residues (9). The presence of a high number of cysteines has been described as an important characteristic of effector proteins secreted to the plant apoplast, conferring greater protein stability by forming disulphide bridges that prevent degradation by plant proteases in the apoplast (Kamoun, 2007).

Examples of well-characterized apoplastic effectors are Avr2, Avr4, Avr4E, and Avr9 from *Cladosporium fulvum*, which are recognized in tomato by resistance genes *Cf-2, Cf-4*, *Cf-4E*, and *Cf-9*, respectively (Joosten *et al*., 1997, 1999, Rooney *et al*., 2005, van den Burg *et al*., 2006). *Avr2* inhibits at least four tomato cysteine proteases including Rcr3 (Rooney *et al*., 2005), *Avr4* protects *C. fulvum* against tomato chitinases during infection (van den Burg *et al*., 2006), Avr4E triggers Cf-4E-mediated HR and *Avr9* has necrosis-inducing activity (Kooman-Gersmann *et al*., 1998). In the case of *Fusarium oxysporum* f.sp *lycopersici* (*Fol*), at least 11 apoplastic effectors were identified and called Secreted In

Xylem (SIX) proteins (Houterman *et al*., 2007, Lievens *et al*., 2009). Avr2 (Six3), Avr3 (Six1) and Six6 contribute to *Fol* virulence (Rep, 2005, Houterman *et al*., 2009).

RFP-tagging revealed that ChEC3 and ChEC89 localize to the surface of biotrophic primary hyphae, but not necrotrophic secondary hyphae. Interestingly, the fluorescent labelling of primary hyphae persisted after the switch to necrotrophy, when the hyphae are in dead plant cells without an intact plasma membrane. This may indicate that these effector proteins may become associated with, or cross-linked onto, the fungal cell wall. However, after plasmolysis of plant cells infected with biotrophic hyphae expressing ChEC89:RFP or ChEC3:RFP, I was able to detect fluorescence in the enlarged plant apoplast between the fungal cell wall and the retracted plant plasma membrane. This suggests ChEC89 and ChEC3 are, at least partially, soluble secreted proteins that are not incorporated into the fungal cell wall or extracellular matrix permanently.

5.3 Putative effector functions

The function of the ChEC3, ChEC6, ChEC36 and ChEC89 effectors is unknown. However, different approaches used in this work suggest that these ChECs can suppress plant defense. Thus, ChEC3 and ChEC89 improved the growth and multiplication of *Pseudomonas syringae* inside *Arabidopsis* leaves using the effector detector vector (EDV) assay. Similarly, it has been shown that the ATR1 and ATR13 effector proteins of *Hyaloperonospora parasitica* are able to increase virulence of *Pst* DC3000 in susceptible *Arabidopsis* plants (Sohn *et al*., 2007). Likewise, the *P. syringae* effector AvrRp2 is able to increase bacterial growth in *Arabidopsis* plants that lack the resistance gene RPS2 (Kim *et al*., 2005, Chen *et al*., 2000). Moreover, in *Phytophthora infestans* a family of RxLR effectors called AVRblb2 have been shown to promote bacterial virulence in the EDV assay (Bozkurt, *et al*., 2011). Recently it was shown that AVRblb2 is localized in the plant plasma membrane around haustoria in infected cells. AVRblb2 targets C14, a host secreted papain-like cysteine protease involved in plant defense by preventing its secretion into the apoplast. *N. benthamiana* plants expressing GFP:AVRblb2 support increased virulence of *P. infestans*, as shown by higher levels of colonization and sporulation relative to control lines (Bozkurt *et al*., 2011). In *Albugo laibachii*, three different kinds of effector proteins were described with the amino acid motifs RxLR, RxLQ

and CHxC. *P. syringae* pv. *tomato* was transformed with constructs containing effectors of each type to be delivered into plant cytoplasm by the EDV system. It was found that both RxLR2 and CHxC effectors were able to enhance bacterial growth, perhaps by suppression of the host resistance mechanisms (Kemen *et al*., 2011).

My results obtained using the EDV assay suggest that ChEC3 and ChEC89 are able to interfere with mechanisms of plant defense against bacteria. Thus, the amount of bacterial growth reached in leaves receiving either of these two effectors was approximately 100-fold greater than in leaves receiving the control protein (YFP). Previously it was shown that *C. higginsianum* is able to suppress callose deposition during the early stages of *Arabidopsis* infection (O´Connell *et al*., 2004). Therefore, it is possible that ChEC3 and ChEC89 are targeting host proteins in the pathway of PAMP-trigged immunity (PTI) which are involved in plant defense against both bacteria and fungi (Sohn *et al*., 2007).

In addition, I found that ChEC3, ChEC6 and ChEC36 can significantly suppress plant cell death induced by ChNLP1. ChEC3 is homolog to the putative effector *CgDN3* from *C. gloeosporioides* (Stephenson *et al*., 2000). *CgDN3* mutants elicited a hypersensitive-like response on the susceptible host plant *Stylosanthes*, leading to the suggestion that CgDN3 can suppress HR cell death, permitting to the pathogen establish/maintain the biotrophic phase. Using the *CgDN3* promoter to drive GFP expression, Stephenson and co-workers (2000) also provided evidence for the early expression of *CgDN3* but not in the necrotrophic stage. *ChEC3* follows the same early pattern of expression and possibly it also functions in maintaining biotrophy by suppressing plant cell death. Since ChNLP1 itself is not expressed at this early infection stage, ChEC3, ChEC6 and ChEC36 may function to suppress cell death triggered by other factors.

