Unveiling and exploiting *Phytophthora capsici* effectors and their host targets, with an emphasis on CRN effector proteins

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List of Abbreviations

3-AT	3-Amino-1,2,4-Triazole
aa	amino acid
ANOVA	ANalysis Of VAriance
ATP	Adenosine TriPhosphate
AVR	AViRulence
BAK1	BRI1-Associated receptor Kinase 1
BiFC	Bimolecular Fluorescence Complementation
BIK1	*Botrytis*-Induced kinase 1
BLAST	Basic Local Alignment Search Tool
Cas9	CRISPR associated protein 9
CC	Coiled-Coil
CD	Cell Death
cDNA	coding DNA
CDPK	Calcium-Dependent Protein Kinase
CF	Culture Filtrate
CMPG1	Cys, Met, Pro, and Gly protein 1
CNL	CC-NB-LRR
Co-IP	Co-ImmunoPrecipitation
Col-0	Columbia-0
CR	Crinkler-RHS-type
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRN	CRinkling and Necrosis
CRT	Compromised for Recognition of TCV
CSP	Cold Shock Protein
DAMP	Damage-Associated Molecular Pattern
DCL	DiCer-Like
DDR	DNA Damage Response
DEG	Differential Expressed Gene

DIC	Differential Interference Contrast
DNA	DeoxyriboNucleic Acid
DPI	Days Post-Infiltration
DTT	DiThioThreitol
DUF	Domain of Unknown Function
EDTA	Ethylene Diamine Triacetic Acid
EFR	EF-Tu receptor
EF-Tu	Elongation Factor Thermo unstable
e.g.	*exempli gratia*
EGF	Epidermal Growth Factor
eGFP	enhanced GFP
EIX	Ethylene-inducing Xylanase
ELR	ELicitin Response
EPIC	Extracellular Cystatin-like Protease Inhibitor
ERCC1	Excision Repair Cross-Complementation group 1
EST	Expressed Sequence Tag
ETI	Effector-Triggered Immunity
ETS	Effector Triggered Susceptibility
EV	Empty Vector
Flg22	22 amino acid motif from bacterial Flagellin
FLIM	Fluorescence Lifetime IMaging
FLS2	Flagellin Sensing 2
FRET	Förster Resonance Energy Transfer
GAL4	GALactosidase 4
GAS1	Glycolipid Anchored Surface protein 1
GC	Germinating Cysts
GFP	Green Fluorescent Protein
GTPase	Guanosine Triphosphate phosphohydrolase
h	hours
H2B	Histone 2B

HhH	Helix-hairpin-Helix
HMM	Hidden Markov Model
HMP1	Haustorial Membrane Protein 1
HR	Hypersensitive Response
HRP	Horse Radish Peroxidase
HRT	HR to TCV
HSE	Heat Shock Element
Hsf	Heat shock factor
HSP	Heat Shock Protein
HTH	Helix-Turn-Helix
i.e.	*id est* (that is)
INF1	INFestin 1
iprDesc	InterPro protein domain Description
LB	Lysogeny Broth
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LFQ	Label-Free Quantification
LK	LimKain b1
LPS	LipoPolySaccharide
LRE	Luciferin-Regenerating Enzyme
LRR	Leucine-Rich Repeat
LSM	Laser Scanning Microscopy
MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen-Activated Protein Kinase
Mcs4	Mitotic catastrophe suppressor 4
MES	2-(N-Morpholino) Ethane Sulfonic acid
MIZ	Msx-Interacting Zinc finger
MPTase	Mitochondrial Protein Translocase
mRFP	monomeric RFP
MUSCLE	MUltiple Sequence Comparison by Log- Expectation
MYB	MYeloBlastosis

Myc	Mycelia
NB(S)	Nucleotide-Binding (Site)
NCD	No Cell Death
NES	Nuclear Exclusion Signal
NLP	Nep1-like effector
NLS	Nuclear Localisation Signals
NPC	Nuclear Pore Complex
NTPase	Nucleoside TriPhosphate hydrolase
OD	Optical Density
OGA	OligoGAlacturonide
OTU	Ovarian TUmour
PAMP	Pathogen-Associated Molecular Pattern
PB	Pea Broth
PCR	Polymerase Chain Reaction
PEP	Plant Elicitor Peptide
PEPR	PEP Receptor
PFAM	Protein FAMilies
P-loop	Phosphate-binding loop
PROPEP	PROPEPtides
PRR	Pattern Recognition Receptor
PSIBLAST	Position-Specific Iterative Blast
PTI	Pattern-Triggered Immunity
PTM	Post-translational modification
pv	pathovar
PVDF	PolyVinylidene DiFluoride
qRT-PCR	quantitative Reverse-Transcriptase PCR
R	Resistance
Ras	Rat sarcoma
REase	Restriction Endonuclease
RFP	Red Fluorescent Protein

RHSP	Retrotransposon Hot Spot Protein
RIN4	RPM-INteracting protein 4
RK	Receptor Kinase
RLK	Receptor-Like Kinase
RLP	Receptor-Like Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
RPM1	Resistance to *Pseudomonas syringae* pv Maculicola 1
RuBisCO	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
SA	Salicylic Acid
SAP	SAF-A/B, Acinus and PIAS
SCP	Sperm-Coating Protein
Sgs1	Slow growth suppressor 1
SiRNA	Small interfering RNA
SIZ	SAP and MIZ domains
SlERF4	*Solanum lycopersicum* Ethylene Responsive transcription factor 4
SLX	Synthetic Lethal of unknown function
SMP	Senescence Marker Protein
SP	Signal Peptide
Spor	Sporangia/zoospore
sRNA	small RNA
SSK1	Suppressor of Sensor Kinase 1
SUMO	Small Ubiquitin-like MOdifier
T3SS	Type III Secretion System
TBS	Tris-Buffered Saline
TCP	Teosinte branched 1, Cycloidea, Proliferating cell
TCV	Turnip Crinkle Virus
TDS	Total dissolved solids
TFA	TriFluoro Acetic acid

TGX	Tris-Glycine eXtended
TIR	Toll/Interleukin-1 Receptor
TNL	TIR-NB-LRR
TRV	Tobacco rattle virus
UBC9	Ubiquitin Carrier Protein 9
Ubl	Ubiquitin-like
Ulp1	Ubiquitin-like protease 1
USA	United States of America
VIGS	Virus-Induced Gene Silencing
WAK1	Wall-Associated Kinase 1
WT	Wild Type
Y2H	Yeast Two-Hybrid
YFP	Yellow Fluorescent Protein
Zn	Zinc

List of Publications (not associated with this thesis)

Motion, G. B.*, **Amaro, T. M. M. M.***, Kulagina, N., and Huitema, E. (2015). Nuclear processes associated with plant immunity and pathogen susceptibility. *Brief. Funct. Genomics* 14, 243–52. doi:10.1093/bfgp/elv013. *Shared first authors

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Declarations

The results presented in this PhD thesis are from investigations conducted by myself. Any work that is not my own is clearly identified with the appropriate references and publications. I hereby declare that I am the author of this work and it has not been previously accepted for any higher degree.