NLPs cause plant cell death by disrupting the plasma membrane of dicotyledoneous plants, producing symptoms similar to those caused by normal host infection. This damage is believed to be perceived by the plant through the release of damage-associated molecular patterns (DAMPs), resulting in the activation of plant immunity

(Matzinger *et al*., 2007, Denoux *et al*., 2008). It has been reported that NLPs activate the expression of genes related to the production of ethylene, phytoalexins, reactive oxygen species and activation of mitogen actived protein kinases (MAPKs). Many of these plant defense responses are also activated by the bacterial PAMP, flagellin flg22 (Felix *et al*., 1999, Gomez *et al*., 1999, Qutob *et al*., 2006). It was reported by Qutob, 2006 and collaborators that there is extensive overlap between the genes expressed by *Arabidopsis* plants in presence of flg22 and NLP1-like proteins from *P. parasitica* (Qutob *et al*., 2006). In addition, NLP1 was able to trigger immune responses as callose deposition, nitric oxide production and ethylene and MAPKs in *Arabidopsis* (Qutob *et al*., 2006). It is possible that ChEC3, ChEC6 and ChEC36-1 interfere with those pathways. Previously one effector, SNE1 (Suppressor of NEcrosis1), from *P. infestans* has been reported to suppress host cell death caused by NLPs from *P. sojae* and *P. infestans* (Kelley *et al*., 2010), which are known to trigger plant defence responses and host cell death in tomato and *Nicotiana* (Qutob *et al*, 2006; Pemberton and Salmond, 2004; Kanneganti *et al*., 2006).

The observations that ChEC3 and ChEC89 were able to increase bacterial growth in wild-type *Arabidospsis* Col-0 plants, while ChEC3, ChEC36-1 and ChEC6 suppressed the cell death triggered by ChNLP1, suggest that these effectors could interfere with plant defense pathways activated against both bacterial and fungal PAMPS.

However, I also obtained evidence that the HR cell death caused by the recognition of the bacterial effector AvrRPS4 by the resistance protein RPS4 may be suppressed by ChEC3, ChEC6 and ChEC36, consistent with their ability to suppress ChNLP1-induced cell death. This finding suggests that these effectors could interfere in both types of plant cell death. The SNE1 (Suppressor of NEcrosis1) effector from *P. infestans* was similarly able to suppress both cell death caused by NLP1 proteins and the HR initiated by Avr-R protein interactions from a broad spectrum of pathosystems, including oomycetes (Avr3a/R3a), bacteria (AvrPto/Pto), fungi (Avr9/Cf9) and viruses (CP/Rx2) (Kelley *et al*., 2010).

Bacterial T3SS effectors from *Pst* such as AvrPtoB (HopAB2), HopE1, HopF2, HopX1 (AvrPphE) and HopAM1 (AvrPpiB1), were able to suppress HopA1-induced HR in *Arabidopsis* when they were expressed in *P. fluorescens* (Jamir *et al*., 2004). Moreover, when *P. fluorescens*, which cannot inject any type III secreted effectors and is not able to elicit an HR, was inoculated into *Arabidospsis* Col-0 plants it triggered callose deposition. When the same strain was used to express AvrPtoB, HopE1, HopF2, HopX1, or HopAM1 into *A. thaliana* Col-0, all of them were able to suppress callose deposition (Guo *et al*., 2009). Therefore, it appears that pathogen effectors can interfere with similar targets necessary for both the ETI and PTI pathways of plant defense.

6. Conclusions and perspectives

In this work it was possible to characterize a set of *C. higginsianum* effectors candidates (ChECs). It was shown that they are specifically expressed during plant infection but not during *in vitro* conditions. This suggests that these effector genes are induced by plant signals and may play roles in the infection process that favour pathogenicity. Important future goals will be to understand the nature of the plant signal(s) inducing effector gene expression and how they are sensed by the pathogen. A better understanding of the mechanisms of host perception by plant pathogenic fungi is likely to provide novel strategies for the control of many economically important crop diseases through chemical intervention or plant breeding.

A novel finding was that ChEC36 localized precisely to the appressorial penetration pore. This localization suggests a new function for appressoria in addition to their long-established roles in adhesion and turgor-driven penetration, namely the local release of effector proteins at a nanoscale interface formed by the penetration pore. In the case of ChEC3 and ChEC89, these effectors were localized in interfacial bodies resembling the BICs found in *M. oryzae*-rice infections. The role of these structures in effector translocation requires further investigation.

Although I could not find any RFP-tagged ChECs in the plant cytoplasm, some were active in suppressing ChNLP1-induced cell death and/or improving bacterial growth when they were delivered or expressed directly in the plant cytoplasm. This raises the possibility that these effectors are indeed translocated into host cells *in vivo*, but addition of the large RFP tag alters their mobility. The use of antibodies raised against native ChEC proteins or

peptides for immunolabelling could be an option for more accurate ChEC localization in future experiments.

The NLP1-induced plant cell death suppression by some ChECs might indicate that they were able to interfere with plant defense pathways activated in response to PAMPs, since NLP1-induced plant defences overlap with plant responses to PAMPs (Qutob *et al*., 2006). Some ChECs may also be involved in the suppression of ETI, because they decreased ion leakage from leaf tissues where the interaction between *AvrRPS4* and *RPS4* had produced an HR response. However, their ability to interfere with ETI now requires further confirmation using different Avr-R gene combinations and different assays, e.g. using the model *Pto–AvrPto* interaction in tomato (Tang *et al*., 1996). Furthermore, it would be interesting to use A*rabidopsis* Ws-0 plants to determine whether the delivery of ChECs through the bacterial T3SS using *P. fluorescens* triggers an HR due to their recognition by the anthracnose resistance genes *RRS1* and *RPS4*.

Future approaches that could be used to study the biological function of the ChECs include: (a) targeted gene knock-outs to test their role in fungal virulence; (b) yeast two-hybrid (Y2H) screens to identify potential plant targets of these effectors; and (c) ChECs expression of the in stable transgenic *Arabidopsis* plants, which could be used in various assays to determine the effect on PTI and ETI responses, including resistance to non-adapted pathogens.

7. Annexes

Annex 1. Primer sequence used

Fluorescent protein tagging of ChECs		
pChEC36-fw		CACCCTGGCTCAACAGTAGTTCCTAATTC
ChEC36-rev	without stop codon	AATAAGCCAAGAAAAGGCATTTAG
pChEC3-fw		CACCACTAACATGCTCTCACGTAGGAACT
ChEC3-rev	without stop codon	ACATTTAAACTTTCCACAGTGTGCT
pCHEC89-fw		CACCTACACTGTAGTAAGCCCACTGTTAC
CHEC89-rev	without stop codon	AGGGCACTAGAGATCAACCCATTGTTA
Cloning of protein coding sequences		
ChEC3 ORF –SP fw	without signal peptide	CACCATGCTCCCTGCCAATAAGCATATAGG
ChEC3 ORF rev	with stop codon	TCAACATTTAAACTTTCCACAG
ChEC36 ORF fw	without signal peptide	CACCATGGTGGTCATTCCTCTCTCTCAAGTTG
ChEC36 ORF rev	with stop codon	TTAAATAAGCCAAGAAAAGGCATTT
CHEC89 ORF fw	without signal peptide	CACCATGCAGTTCTTCAACGCCATCATCGTTTTCG
CHEC89 ORF rev	with stop codon	TTAAGGGCAGCTCCAGTAGG
ChEC6 ORF-SP fw	without signal peptide	CACCATGTCGCCTGTCTCTGAGCGCGCCATTGG
ChEC6 ORF-SP rev	with stop codon	TTAGCCGAGGACTTGCTTGGGATCAGC

Annex 2. Primers used for qRT-PCR

Name	Forward	Reverse	Amplicon length (bp)	Efficiency (%)
α tubulin	GAGCGCCCTAACTACGAGAA	CGAAGCAGGACATGGTCATC	232	103
Actin	CCCCAAGTCCAACAGAGAGA	CATCAGGTAGTCGGTCAAGTCA	238	100
ChEC3	CGCTCTTCCCTTTACAACCA	ATATTCCACGCCCACACATT	153	106
ChEC6	CGCCATTCTTGCCATCATT	GAGGACTTGCTTGGGATCAG	256	106
CHEC89	TGGAACACCGGCAACTATG	TAAGGGCAGCTCCAGTAGG	119	106
ChEC36	TTTGTGCCAACAACGAAGTC	ATTGGGTCTGTCCTCCATTG	106	115

References

Adam, L., Ellwood, S., Wilson, I., Saenz, G., Xiao, S., Oliver, R. P., Turner, J. G., and Somerville, S. 1999. Comparison of *Erysiphe cichoracearum* and *E. cruciferarum* and a survey of 360 *Arabidopsis thaliana* accessions for resistance to these two powdery mildew pathogens. Mol. Plant-Microbe Interact. 12: 1031-1043.

Bailey, J. A., Jeger, M., J, editors. 1992. Colletotrichum: biology, pathology and control. Wallingford: Commonwealth Mycological Institute; 1992. p. 388.

Bartetzko, V., Sonnewald, S., Vogel, F., Hartner, K., Stadler, R., Hammes, U.Z., and Börnke, F. 2009. The *Xanthomonas campestris* pv. *vesicatoria* type III effector protein XopJ inhibits protein secretion: evidence for interference with cell wall–associated defense responses. Mol. Plant-Microbe Interact. 22: 655–664.

Bechinger, C., Giebel, K. F., Schnell, M., Leiderer, P., Deising, H. B. and Bastmeyer, M. 1999. Optical measurements of invasive forces exerted by appressoria of a plant pathogenic fungus. Science. 285:1896-1899.

Birker, D., Heidrich, K., Takahara, H., Narusaka, M., Deslandes, L., Narusaka, Y., Reymond, M., Parker, J. E. O'Connell, R. 2009. A locus conferring resistance to *Colletotrichum higginsianum* is shared by four geographically distinct *Arabidopsis* accessions. Plant J. 60: 602-613.

Bhattacharjee, S., Hiller, N.L., Liolios, K., Win, J., Kanneganti, T. D., Young, C., Kamoun, S., Haldar K. 2006. The malarial host-targeting signal is conserved in the Irish potato famine pathogen. PLoS Pathogens 2: e50.

Block, A., Li, G., Qing Fu, Z., and Alfano, J. R. 2008. Phytopathogen type III effector weaponry and their plant targets. Curr. Opin. Plant. Biol. 11: 396–403.

Boch, J. and Bonas, U. 2010. *Xanthomonas* AvrBs3 family-Type III effectors: discovery and function. Annu. Rev. Phytopathol. 48: 419–36.

Boller, T and Felix G. 2009. A renaissance of elicitors: perception of microbe associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev. Plant. Biol. 60: 379–406.