Tiago Miguel Marques Monteiro Amaro

We certify that Tiago Amaro has fulfilled the relevant ordinance and regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

Dr. Edgar Huitema, School of Life Sciences, University of Dundee

Summary

Phytophthora capsici is a devastating plant pathogen for which virulence is aided by the secretion of effectors, including cytoplasmic effectors from the CRN (CRinkling and Necrosis) family.

CRN effectors were first described in oomycetes by their capacity to induce host cell death. Nevertheless, despite recent efforts aiming to elucidate CRN virulence functions, the virulence relevance of CRN mediated cell death remains unknown. In this thesis, by performing a PCR based random mutagenesis screen on *P. capsici* CRN effector PcCRN83_152, we showed that PcCRN83_152 cell death is not required for its virulence function. In addition, we demonstrated that PcCRN83_152 interacts with nuclear proteins from the host plants *N. benthamiana* (NbSIZ1 and NbSLX1) and tomato (SlSIZ1Δ867), which we connected to plant immunity processes and PcCRN83_152 mediated phenotypes.

Besides increasing our knowledge on *P. capsici* CRN effectors, in this thesis we also aimed for the identification of new *P. capsici* effectors. Using a proteomics-based approach, we identified a set of candidate effectors from *P. capsici* that would not be identified using conventional genomic- and transcriptomic-based studies.

In sum, in this thesis we summarise our current understanding of CRN effectors and re-assess some basic assumptions regarding this protein family. Moreover our results pointing to CRN virulence functions independent of cell death phenotypes provide a new conceptual framework for studies aimed at unveiling the virulence functions of cell death inducing CRNs. This knowledge

complemented with the identification of PcCRN83_152 plant targets provides great leads to uncover PcCRN83_152 virulence functions. Moreover, the identification of new candidate effectors form *P. capsici* could be important towards a more global understanding of *P. capsici* virulence mechanisms.

Chapter 1. General introduction

Plant pathogens hamper crop production worldwide

The advent of agriculture is one of the most important developments that drove the evolution of human culture. The establishment of agriculture led to sedentism (a transition from a nomadic population being placed into more permanent settlements) and consequently, to investments in architecture and growth of community population sizes (Fuller, 2010). Even today, agriculture remains a crucial pillar upon which the existence of modern human societies rests. However, global population growth has placed greater demands on the food supply whilst yields are under significant pressure, diminishing access to affordable food sources.

In the last 50 years, the human population more than doubled while cultivated land area only increased by 30%. Fortunately, most of the developing world has overcome chronic food deficiency as the direct beneficiary of the green revolution (1966-1985). This revolution massively enhanced crop yields by a combination of investment on crop research, and improvements on agriculture practices and infrastructures (Pingali, 2012). Nonetheless, predictions for the year 2050 point to an increase of the world population to 9.3 billion people. Moreover, population income is predicted to increase, what is usually coupled

with a less sustainable diet favouring consumption of livestock over cereals. Land competition for biofuel production and the uncertainty brought up by climate change are also part of an increasingly complex situation, negatively affecting the food supply (Fischer et al., 2014; Newbery et al., 2016).

Today, the widely held view is that new food security challenges are rising. One major issue on food security is connected with crop losses caused by plant pests such as weeds, microbial pathogens and animal parasites. These pests are responsible for significant crop losses that can lead to, for example, 31% of losses in maize and 50% in cotton production (Oerke, 2006). Plant pathogens account for 16% of these losses although these values depend greatly on the crop and on the control measures used (Oerke, 2006).

There are several examples of crop pathogens that hampered and still threaten food supplies worldwide. A famous example is *Phytophthora infestans* that caused the Irish potato famine in the late 1840's. This pathogen caused 600,000 deaths in Ireland and prompted the emigration of over one million people in a five year period, from 1846 to 1851 (Zadoks, 2008).

Another famous example is the fungal pathogen *Fusarium oxysporum,* responsible for Panama disease epidemics that completely destroyed banana plantations of the Gros Michel cultivar in the 1950s (Ploetz, 2000). Even though the resistant cultivar "Cavendish" was successfully deployed and used for decades, new *Fusarium oxysporum* races have started to emerge in the 1990's, able to break Cavendish resistance and thereby renewing this threat to banana production. These developments have prompted the need for new and more durable resistance against this pathogen (Ploetz, 2015).

Another pathogenic fungus, *Magnaporthe oryzae*, is considered of great economic importance and consequently, is a pathogenic microbe whose biology has been intensely studied. *M. oryzae* causes rice blast (Dean et al., 2012), a disease that has persisted for decades despite significant effort focussed on the deployment of genetically encoded resistances through conventional breeding approaches (Miah et al., 2013). Given that rice is the most consumed food crop, *M. oryzae* incited losses continue to create great problems, directly affecting more than half of the world's population (Khush, 2005).

In addition to their immense impact on food security, plant pathogens also are a menace to natural ecosystems. For example, pathogens of trees have a high environmental impact as forests harbour more than 50% of terrestrial biodiversity (Neale and Kremer, 2011). For instance, *Phytophthora cinnamomi* causes severe diseases in chestnut (*Castanea dentata*) and it was connected to causing root rot and decline in the South African shrub forest fynbos (von Broembsen and Kruger, 1985; Burgess et al., 2016). Another example is *Phytophthora ramorum* which is causing destruction in evergreen oak, tanoak and larches around the world (Brasier and Webber, 2010). It is therefore clear that collectively, pathogens form a grave threat to food production systems and our natural environment.

Plant Immune system

As we can see by the examples above, plants need to defend themselves against a variety of would be microbes, implying the presence of an immune system. Initial efforts to study plant immunity concluded that plants are capable

of recognising pathogens and initiating a defence response called hypersensitive response (HR) (Mur et al., 2008; Stakman, 1915).

H. M. Ward showed that during infection of wheat cultivars by leaf rust (*Puccinia dispersa*) different outcomes were observed. In a successful infection, vigorous fungal growth was achieved without serious injury to the host, while in other cases, the fungus was thought to induce host cell death whilst growing very little (Ward, 1902). E. C. Gibson noted that the rust fungus (*Puccinia chrysanthemi*) was able to penetrate and gain access to practically every plant tested. Notwithstanding, and importantly, it was observed that after entry, infection often resulted in an incompatible interaction where the fungus was unable to grow and thrive, a finding which was found to be connected to the presence of localised host cell death (Gibson, 1904). Similar results were obtained by D. Marryat in the wheat–*Puccinia glumarum* (leaf yellow rust) pathosystem (Marryat, 1907).