Bos, J. I. B., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I. Taylor, R. M., Zhendong, T., Engelhardt, S., Vetukuri, R. R., Harrower, B., Dixelius, CV., Bryan, G., Sadanandom, A., Whisson, S. C., Kamoun, S. and Birch, P. R. J. 2010. *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. Proc Natl Acad Sci USA 107: 9909-9914.

Bozkurt, T. O., Schornack, S., Win, J., Shindo, T., Ilyas, M., Oliva, R., Cano, L.M., Jones, A. M. E., Huitema, E., Renier A. L. van der Hoorn, and Kamoun, S. 2011. *Phytophthora infestans* effector AVRblb2 prevents secretion of a plant immune protease at the haustorial interface. Proc. Natl. Acad. Sci. USA. 108: 20832-20837.

Catanzariti, A.M., Dodds, P.N., Lawrence, G.J., Ayliffe, M.A., Ellis, J.G. 2006. Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. Plant Cell. 18: 243–56.

Chakrabarti, A., Rep, M., Wanga, B., Ashtona, A., Doddsa, P. and Ellisa, J. 2011. Variation in potential effector genes distinguishing Australian and non-Australian isolates of the cotton wilt pathogen *Fusarium oxysporum* f.sp. *vasinfectum*. Plant Pathol. 60: 232–243

Chen, Z., Kloek, A.P., Boch, J., Katagiri, F., and Kunkel, B.N. 2000. The *Pseudomonas syringae* avrRpt2 gene product promotes pathogenicity from inside the plant cell. Mol. Plant-Microbe Interact. 13:1312–1321.

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.

Chytilova, E., Macas, J and Galbraith, D.W. 1999. Green Fluorescent Protein Targeted to the Nucleus, a Transgenic Phenotype Useful for Studies in Plant Biology. Ann. Bot-london.. 83: 645-654.

Crous, P.W., Gams, W., Stalpers, J.A., Robert, V. & Stegehuis, G. 2004. MycoBank: an online initiative to launch mycology into the 21st century. Stud. Mycol. 5: 19-22.

Dangl, J. L. and Jones, J. D. G. 2001. Plant pathogens and integrated defence responses to infection. Nature. 411:826-833.

DebRoy, S., Thilmony, R., Kwack, Y.B., Nomura, K., and He, S.Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. Proc. Natl. Acad. Sci. USA. 101: 9927–9932.

Deising, H. B., Werner, S. and Wernitz, M. 2000. The role of fungal appressoria in plant infection. Microbes Infect. 2: 1631-1641.

De Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M. H. A. J., Thomma B. P. H. J. 2010. Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. Science. 329: 953-955.

Denoux, C., Galletti, R., Mammarella, N., Gopalana, S., Werck, D., De- Lorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J. 2008. Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. Mol. Plant. 1: 423-445.

Deslandes, L., Olivier, J., Peeters, N., Feng, D., Khounlotham, M., Boucher, C., Somssich, I., Genin, S. and Marco, Y. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. USA. 100: 8024–8029.

Dickman, M. B., Ha, Y.S., Yang, Z., Adams, B., and Huang, C. 2003. A Protein Kinase from *Colletotrichum trifolii* Is Induced by Plant Cutin and Is Required for Appressorium Formation. Mol. Plant. Microb. Interac. 16: 411–421.

Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Ayliffe, M. A., Ellis, J. G. 2004. The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell. 16: 755–68.

Doehlemann, G., van der Linde, K., Aßmann, D., Schwammbach, D., Hof, A., Mohanty, A., Jackson, D., Kahmann, R. 2009. Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. PLoS Pathogens 5 e1000290. doi:10.1371/journal.ppat.1000290.

Doehlemann, G., Wahl, R., Horst, R. J., Voll, L.M., Usadel, B., Poree, F., Stitt, M., Pons-Kühnemann, J., Sonnewald, U., Kahmann, R. and Kämper, J. 2008. Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. Plant J. 56:181–195.

Felix, G., Duran, J. D., Volko, S. And Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18: 265–276.

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

Flaishman, M.A. and Tolatrukudy, P.E. 1994. Timing of fungal invasion using host's ripening hormone as a signal. Proc. Nati. Acad. Sci. USA. 91: 6579-6583.

Flor, H.H. 1942. Inheritance of pathogenicity in *Melampsora lini*. Phytopathology 32: 653–669

Frey, T.J., Weldekidan, T., Colbert, T., Wolters, P.J.C.C. and Hawk, J.A. 2011. Fitness evaluation of *Rcg1*, a locus that confers resistance to *Colletotrichum graminicola* (Ces.) GW Wils. using near-isogenic maize hybrids. Crop. Sci .51: 1551-1563.

Gaulin E, Drame N, Lafitte C, Torto-Alalibo T, Martinez Y, Ameline- Torregrosa C, Khatib M, Mazarguil H, Villalba-Mateos F, Kamoun S et al. 2006. Cellulose binding domains of a *Phytophthora* cell wall protein are novel pathogen-associated molecular patterns. Plant Cell. 18: 1766-1777.

Gehrig, H., Schussler, A., and Kluge, M. 1996. Geosiphon pyriforme, a fungus forming endocytobiosis with Nostoc (cyanobacteria), is an ancestral member of the Glomales: evidence by SSU rRNA analysis. J. Mol. Evol. 43: 71–81.

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

Gomez-Gomez, L. and Boller, T. 2000. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol. Cell. 5: 1003–1011.