An appreciation for cell death and its connection to plant resistance arose from the work of E. C. Stakman, investigating the responses of various cereal crops to the black stem rust fungus (*Puccinia graminis*) (Stakman, 1915). He also observed cell death upon pathogen infection and subsequently defined HR as “abnormally rapid death of the host plant cells when attacked by rust hyphae. It is used in this sense without any implication as to the exact physiological nature of the phenomenon, referring, therefore, only to the facts substantiated by visual evidence” (Stakman, 1915).

Perhaps one of the most important discoveries in the plant-microbe interactions field was the observation that resistance and susceptible host-pathogen interactions have a genetic basis. Studies conducted by Harrold Flor on the

inheritance of pathogenicity and avirulence in the flax rust fungus (*Melampsora lini*), suggested that for each dominant gene conferring resistance in the plant there is a corresponding dominant gene in the parasite that conditions avirulence, forming the gene-for-gene hypothesis (Flor, 1955, 1971). These findings implied both host- and pathogen-encoded factors that determine interaction outcomes. After the formulation of this hypothesis great efforts have been undertaken to identify and characterise these gene pairs culminating on the cloning of the first pathogen gene that conditions avirulence or incompatibility (Staskawicz et al., 1984) and the first plant resistance gene (R gene) (Martin et al., 1993).

These crucial findings on the nature of HR, the gene-for-gene hypothesis, and on avirulence as well as R genes, drove huge efforts on characterising the plant immune system and the mechanisms deployed by microbes to overcome it, resulting in the recent and exciting developments I will now describe.

Plants recognise microbe conserved patterns to mount defence responses

When trying to infect plants, microbes first have to overcome physical and chemical barriers deployed by the plant. These include waxy cuticular layers, strong cell walls and the deployment of anti-microbial compounds (Dangl and Jones, 2001; Malinovsky et al., 2014). Perhaps not surprisingly, some microbes have evolved factors (e.g. lytic enzymes, detoxification enzymes, inhibitors) that are able to degrade these physical barriers and allow microbial access to host tissues or cells (Misas-Villamil and van der Hoorn, 2008).

To counter a vast number of potential microbial parasites able to compromise physiological barriers, plants have evolved an adaptive immune system, capable of mounting finely tuned responses to a given biotic threat. In a first layer of adaptable immunity, plants detect Microbe, Pathogen or Damage-Associated Molecular Patterns (MAMPs, PAMPs or DAMPs), triggering what is generally called Pattern-triggered immunity (PTI) (Bigeard et al., 2015; Boller and Felix, 2009; Derevnina et al., 2016; Jones and Dangl, 2006; Muthamilarasan and Prasad, 2013; Zhang and Zhou, 2010). PAMPs are considered to be small, essential and conserved molecules that are associated with microbes and are in effect, non-self immunogens from a plant perspective. However, given that PAMPs occur in non-pathogenic microbes the term PAMP is a misnomer and it was suggested that the term MAMP should be used instead (Ausubel, 2005; Mackey and McFall, 2006). Notwithstanding, despite acknowledging the reasons for this differentiation, and as we are studying plant pathogens, here the term PAMP will be used. Classical examples of PAMPs include peptidoglycans and lipopolysaccharides from bacteria cell wall envelopes, a peptide from bacterial flagellin (flg22), chitin from fungal cell walls as well as glucans and glycoproteins from oomycetes (Boller and Felix, 2009; Nurnberger et al., 2004; Raaymakers and Ackerveken, 2016).

The recognition of PAMPs, which by definition precedes PTI, is performed by plant proteins named pattern recognition receptors (PRRs) (Medzhitov and Janeway, 1997; Nicaise et al., 2009). All plant PRRs identified to date are plasma membrane-associated receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Both RLKs and RLPs contain an extracellular domain for ligand binding and a single transmembrane domain, anchoring these proteins in the host cell membrane. The difference between RLKs and RLPs remains on

the presence of a cytosolic kinase domain on RLKs (Macho and Zipfel, 2014; Trdá et al., 2015). Interestingly, while some PAMPs, as bacterial flagellin, induce responses in a wide range of plant species, perception of other PAMPs, such as the bacterial cold shock protein (CSP) and elongation factor (EF-Tu) seem to be restricted to the orders of Solanales and Brassicales, respectively, reflecting possibly a differential presence of particular PRRs (Thomma et al., 2011; Zipfel et al., 2006). This raises the possibility of engineering plant resistance by interfamily transfer of PRRs (Dodds and Rathjen, 2010). For instance, expression of EFR, the *Arabidopsis* receptor of EF-Tu (Zipfel et al., 2006), in tomato and *Nicotiana benthamiana* turns these plants more resistant against various bacterial pathogens (Lacombe et al., 2010).

PAMP/PRR signalling complexes have been intensively studied (Trdá et al., 2015). One of the best characterised PAMP/PRR combinations is the flg22/FLS2 pair, which features the recognition of flg22, a conserved 22 amino acid motif of bacterial flagellin, by the RLK FLS2, a PRR initially identified in *Arabidopsis thaliana* (Felix et al., 1999; Gómez-Gómez and Boller, 2002). The binding of flg22 triggers FLS2 association with a leucine-rich repeat receptor-like kinase (LRR-RLK) protein named BAK1, that has been shown to bind other PRRs and to be a major component of plant immunity (Chinchilla et al., 2007; Roux et al., 2011; Schulze et al., 2010). Flg22 presence was also shown to induce the dissociation of FLS2 from the receptor-like cytoplasmic kinase BIK1 (botrytis-induced kinase 1). BIK1 was shown to be phosphorylated upon flagellin perception and to mediate the phosphorylation of both FLS2 and BAK1 in a process required for the propagation of flagellin induced defence signalling (Lu et al., 2010; Zhang et al., 2010). Downstream of FLS2-BAK1 complex formation, mitogen-activated protein kinase (MAPK) (Asai et al., 2002) and

calcium-dependent protein kinase (CDPK) cascades (Boudsocq et al., 2010) rapidly translate flg22 perception into various defence responses such as oxidative burst, callose deposition, ethylene production and global transcriptional changes involving WRKY transcription factors (Asai et al., 2002; Boller and Felix, 2009; Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2002; Robatzek and Wirthmueller, 2013). After elicitation, FLS2 is internalized into endosomal vesicles and degraded in a process involved in the regulation of flagellin mediated signalling (Beck et al., 2012; Robatzek et al., 2006).

In oomycetes, the best studied PAMPs are proteins from the elicitin family, with the *P. infestans* elicitin INF1 being the best known member (Kamoun et al., 1997). Elicitins are thought to work on mediating sterol uptake in *Phytophthora* as they harbour sterol carrier activity and *Phytophthora* species are unable to synthesise sterols (Derevnina et al., 2016; Ponchet et al., 1999). INF1, when present in plants, induces the accumulation of reactive oxygen species (ROS) and plant cell death (Chaparro-Garcia et al., 2011; Kamoun et al., 1997). In tomato, INF1 was shown to activate jasmonic acid and ethylene mediated defence signalling pathways and to induce resistance to infections of *Ralstonia solanacearum* (the cause of bacterial wilt disease in tomato) (Kawamura et al., 2009). The recognition of INF1 and other oomycete elicitins was recently shown to be mediated in potato by the receptor-like protein ELR in a BAK1 dependent manner (Chaparro-Garcia et al., 2011; Du et al., 2015). Importantly, potato transgenic plants over-expressing ELR showed enhanced resistance to two *P. infestans* strains, suggesting that deployment of PRRs targeting elicitins may aid current resistance breeding efforts (Du et al., 2015)

Besides the detection of PAMPs, the detection of DAMPs is also though to lead to PTI-like responses. In contrast to PAMPs, DAMPs are endogenous host molecules that originate as the result of mechanical and cellular damage associated with pathogen ingress (Boller and Felix, 2009; Choi and Klessig, 2016). Classic examples of DAMPs are plant cell wall fragments released by microbial enzymes as for instance the oligogalacturonides (OGAs). OGAs are produced by incomplete digestion of plant cell wall pectin and were shown to be recognised by the *Arabidopsis* RLK named WAK1 (Brutus et al., 2010). Moreover, *Arabidopsis* plants with increased levels of OGAs displayed increased resistance to the plant pathogens *Botrytis cinerea*, *Pectobacterium carotovorum* and *Pseudomonas syringae* (Benedetti et al., 2015).

Plant elicitor peptides (PEPs) form also a relatively well-studied family of DAMPs. These peptides originate from pro-peptides (PROPEPs), whose expression is induced by wounding or PAMP perception (Bartels et al., 2013; Huffaker et al., 2006). The *Arabidopsis* LRR-RK receptors PEPR1 and PEPR2 recognise these peptides (Krol et al., 2010) and initiate BAK1 dependent signalling cascades akin to those triggered by PAMP perception (Krol et al., 2010; Lori et al., 2015; Schulze et al., 2010). Nevertheless, while PAMP and DAMP perception and transduction seem dependent on conserved signalling pathways and share defence outcomes, PEPs were shown to enhance plant immunity in *Arabidopsis bak1*-knockout lines, compromised in PTI signalling. These results point to the existence of additional and hitherto unknown events that help initiate PEP induced defence signalling (Yamada et al., 2016).

Pathogen Effectors supress plant immunity

By definition, a successful plant pathogen needs to overcome basal defences activated upon PAMP perception. It is believed that pathogens achieve this by the secretion of effector molecules. Effectors are defined as secreted molecules that alter host-cell processes in order to promote the microbe lifestyle(Hogenhout et al., 2009; Kamoun, 2007; Win et al., 2012). Effectors have been the subject of several studies (Asai and Shirasu, 2015; Bozkurt et al., 2012; Kay and Bonas, 2009; Oliveira-Garcia and Valent, 2015; Selin et al., 2016), however in this section I focus on examples of effectors that work on modulating plant immunity, namely on the suppression of PTI responses.

Several effectors from a diverse range of plant pathogens have been shown to aid pathogen virulence by targeting various stages of the PTI signalling process (Bigeard et al., 2015; Oliveira-Garcia and Valent, 2015; Zheng et al., 2014). For instance, despite not sharing any sequence similarity, the effectors AvrPto and AvrPtoB from *Pseudomonas syringae* are both capable of blocking PTI responses by interfering with the kinase activities of plant immunity related RLKs, including FLS2 and BAK1 (Cheng et al., 2011; Gravino et al., 2016; Xiang et al., 2008). Downstream of PAMP recognition, the *Xanthomonas euvesicatoria* effector XopD also works, supressing PTI responses. XopD is a SUMO protease that works in the plant nucleus on supressing the transcription of defence related genes by deSUMOylating the tomato transcription factor SlERF4. SlERF4 deSUMOylation leads to a decrease on SlERF4 levels and a consequent inhibition of the expression of ethylene related defence genes under SlERF4 control (Kim et al., 2013). The fact that pathogen encoded effectors play such crucial roles on supressing PTI responses demonstrates the threat these responses pose to pathogen ability to infect and reproduce.

Moreover, it demonstrates the need for an in-depth understanding of effector functions when aiming to enhance plant resistance against plant pathogenic microbes.

Plants recognise pathogen effectors, or their activities, to enhance plant resistance

In the fight against pathogenic microbes, plants have evolved the capacity to detect effectors or their activities, often leading to HR responses and an immunity state named effector-triggered immunity (ETI) (Jones and Dangl, 2006). The genes responsible for these recognition events are named R genes and mostly encode for cytoplasmic nucleotide-binding and leucine-rich repeat (NB-LRR) proteins (Flor, 1971; Lee and Yeom, 2015). Most NB-LRRs can be classified according to their N-terminal domains into: Toll/interleukin-1 receptor (TIR)–NB-LRRs (TNLs) and coiled-coil (CC)-NB-LRRs (CNLs) (McHale et al., 2006). Both N-terminal domains (TIR and CC) are thought to be involved in the formation of homo-dimers, a process that is essential for the activation of plant defence responses (Takken and Goverse, 2012). The C-terminal LRR domain is proposed to adopt an arc-shaped confirmation involved in protein-protein interactions. LRR domains are believed to work on mediating the recognition of pathogen effectors or their functions (Lee and Yeom, 2015; Lukasik and Takken, 2009; Padmanabhan et al., 2009).

To date, several R proteins have been identified and shown able to detect specific pathogen effectors (Dangl and Jones, 2001; van Ooijen et al., 2007). One famous example is the potato R protein named R3a (Huang et al., 2005). R3a is capable of recognising the *P. infestans* effector Avr3A. Interestingly

Avr3a has two isoforms (Avr3aKI and Avr3aEM), but R3a is only capable of recognising the Avr3aKI isoform (Armstrong et al., 2005). Nevertheless, both isoforms are able to suppress INF1 induced cell death, presumably through a direct interaction with the E3 ubiquitin ligase CMPG1, which results in its stabilisation (Bos et al., 2006, 2009, 2010).

Another well characterised R protein is CF4 from tomato that confers resistance against the biotrophic fungus *Claudosporium fulvum* by mediating the recognition of the effector Avr4 (Joosten et al., 1994; Thomas et al., 1997). CF4 is an extracellular membrane-anchored glycoprotein that recognises Avr4 in the apoplast where it acts on binding chitin from the fungal cell wall, shielding it against the action of host-derived chitinases (van den Burg et al., 2006; Kohler et al., 2016).