Gómez-Gómez, L., Felix, G. and Boller, T. 1999. A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J. 18: 277–284.

Grouffaud S, van West P, Avrova AO, Birch PRJ, Whisson SC. 2008. *Plasmodium falciparum* and *Hyaloperonospora parasitica* effector translocation motifs are functional in *Phytophthora infestans*. Microbiology 154: 3743–3751.

Guo, M., Tian, F., Wamboldt, Y., and Alfano, J.R. 2009.The Majority of the Type III Effector Inventory of *Pseudomonas syringae* pv. tomato DC3000 Can Suppress Plant Immunity. Mol. Plant-Microbe Interact. 22: 1069 –1080.

Gust AA, Biswas R, Lenz HD, Rauhut T, Ranf S, Kemmerling B, Gotz F, Glawischnig E, Lee J, Felix G et al. 2007. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in Arabidopsis. J. Biol. Chem.

282: 32338-32348.

Hahn, M. and Mendgen, K. 1997. Characterization of In Planta–Induced Rust Genes Isolated from a Haustorium-Specific cDNA Library. Mol. Plant-Microbe Interact. 10: 427–437.

He, P., Chintamanani, S., Chen, Z., Zhu, L., Kunkel, B.N., Alfano, J.R., Tang, X., and Zhou, J.M. 2004. Activation of a COI1-dependent pathway in *Arabidopsis* by *Pseudomonas syringae* type III effectors and coronatine. Plant J. 37:589–602.

Heath MC. 1997. Signalling between pathogenic rust fungi and resistant or susceptible host plants. Ann. Bot-london. 80: 713–720.

Herman, P.L. and Marks, M. D.1989. Trichome Development in *Arabidopsis thaliana*. II. Isolation and Complementation of the GLABROUSI Gene. Plant Cell. 1: 1051-1055.

Holub, E. B., Brose, E., Tor, M., Clay, C., Crute, I. R., and Beynon, J. L. 1995. Phenotypic and genotypic variation in the interaction between *Arabidopsis thaliana* and *Albugo candida*. Mol. Plant-Microbe Interact. 8: 916-928.

Houterman, P. M., Ma, L., van Ooijen, G., de Vroomen, M. J., Cornelissen, B. J., Takken, F. L., Rep, M. 2009. The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. Plant J. 58: 970-978.

Houterman, P. M., Speijer, D., Dekker, H. L., De Koster, C. G., Cornelissen, B. J. C., Rep, M. 2007. The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. Mol. Plant. Pathol. 8:215–21.

Huser, A., Takahara, H., Schmalenbach, W. and O'Connell, R. 2009. Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis. Mol. Plant-Microbe Interac. 22: 143-156.

Jabs T, Tschöpe M, Colling C, Hahlbrock K, ScheelD. 1997. Elicitor-stimulated ion fluxes and O2– from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. USA. 94: 4800–4805.

Jamir, Y., Guo, M., Oh, H.-S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A., and Alfano, J. R. 2004. Identification of *Pseudomonas syringae* type III effectors that suppress programmed cell death in plants and yeast. Plant J. 37: 554-565.

Jia Y, McAdams SA, Bryan GT, Hershey HP, Valent B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO Journal. 19: 4004–4014.

Jones, J. D. G. and Dangl, J. L. 2006. The plant immune system. Nature. 444: 323-329.

Joosten, M. H.A. J., De Wit, P. J. G. M. 1999. The tomato-*Cladosporium fulvum* interaction: a versatile experimental system to study plant-pathogen interactions. Annu Rev. Phytopathol. 37:335–67

Joosten, M. H. A. J., Vogelsang, R., Cozijnsen, T. J., Verberne, M.C., DeWit, P. J. G. M. 1997. The biotrophic fungus *Cladosporium fulvum* circumvents Cf-4-mediated resistance by producing unstable AVR4 elicitors. Plant Cell. 9:367–79

Kale, S.D., Gu, B., Capelluto, D., Dou, D., Feldman, E., Rumore, A., Arredondo, F.D., Hanlon, R., Fudal, I., Rouxel, T., Lawrence, C.B., Shansend, W. and Tyler, B.M.2010 External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142:284–295.

Kamoun, S. 2006. A catalogue of the effector secretome of plant pathogenic oomycetes. Annu. Rev. Phytopathol. 44: 41-60.

Kamoun, S. 2007. Groovy times: filamentous pathogen effectors revealed. Curr. Opin. Plant Biol. 10: 358–365.

Kang S., Lebrun, M. H., Farrall, L. and Valent, B. 2001. Gain of virulence caused by insertion of a Pot3 transposon in a *Magnaporthe grisea* avirulence gene. Mol. Plant-Microbe Interac. 14: 671-674

Kanneganti, T.-D., Huitema, E., Cakir, C. and Kamoun, S. 2006. Synergistic interactions of the plant cell death pathways induced by *Phytophthora infestans* Nep1-like protein PiNPP1.1 and INF1 elicitin. Mol. Plant-Microb Interac. 19: 854-863.

Kelley, B. S., Lee, S.-J., Damasceno, C. M. B., Chakravarthy, S., Kim, B.-D., Martin, G. B. and Jocelyn K. C. Ros, J. K. C. 2010. A secreted effector protein (SNE1) from *Phytophthora infestans* is a broadly acting suppressor of programmed cell death. Plant J. 62: 357-366.

Kemen, E., Gardiner, A., Schultz-Larsen, T., Kemen, A. C., Balmuth, A.L., et al. 2011 Gene gain and loss during evolution of obligate parasitism in the white rust pathogen of *Arabidopsis thaliana*. PLoS Biol 9 e1001094.