Notwithstanding these examples, the recognition of a single effector by a single R protein does not always occur following the gene-for-gene model developed by Harold Flor (Flor, 1971). Recently it was shown that R proteins work in pairs (Eitas and Dangl, 2010) that are able to recognise diverse pathogen effectors. These and other observations have led to the proposition of the guard hypothesis (Van Der Biezen and Jones, 1998). In this hypothesis, R proteins are thought to work by “guarding” specific host factors, triggering a defence response upon the detection of their modification. In this model, specific effector-mediated modifications trigger conformational changes in an associated R-protein, thereby initiating defence responses. Given that target modification rather than effector recognition underpins ETI, R proteins can condition resistance in the presence of diverse effectors on the basis of a specific but yet shared modification (Van Der Biezen and Jones, 1998; Dangl and Jones, 2001;

Marathe and Dinesh-Kumar, 2003). A good illustration of this model comes from the *Arabidopsis* protein RIN4. RIN4 was shown to interact with an *Arabidopsis* R protein RPM1 and with two unrelated *Pseudomonas syringae* effectors AvrRpm1 and AvrB (Mackey et al., 2002). AvrRPm1 and AvrB were shown to induce phosphorylation of RIN4 to supress basal plant defences. This modification of RIN4 is recognised by RPM1, leading to immunity in *Arabidopsis* (Mackey et al., 2002). Another *P. syringae* effector AvrRpt2 targets RIN4, mediating its degradation in a process which is recognised by the R protein RPS2 (Axtell et al., 2003; Mackey et al., 2003). Thus, RIN4 is a key host immunity protein that is targeted by multiple effectors and that is guarded by at least two R proteins.

The observation that R proteins were “guarding” effector mediated changes in the host gave rise to the decoy model to describe R protein functions (van der Hoorn and Kamoun, 2008). Importantly, the guard model predicts the existence of two opposing selective pressures. On the one hand, it would be beneficial for the plant to enhance binding of the effector to its host target, which would in turn enhance R protein mediated recognition. On the other hand, enhanced binding of a pathogen effector to its host target is also prejudicial for the plant as it could mean improved effector function. Thus, a model where plants would develop a decoy protein, still capable to bind the effector but with no importance for immunity, arose (van der Hoorn and Kamoun, 2008). AvrPto and AvrPtoB are two effectors from the plant pathogen *Pseudomonas syringae* that work on supressing PTI-associated kinases. While AvrPto blocks kinase activities by binding kinase catalytic clefts, AvrPtoB possesses an ubiquitin E3 ligase domain that mediates ubiquitination of the targeted kinases leading to their proteasome-mediated degradation (Ntoukakis et al., 2014). Both these effectors

are detected by the R protein Pto which encodes a serine/threonine protein kinase, which is capable of avoiding AvrPtoB-mediated proteasomal degradation by mediating the phosphorylation and consequent inactivation of its E3 ubiquitin ligase domain (Martin et al., 1993; Ntoukakis et al., 2009). In the presence of AvrPto and AvrPtoB, Pto forms a complex with the NB-LRR Prf leading to the activation of Prf-mediated ETI (Ntoukakis et al., 2013). While tomato transgenic lines stably expressing Pto featured enhanced resistance against *Pseudomonas syringae* pv. *tomato*, this resistance was shown to be completely dependent of Prf functions (Balmuth and Rathjen, 2007). Thus, it is clear that Pto constitutes a kinase decoy deployed by the plant to trap AvrPto and AvrPtoB effectors leading to subsequent ETI-associated responses (Ntoukakis et al., 2014).

The ZigZag model

Efforts to reconcile our understanding on the components and mechanisms that help determine plant-pathogen interaction outcomes with models describing host-pathogen co-evolution led to the development of the zigzag model (Jones and Dangl, 2006). This model asserts that plants are capable of mounting basal defences upon the recognition of PAMPs by pattern recognition receptors (PRRs). Recognition and subsequent signalling allows cells to activate cellular processes that heighten defence, collectively increasing immunity to would-be pathogens. This cellular reprogramming and basal defence is named PAMP triggered immunity (PTI) and provides a robust defence against the vast majority of microbial attacks. Pathogens evolved effectors that can work as suppressors of PTI responses leading to a susceptible state called “effector

triggered susceptibility" (ETS). However, plants, in this evolutionary battle, evolved another family of receptors, named resistance or R proteins, that are capable of recognising effectors and initiate the establishment of an enhanced immunity state called "effector triggered immunity" (ETI) (Jones and Dangl, 2006). While the zigzag model is a good way of illustrating the evolutionary fight occurring during plant-microbe interactions, this model does not fit or explain all the experimental knowledge acquired to date. The value of the zigzag theory is its ability to serve as an "expository" model, not possessing a predictive function on plant pathogenic outcome (Pritchard and Birch, 2014).

Some of the criticism around the zigzag model arises from the difficult distinction between some of its basic components, as the distinction between PAMPs and effectors and consequently between PTI and ETI is not always clear (Thomma et al., 2011). PAMPs are generally considered to be conserved pathogenic proteins that despite their importance for the pathogen do not modulate host processes. However, some considered PAMPs do not contain these characteristics. The PAMP PEP-13, a surface-exposed fragment of a cell wall transglutaminase, is only present in *Phytophthora* species (Brunner et al., 2002). In addition, bacterial lipopolysaccharide (LPS) envelopes from the symbiont *Sinorhizobium meliloti*, described as PAMPs in *Arabidopsis* (Zeidler et al., 2004), were shown to supress defence responses in *Medicago truncatula* (Tellstrom et al., 2006). In contrast, some effectors have PAMP characteristics as, for instance, the Nep1-like effectors (NLPs) that are conserved among bacteria, fungus and oomycetes (Gijzen and Nürnberger, 2006; Kamoun, 2006; Thomma et al., 2011). Also, the features that distinguish PTI and ETI are increasingly difficult to define. Being both responsible for upregulation of conserved defence responses, PTI is thought to be a weaker response, while

ETI is connected with HR induction (Jones and Dangl, 2006). However, bacterial flagellin, containing one of the best characterised PAMPs (flg22), was demonstrated to be able to cause HR in *Arabidopsis* (Naito et al., 2007).

A new model to describe plant-microbe interactions has been proposed and named the state-based model (Pritchard and Birch, 2011). This model is based on a systems biology approach and defines plant pathogenic interactions as a balance between healthy and disease states. A fine understanding of the immune networks that define these two states, could lead to a prediction of the outcome of any infection (Pritchard and Birch, 2011). However our knowledge of plant-pathogenic interactions, in particular on the networks that govern immunity and susceptibility, are insufficient for an effective use of the predictive capabilities of this model.

The Oomycete lineage

Oomycetes form a large family of eukaryotic organisms. Despite being phylogenetically more closely related to brown algae and diatoms, oomycetes were initially thought to be fungi due to their similar morphology and filamentous growth (Baldauf et al., 2000; Latijnhouwers et al., 2003; Simpson and Roger, 2004). However, common morphological characteristics do not extend on the biochemical and structural level. For instance, while chitin is the major component of fungal cell walls, oomycete cell walls are on their majority composed by cellulose (Fawke et al., 2015; Money et al., 2004; Richards et al., 2006). Moreover, the availability of genetic and genome level information in a wider range of organisms has enabled researchers to conclusively demonstrate that the oomycetes represent a distinct lineage of filamentous Eukaryotes.

Oomycetes can be found in a great variety of environments and geographic locations (Thines and Kamoun, 2010) and have been described mostly as organisms with a pathogenic lifestyle. Nevertheless, saprophytic aquatic oomycetes have been described (Riethmüller et al., 2006). Pathogenic oomycetes can infect a variety of hosts. For example, *Aphanomyces astaci* is the causal agent of the crayfish plague that has been devastating European species of freshwater crayfish in their natural environment (Edgerton et al., 2004; Phillips et al., 2008). In addition, the oomycete *Pythium insidiosum* is able to infect mammals, particularly horses, dogs and humans, in tropical and sub-tropical areas (Gaastra et al., 2010). Yet, most of oomycetes are plant pathogenic with some of them severely hampering crop production worldwide. Thus, most of the research on oomycetes has been focused on plant pathogenic species (Kamoun, 2003; Thines and Kamoun, 2010).

One of the most devastating and better studied genera of plant pathogenic oomycetes is the *Phytophthora* genera with more than 100 species identified (Kroon et al., 2012). As mentioned above, a famous example of a devastating *Phytophthora* pathogen is *Phytophthora infestans* that caused the Irish potato famine in the late 1840's (Zadoks, 2008). Despite having been identified as a pathogen over 150 years ago and the numerous studies focusing on its biology, many unanswered questions remain regarding *P. infestans* pathogenicity and no durable resistance has been achieved against this pathogen (Fry et al., 2015). Another example is *P. sojae*, the causal agent of soybean root rot. This pathogen is responsible for crop losses worth more than one billion dollars worldwide (Tyler, 2007). *Phytophthora* species do not affect only crop systems. They are also a threat to environmental important species. For instance, as mentioned before, *P. ramorum* and *P. cinamoni* are able to infect trees

threatening the integrity and biodiversity of forest ecosystems (Brasier and Webber, 2010; Hardham, 2005; Rizzo et al., 2005).

Phytophthora capsici

Phytophthora capsici was identified on chilli pepper (*Solanum capsicum*) in New Mexico. Since its identification, *P. capsici* was shown to be a broad host range pathogen being able to infect, among others, *Solanaceae* (such as tomato and pepper) and *Cucurbiceae* (such as pumpkin and melon) plants (Granke et al., 2012; Hausbeck and Lamour, 2004b; Lamour et al., 2012b). *P. capsici* was also shown to have a worldwide distribution being identified in North and South America, Asia, Africa and Europe (Dunn et al., 2010; Gobena et al., 2012; Hurtado-Gonzáles et al., 2008; Hwang and Kim, 1995; Li et al., 2012; Meitz et al., 2010; Silvar et al., 2006). Not surprisingly, taking into account the broad host range and worldwide distribution, *P. capsici* infections have been estimated to threaten over one billion dollars' worth of plant crops every year (Lamour et al., 2012b).

Phytophthora capsici is heterothallic, requiring two mating types (A1 and A2) to fulfil the sexual stage of its life cycle (Ko, 1988). The spores from this sexual stage are named oospores and are capable of surviving in the soil for many months due to their thick cell walls (Bowers, 1990). The relevance of the sexual stage in *P. capsici* life cycle appears to differ by geographical location. While in Argentina, Peru and across most of China, clonal lineages are prevalent (Gobena et al., 2012; Hu et al., 2013; Hurtado-Gonzáles et al., 2008), in the USA, South Africa and northern provinces of China outcrossing is frequent (Dunn et al., 2010; Gobena et al., 2012; Kamoun et al., 2015; Meitz et al.,

2010). Sexual reproduction in these geographical locations increases the levels of *P. capsici* genotypic diversity, hampering effective resistance breeding efforts. Besides sexual reproduction, *P. capsici* is also capable of asexual reproduction via the formation of sporangia. *P. capsici* sporangia is dislodged by irrigation or rain and is capable of germinating directly or, when immersed in water, to release 20 to 40 motile zoospores (Hausbeck and Lamour, 2004b).

P. capsici spores are disseminated in field waters and when in contact with plants they germinate allowing the formation of germ tubes, the penetration of the plant cuticle and the formation of an appressorium. Following penetration, *P. capsici* exhibits hyphal growth between living plant cells and the formation of specialised structures (haustoria) that invaginate host cells and support pathogen growth (biotrophy). *P. capsici* infection ends with tissue collapse (necotrophy) and the development of sporangia, marking the start of a new disease cycle. Due to these two separate states of biotrophic and necrotrophic infection, *P. capsici* is considered a hemi-biotroph (Hausbeck and Lamour, 2004a; Jupe et al., 2013; Lamour et al., 2012b; Schlub, 1983).

As for other *Phytophthora* species, current breeding strategies aiming for *P. capsici* resistance are mostly based on the introgression, improvement and identification of R genes (Chapman et al., 2014; Jupe et al., 2012; Segretin et al., 2014; Tan et al., 2010; Zhu et al., 2012). However, identified genetic resistance to *P. capsici* remains confined to few pepper varieties and wild tomato species without the identification of the R genes mediating these resistances (Quesada-Ocampo and Hausbeck, 2010; Walker and Bosland, 1999; Xu et al., 2016). Moreover, R gene based resistance is often reported to be rapidly overcome by *Phytophthora* species (Fry, 2008). With this little

knowledge on genetic resistance, growers have resorted to the use of fungicides to control *P. capsici* infections, most commonly metalaxyl and mefenoxam (Hausbeck and Lamour, 2004b). However, *P. capsici* has proven to be able to gain insensitivity to fungicidal treatments (Lamour and Hausbeck, 2003). These facts point to a need for a greater understanding of plant-*Phytophthora capsici* interactions in order to achieve durable resistances.

In this work I aimed for an improved characterisation of the virulence mechanisms of *P. capsici* by studying the functions of *P. capsici* encoded effectors. Our efforts on expanding the current understanding of the virulence functions of the cell death mediated by Crinkling and Necrosis (CRN) effectors, using PcCRN83_152 as a model (Chapter 3), coupled with the discovery of PcCRN83_152 host targets (Chapter 4) improve our comprehension on the relevance of this family of effectors towards *P. capsici* virulence. Moreover, a proteomics-based characterisation of the *P. capsici* secretome (Chapter 5) allowed the identification of new candidate effectors from this devastating plant pathogen. In sum, this work provides new insights on the effector complement of *P. capsici* and on the function of *P. capsici* CRN effectors, namely of PcCRN83_152, that could prove to be important for developing new pathogen-informed crop improvement strategies.

Chapter 2. A perspective on CRN proteins in the genomics age: Evolution, Classification, Delivery and Function revisited.

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Introduction

Pests and pathogens form some of the greatest threats to global food production, constraining crop productivity in an age that features significant growth of the world's human population (Newbery et al., 2016; Oerke, 2006). Amongst the biotic threats that wreak havoc on plants destined for consumption, the Oomycota form a distinct lineage of water-dwelling Eukaryotic microbes, many of which form parasitic interactions with plants. Amongst them, members of the *Phytophthora* genus rank amongst the most devastating pathogens, collectively affecting virtually every dicotyledonous crop plant (Fawke et al., 2015; Lamour et al., 2007).