Kemen, E., Kemen, A. C., Rafiqi, M., Hempel, U., Mendgen, K., Hahn, M., and Voegele, R.T. 2005. Identification of a protein from rust fungi transferred from haustoria into infected plant cells. Mol. Plant-Microbe Interact. 18: 1130–1139.

Khang, C. H., Berruyer, R., Giraldo, M. C., Kankanala, P., Park, S. Y., Czymmek, K., Kang, S. and Valent, B. 2010. Translocation of *Magnaporthe oryzae* effectors into rice cells and their subsequent cell-to-cell movement. Plant Cell. 22:1388-1403.

Kim, M., Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J. and Mackey, D. 2005. Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis*. Cell. 121: 749–759

Kleemann, J., Takahara, H., Stueber, K. and O'Connell, R. 2008. Identification of soluble secreted proteins from appressoria of *Colletotrichum higginsianum* by analysis of expressed sequence tags. Microbiology 154:1204-1217.

Kleemann, J. 2010. Identification and functional characterization of secreted effector proteins of the hemibiotrophic fungus *Colletotrichum higginsianum*. Inaugural dissertation. Cologne University p133.

Kleemann, J., Rincon-Rivera, L.J., Takahara, H., Neumann, U., van Themaat, E.,V.L., van der Does, H.C., Hacquard, S., Stüber, K., Will1, I., Schmalenbach, W., Schmelzer, E., O'ConnelL, R.J. 2012. Sequential Delivery of Host-Induced Virulence Effectors by

Appressoria and Intracellular Hyphae of the Phytopathogen *Colletotrichum higginsianum*. PLoS Pathog 8(4): e1002643.

Kobayashi I, Kobayashi Y, Hardham A.R. 1994. Dynamic reorganization of microtubules and microfilaments in flax cells during the resistance response to flax rust infection. Planta. 195: 237–247.

Koch, E., and Slusarenko, A. J. 1990. Fungal pathogens of *Arabidopsis thaliana* (L.) Heyhn. Bot .Helv. 100: 257-268.

Koh, S., André, A., Edwards, H., Ehrhardt, D., and Somerville, S. 2005. *Arabidopsis thaliana* subcellular responses to compatible *Erysiphe cichoracearum* infections. Plant J. 44: 516–529.

Kooman-Gersmann, M., Vogelsang, R., Vossen, P., Van Den Hooven, H.W., Mahe, E., et al. 1998. Correlation between binding affinity and necrosis-inducing activity of mutant AVR9 peptide elicitors. Plant Physiol. 117:609–18.

Latunde-Dada, A.O. 2001. *Colletotrichum*: tales of forcible entry, stealth, transient confinement and breakout. Mol. Plant Pathol. 2: 187-198.

Lievens, B., Houterman, P. M. and Rep, M. 2009 Effector gene screening allows unambiguous identification of *Fusarium oxysporum* f. sp. *lycopersici* races and discriminationfrom other formae speciales. FEMS Microbiol. Lett. 300 : 201–215.

Liu, W., Zhou, X., Li, G., Li, L., Kong, L., Wang, C., Zhang, H., Xu, J.R. 2011. Multiple plant surface signals are sensed by different mechanisms in the rice blast fungus for appressorium formation. PLoS Pathog 7(1): e1001261. doi:10.1371/journal.ppat.1001261

Marion, J., Bach, L., Bellec, Y., Meyer, C., Gissot, L., Faure, J.-D. 2008. Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of *Arabidopsis* seedlings. Plant J. 56: 169–179.

Martin, G.B., Bogdanove, A. J. and Sessa, G. 2003. Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. 54: 23–61.

Matzinger, P. 2007. Friendly and dangerous signals: is the tissue in control? Nat. Immunol. 8: 11-13.

Mendgen, K. and Hahn, M. 2002. Plant infection and the establishment of fungal biotrophy. Trends in Plant Sci. 7: 352-356.

Micali, C.O., Neumann, U., Grunewald, D., Panstruga, R., O'Connell, R. 2011. Biogenesis of a specialized plant–fungal interface during host cell internalization of *Golovinomyces orontii* haustoria. Cell Microbiol. 13: 210–226.

Mitchell-Olds, T., and J. Schmitt. 2006. Genetic mechanisms and evolutionary significance of natural variation in *Arabidopsis*, Nature. 441: 947-952.

Miya, A., Albert, P., Shinya, T., Desaki, T., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. USA. 104: 19613–19618

Money, N.P. 1999. Biophysics: fungus punches its way in. Nature. 401: 332-333.

Money, N.P. and Howard, R.J. 1996. Confirmation of a link between fungal pigmentation, turgor pressure and pathogenicity using a new method of turgor measurement. Fungal Genet. Biol. 20: 217–227.

Morgan, W. and Kamoun, S. 2007. RXLR effectors of plant pathogenic oomycetes. Curr. Opin. Microbiol. 10: 332–338

Mosquera, G., Giraldo, M. C., Khang, C. H., Coughlan, S. and Valent, B. 2009. Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. Plant Cell. 4: 1273–1290.

Narusaka, Y., Narusaka, M., Park, P., Kubo, Y., Hirayama, T., Seki, M., Shiraishi, T., Ishida, J., Nakashima, M., Enju, A., Sakurai, T., Satou, M., Kobayashi, M., and Shinozak, K. 2004. RCH1, a Locus in *Arabidopsis* that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. Mol. Plant-Microbe Interact. 17: 749–762.

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M. and Narusaka, Y. 2009. RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. Plant J. 60: 218–226.

Newman, M.A., Dow, J.M., Molinaro, A., Parrilli, M. 2007. Invited review: priming, induction and modulation of plant defence responses by bacterial lipopolysaccharides. J. Endotoxin. Res. 13:69-84.

Nurnberger, T., Brunner, F., Kemmerling, B., and Piater, L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198: 249–266.

O'Connell, R.J. et al. 2000. Dissecting the cell biology of *Colletotrichum* infection processes. In Colletotrichum: Host specificity, Pathology, and Host–Pathogen Interaction (Prusky, D. et al., eds), pp. 57–77, APS Press

O'Connell, R. J., Herbert, C., Sreenivasaprasad, S., Khatib, M., Esquerré-Tugayé, M.-T., Dumas, B. 2004. A novel *Arabidopsis-Colletotrichum* pathosystem for the molecular dissection of plant-fungal interactions. Mol. Plant-Microb Interact. 17: 272-282.

Ottmann, C., Luberacki, B., Kuefner, I, Koch, W., Brunner, F., Weyand, M., Mattinen, L., Pirhonen, M., Anderluh, G., Seitz, H. U., Nuernberger, T. and Oecking, C. 2009. A common toxin fold mediates microbial attack and plant defense. Proc. Natl. Acad. Sci. USA. 106: 10359-10364.

Pemberton, C.L., Salmond, G.P. 2004. The Nep1-like proteins: a growing family of microbial elicitors of plant necrosis. Mol. Plant Path. 5: 353–359.

Perfect, S. E., O´Connell, R. J., Green, E.F., Doering, C., Green, J.R. 1998. Expression cloning of a fungal proline rich glycoprotein specific to the biotrophic interface formed in the *Colletotrichum*-bean interaction. Plant J. 15: 273-279.

Pitzschke, A., Schikora, A. and Hirt, H. 2009. MAPK cascade signalling networks in plant defence. Curr. Opin. Plant Biol. 12: 421–426.

Podila, G.K., Rogers, L.M. and Kolattukudy, P. E. 1993. Chemical Signals from Avocado Surface Wax Trigger Germination and Appressorium Formation in *Colletotrichum gloeosporioides* Plant Physiol. 103: 267-272.

Qutob, D., Kamoun, S. and Gijzen, M. 2002. Expression of a *Phytophora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant J. 32: 361-373.

Qutob, D., Kemmerling, B., Brunner, F., Kuefner, 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 Nuernberger, T. (2006). Phytotoxicity and innate immune responses induced by Nep1-like proteins. Plant Cell. 18: 3721-3744.

Rafiqi, M., Gan, P. H.P., Ravensdale, M., Lawrenceb, G.J., Ellis, J., Jones, D.A., Hardham, A.R., and Doddsb, P.N. 2010. Internalization of flax rust avirulence Proteins into flax and tobacco cells can occur in the absence of the pathogen. Plant Cell. 22: 2017–2032.

Rep, M., Meijer, M., Houterman, P. M., Van Der Does, H. C., Cornelissen, B. J. C. 2005. *Fusarium oxysporum* evades I-3-mediated resistance without altering the matching avirulence gene. Mol. Plant-Microbe Interact. 18:15–23.

Reymond, P., and Farmer, E.E. 1998. Jasmonate and salicylate as global signals for defense gene expression. Curr. Opin Plant Biol. 1: 404–411.

Ridout, C. J., Skamnioti, P., Porritt, O., Sacristan, S., Jones J. D. G. and Brown, J. K. M. 2006. Multiple avirulence paralogues in cereal powdery mildew fungi may contribute to parasite fitness and defeat of plant resistance. Plant Cell. 18: 2402-2414.

Rizhsky, L., Shulaev, V. and Mittler, R. 2004. Measuring programmed cell death in plants. In: Apoptosis Methods and Protocols Methods in Molecular Biology. Brady, J.H.M Institute of Child Health,University College London,London, UK Humana Press. pp. 179-189.

Roetschi, A., Si-Ammour, A., Belbahri, L., Mauch, F., and Mauch-Mani, B. 2001. Characterization of an Arabidopsis-Phytophthora pathosystem: Resistance requires a functional PAD2 gene and is independent of salicylic acid, ethylene and jasmonic acid signalling. Plant J. 28:293-305.

Rooney, H.C., Van't Klooster, J.W., van der Hoorn, R.A., Joosten, M.H., Jones, J.D., and de Wit, P.J. 2005. *Cladosporium* Avr2 inhibitstomato Rcr3 protease required for Cf-2-dependent disease resistance. Science. 308: 1783-1786.

Rose, J.K., Ham, K.S., Darvill, A.G., Albersheim, P. 2002. Molecular cloning and characterizationof glucanase inhibitor proteins: coevolution of a counter defense mechanism by plant pathogens. Plant Cell. 14: 1329-45.

Rose, L.E., Bittner-Eddy, P.D., Langley, C.H., Holub, E.B., Michelmore, R.W., and Beynon, J.L. 2004. The maintenance of extreme amino acid diversity at the disease resistance gene, RPP13, in *Arabidopsis thaliana*. Genetics. 166: 1517-1527.

Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y. Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. 2010. Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. Plant J. 64: 204-214.

Sohn, K. H., Lei, R., Nemri, A. and Jones, J. D. G. 2007. The downy mildew effector proteins ATR1 and ATR13 promote disease susceptibility in *Arabidopsis thaliana*. Plant Cell. 19: 4077-4090.