Studies on the effector biology within oomycetes have led to the identification of vast effector repertoires, some of which act inside the plant cell (Hein et al., 2009; Schornack et al., 2009). Within the *Phytophthora* genus, two predominant classes of cytoplasmic effectors have been identified and studied, namely the RXLR and CRN effector protein families. Both protein classes feature modular

architectures, featuring motifs or domains required for delivery situated at the N-terminus (the RXLR motif for RXLR effectors and the LXLFLAK motif for CRN proteins), followed by C-terminal domains that carry effector functions (Whisson et al., 2007; Schornack et al., 2010). The identification of RXLR proteins within *Phytophthora* and the realisation that some members of this family act as avirulence (*Avr*) factors in the presence of specific (intracellular) receptor-like R genes have prompted and driven the discovery of a plethora of effector targets, virulence functions and molecular strategies within this family (Bozkurt et al., 2012; Schornack et al., 2009). These results have led to the view that the RXLR effectors comprise a large repertoire of fast evolving genes, whose products target nearly every subcellular compartment and are confined to a relatively small group of oomycete pathogens (Anderson et al., 2015). The increasing availability of pathogen genomes has not only led to an appreciation of the vast effector arsenals pathogens deploy, but also presented the field with a number of questions, some of which have remained unanswered. One observation for example is that in contrast to the RXLRs, the CRN protein family is widespread across the oomycete lineage (Schornack et al., 2010; Stam et al., 2013b; Zhang et al., 2016). This has raised the possibility that, besides the RXLR protein family, other cytoplasmic effectors, such as the CRNs, exist and have equivalent important roles in triggering host susceptibility. If true, the CRN effector family exemplifies the need to study lesser-known effector classes to fully understand pathogen biology. In this chapter we will summarise the current state of art on CRN research, explore the biology of these proteins, define open questions and propose ways to improve our knowledge on CRN function towards immunity associated processes in plants.

CRNs are part of a large and conserved eukaryotic protein family

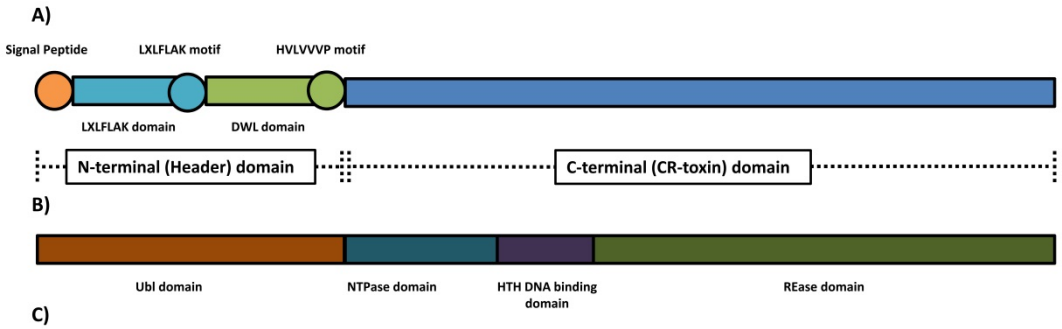
CRN effectors were first identified in the plant pathogenic oomycete *Phytophthora infestans* where they were found to cause a CRinkling and Necrosis (CRN) phenotype when systemically expressed in plant tissue (Torto et al., 2003). In that study, high throughput cloning was conducted of *P. infestans*-derived cDNA clones, which were identified in an Expressed Sequence Tag (EST) sequencing approach and found to have a predicted signal peptide. Subsequent application of a high-throughput functional expression assay *in planta* led to the identification of proteins that induce cell death upon expression in plants, two of which (CRN1 and CRN2), were found to be related on the sequence level (Torto et al., 2003). Since their discovery in *P. infestans*, equivalent studies in other oomycete pathogens revealed that in contrast to the RXLR protein family, CRN coding genes are widespread in the oomycete lineage. Transcriptome sequencing in the phylogenetically distinct pathogen *Aphanomyces euteiches* for example, also identified CRN effectors, thereby extending their known occurrence beyond the *Phytophthora* genus (Gaulin et al., 2008). These results suggest that CRNs are an ancient class of conserved oomycete effector proteins. Consistent with this finding, subsequent genome analyses have unveiled CRN coding genes in all plant pathogenic oomycetes sequenced to date (Adhikari et al., 2013; Derevnina et al., 2015; Haas et al., 2009; Kemen et al., 2011; Lamour et al., 2012a; Links et al., 2011; Sharma et al., 2015; Stam et al., 2013b) although in some genomes, gene family expansion seems to have taken place (Haas et al., 2009; Stam et al., 2013b). Interestingly, CRN-like proteins were also identified in the two basal fungal species *Batrachochytrium dendrobatidis* and *Rhizophagus irregularis*. These results suggest either a horizontal transfer event between organisms or

that all these genes were already present in early eukaryote progenitors (Lin et al., 2014; Sun et al., 2011a). Regardless of their history, the presence of CRNs in the pathogenic fungus *Batrachochytrium dendrobatidis* and their absence in its closest relative, a non-pathogenic chytrid fungus *Homolaphlyctis polyrhiza,* suggest that these effectors are retained in pathogens and thus form a link with pathogenic processes (Joneson et al., 2011). Recently a comprehensive study employed sequence analysis, structure comparison and comparative genomics to assess CRN occurrence across the Eukaryote taxon (Zhang et al., 2016). This revealed that CRN-like proteins are not only widespread in parasitic organisms, but also occur in free living eukaryotes and land plants that are not known to have a pathogenic lifestyle, seemingly invalidating the link between CRN presence and pathogenicity (Zhang et al., 2016). It was suggested however that CRN like proteins were initially deployed to resolve inter-organismal conflicts, after which in some host-pathogen interactions, these proteins were co-opted as effectors (Zhang et al., 2016).

CRNs modular structure

The first conserved regions identified in CRN proteins were found to be situated at the N-terminus, featuring a highly conserved LXLFLAK motif (Figure 1A) (Win et al., 2007). Aiming to study the evolution of RXLR effectors, it was observed that 16 *Hyaloperonospora parasitica* effectors showed similarity to CRN proteins, prompting the discovery that the RXLR motif was coupled to the LXLFLAK amino acid sequence (Win et al., 2007). This observation then led to the suggestion that both RXLR and LXFLAK domains are analogous and possibly involved in host targeting. This then implied that CRN proteins are

modular with domains that execute distinct functions, i.e. host targeting and signalling perturbation. Subsequent sequence analyses of CRN proteins identified in three *Phytophthora* genomes confirmed this notion whilst extending this rule to the entire CRN protein family (Haas et al., 2009). From this it was proposed that the CRNs form a family of modular proteins with a highly conserved N-terminal domain of around 130 amino acids, presumed to specify trafficking and containing both an LXLFLAK motif and diversified DWL domains (defined by the presence of the HVLVXXP motif). In this model, the highly conserved HVLVXXP motif marks the end of the N-terminal region as it is considered a recombination hotspot where C-terminal regions, carrying effector functions are linked up (Figure 1A) (Haas et al., 2009). In line with the expectation that effector families and their functions are diverse, subsequent computational analyses on CRN coding genes identified in *P. infestans*, *P. ramorum*, and *P. sojae* allowed the identification of 36 conserved C-terminal sub-domains. Expression of the C-terminal domains from the previously described CRN2 and four other CRNs led to cell death in *N. benthamiana* plants, suggesting that effector functions are diverse and located at the C-terminus. Given that the N-terminus (and predicted signal peptides) were found to be dispensable for cell death induction and CRN effectors thus seemingly acted inside plant cells, it was suggested that CRN N-termini specify the secretion and translocation of effector domains into the host (Haas et al., 2009).