Sohn, K.H., Zhang, Y. and Jonathan Jones, D.G. 2009. The *Pseudomonas syringae* effector protein, AvrRPS4, requires in planta processing and the KRVY domain to function. Plant J. 57: 1079-1091.

Stephenson, S. A., Hatfield, J., Rusu, A. G., Maclean, D. J., Manners, J. M. 2000. CgDN3: an essential pathogenicity gene of *Colletotrichum gloeosporioides* necessary to avert a hypersensitive-like response in the host *Stylosanthes guianensis*. Mol. Plant-Microb. Interact. 13: 929-941.

Stergiopoulos, I. and de Wit, P. J. G. M. 2009. Fungal effector proteins. Annu. Rev. Phytopathol. 47: 233-263.

Takahara, H., Dolf, A., Endl, E., O'Connell, R. 2009. Flow cytometric purification of *Colletotrichum higginsianum* biotrophic hyphae from *Arabidopsis* leaves for stage-specific transcriptome analysis. Plant J. 59: 672–683.

Takken, F., Rep, M. 2010. The arms race between tomato and *Fusarium oxysporum*. Mol. Plant Pathol. 11: 309-314.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D. A., Jia, Y., Martin, G.B. Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase. 1996. Science. 274: 2060-2062.

Thara, V.K., Fellers, J. P. and Zhou, J.M. 2003. In planta induced genes *of Puccinia triticina* 2003. Mol. Plant. Path. 4: 51–56.

Thilmony, R., Underwood, W., He, S. Y. 2006. Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. Plant J. 46: 34-53.

Thomas, W.J., Thireault, C. A., Kimbrel, J.A. and Chang, J.F. 2009. Recombineering and stable integration of the *Pseudomonas syringae* pv. *syringae* 61 hrp/hrc cluster into the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-1. Plant J. 60: 919–928.

Thomma, B. P. H. J., Nelissen, I., Eggermont, K., and Broekaert, W. F. 1999. Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicola*. Plant J. 19:163-171.

Tu, J.C. 1985. An improved Mathur's medium for growth, sporulation, and germination of spores of *Colletotrichum lindemuthianum*. Microbios. 44: 87-93.

Ushimaru, T., Terada, H., Tsuboi, K., Kogou, Y., Sakaguchi, A., Tsuji, G., and Kubo, Y. 2010. Development of an efficient gene targeting system in *Colletotrichum higginsianum* using a non-homologous end-joining mutant and *Agrobacterium tumefaciens*-mediated gene transfer. Mol Genet and Genomics. 284: 357-371.

Van den Burg, H. A., Westerink, N., Francoijs, K. J., Roth, R., Woestenenk, E., et al. 2003. Natural disulfide bond-disrupted mutants of AVR4 of the tomato pathogen *Cladosporium fulvum* are sensitive to proteolysis, circumvent Cf-4-mediated resistance, but retain their chitin binding ability. J. Biol. Chem. 278: 27340-27346.

Van Den Burg, H. A., Harrison, S. J., Joosten, M. H. A. J., Vervoort, J., DeWit, P. J. G. M. 2006. *Cladosporium fulvum* Avr4 protects fungal cell walls against hydrolysis by plant chitinases accumulating during infection. Mol. Plant-Microbe Interact. 19:1420–30

Van den Hooven, H. W., van den Burg, H. A., Vossen, P., Boeren, S., de Wit, P. J. G. M. and Vervoort, J. 2001. Disulfide bond structure of the AVR9 elicitor of the fungal tomato pathogen *Cladosporium fulvum*: evidence for a cystine knot. Biochemistry-US. 40: 3458-3466.

Van der Hoorn, R. and Kamoun, S. 2008. From guard to decoy: A new model for perception of plant pathogen effectors. Plant Cell .20: 2009–2017.

Van Esse, H. P., Fradin, E., de Groot, P., de Wit, P., and Thomma, B. 2009. Tomato transcriptional responses to a foliar and a vascular fungal pathogen are distinct. 2009. Mol. Plant-Microbe Interact. 22: 245–258.

Van Kan, J. A. L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 11: 247-253.

Viaud, M. C., Balhadère, P.V. and Talbot, N.J. 2002. A *Magnaporthe grisea* cyclophilin acts as a virulence determinant during plant infection. 2002. Plant Cell. 14: 917–930.

Veneault-Fourrey, C., Laugé, R. and Langin, T. 2005. Nonpathogenic strains of *Colletotrichum lindemuthianum* trigger progressive bean defense responses during appressorium-mediated penetration. Appl. Environ. Microb. 71: 4761-4770.

Voegele, R.T. and Mendgen, K.W. 2011. Nutrient uptake in rust fungi: how sweet is parasitic life? Euphytica 179: 41–55.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33: 949–956.

Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I. K., Pritchard, L. and Birch, P. R. J. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature. 450: 115-118.

Yaenoa, T., Lib, h., Chaparro-Garcia, A., Schornackc, S., Koshibab, S., Watanabe, S., Kigawa, T., Kamoun, S., and Shirasu, K. 2011. Phosphatidylinositol monophosphate-binding interface in the oomycete RXLR effector AVR3a is required for its stability in host cells to modulate plant immunity. Proc. Natl. Acad. Sci. USA 108: 14381-14382.

Zipfel, C., and Felix, G. 2005. Plants and animals: a different taste for microbes? Curr. Opin. Plant Biol. 8: 353–360.