C)

	N-terminal domain	C-terminal domain
***P. infestans* (142)**	Ubl1(115)	REase (50)
		NTPase + HTH + REase (48)
		REase + Kinase (14)
		NTPase + HTH + REase + HHH (1)
		SF-Toxin + CR-HNH (1)
		CR-HNH (1)
	Ubl2 (27)	CR-Trypsin (13)
		NTPase + HTH + REase + HHH (10)
		NTPase + HTH + REase (2)
		Tox-MPTase (2)
***P. sojae* (63)**	Ubl1 (59)	SF-Toxin + CR-HNH (32)
		NTPase + HTH + REase (14)
		SF-Toxin + CR-HNH +OTU + Znbinding + Polymerase + HNH + Polymerase (12)
		REase + OTU + Znbinding + Polymerase + HNH + Polymerase (1)
	Ubl2 (4)	NTPase + HTH + REase (4)

Figure 1 CR(N) structure analysis. CRN effectors are modular proteins with an N-terminus thought to be responsible for CRN secretion and translocation into the host and a C-terminus responsible for CRN virulence function(s). A) CRN N-termini were thought to contain a conserved structure featuring: a signal peptide for secretion; an LXLFLAK domain containing the respective LXLFLAK motif connected with translocation; and a DWL domain that ends in a conserved HVLVVVP motif that marks the end of CRN N-terminus and is thought to be a hot spot for recombination events. In contrast, CRN C-termini were shown to exhibit a large variety of domain structures (not depicted here). B) Zhang et al., 2016 redefined CRN structure. CRN N-termini (renamed header domains) from the two *Phytophthora* species analysed (*P. infestans* and *P. sojae*) all feature an Ubiquitin like (Ubl) domain that is thought to be responsible for secretion and translocation into the host cell. CRN C-termini (also named CR-toxin domains) feature distinct domain architectures, having enzymatic origins. The majority of *Phytophthora* CRN C-termini contained the depicted domain structure (NTPase+HTH+REase). C) Summary of domain architectures predicted to occur in *Phytophthora* (from Zhang et al., 2016). The number of CRN proteins with each given domain architecture/composition are indicated between brackets.

CRN gene expression and regulation

To allow successful host colonisation, the expression of pathogen genes requires great coordination and thus extensive regulation. This also appears true for effector gene expression, with dynamic and stage-specific changes in effector transcript levels demonstrated repeatedly. Microarray analyses of *P. infestans* mycelia revealed that 98% of all annotated CRNs are expressed and 66% of those were amongst the top 10% when assessed for array signal

intensities. These results were similar to those found for RXLR effectors, where 66% of the genes were expressed and 4 % were in the top 10% (Haas et al., 2009). Another study showed similar results, indicating that CRNs were expressed to a higher level than RXLR effectors (Shen et al., 2013). Besides high levels of expression, CRN coding genes were also differentially expressed during infection. CRNs from *P. capsici* could be divided in two groups according to their expression patterns: Class 1, forming a group that are upregulated in the early and late stages of infection, while Class 2 CRN gene expression gradually increases to peak in the late infection stages (Stam et al., 2013b).

Whilst CRN gene expression appears to be regulated during the infection process, the principal transcription factors remain to be identified. One possible mechanism of post-transcriptional regulation was unveiled recently in *P. infestans*, when sequencing of small non-coding RNAs led to the discovery of families of sRNAs that were predicted to target CRN coding genes (Vetukuri et al., 2012). Although Dicer-like (DCL) proteins were implicated in the generation of sRNAs by means of gene silencing, the effect of sRNA abolishment on CRN gene expression or pathogen virulence was not assessed (Vetukuri et al., 2012). Further studies will be required to firmly implicate sRNAs in CRN gene regulation in *P. infestans* and other oomycete pathogens. Besides (post) transcriptional regulation, translational control and post-translational modifications form important means by which level of functional effector proteins could be controlled. A quantitative phospho-proteomics study in *P. infestans* revealed that CRN proteins are phosphorylated across distinct life cycle stages (Resjö et al., 2014). Although phosphorylation of CRN8 had previously been demonstrated and implicated in virulence function (van Damme et al., 2012), this study revealed that other CRNs, lacking a kinase domain, are

also phosphorylated on residues that are widely conserved within the CRN protein family (Resjö et al., 2014). Whilst phosphorylation of residues was found to be widespread and target conserved domains within the CRN protein family, functional relevance remains to be established. It is likely however that use of this and possibly other PTMs, not only help regulate protein function, but also direct events required for secretion, delivery and stability. The study of the kinases responsible as well as their targets will undoubtedly reveal mechanisms required for CRN delivery and function.

Evidence supporting translocation of CRN proteins into host cells

Per definition, cytoplasmic effectors need to reach the cell interior to function towards their host target(s). Whilst computational and deletion analyses pointed at a role for CRN C-terminal domains inside the host cell and implicated N-termini in delivery, more concrete evidence emerged from functional studies in *Phytophthora capsici*. Using a *Phytophthora* transformation approach, constructs carrying the *P. infestans* AVR3a coding gene were first introduced in *P. capsici* and resulting strains used to infect transgenic *N. benthamiana* leaves expressing the potato resistance protein R3a. Whilst expression of AVR3a led to avirulence in these assays, strains that expressed AVR3a versions with a mutated RXLR motif, remained virulent, mirroring results in *P. infestans* (Whisson et al., 2007) and suggesting that AVR3a translocation conditions avirulence in the *P. capsici-N. benthamiana* system (Schornack et al., 2010). The ability of R3a to detect translocation in these assays was then used to show that the N-termini of CRN2, CRN8 and CRN16 mediate host-trafficking of the AVR3a C-terminus, evidenced by avirulent outcomes in infection assays on

R3a expressing leaves, but not in the absence of R3a (Schornack et al., 2010). Importantly, equivalent experiments using *P. capsici* strains expressing CRN-AVR3a fusion proteins in which the N-terminal LFLAK motif was mutated to LAAAA, led to infection. These results suggested that the LXLFLAK motif helps CRN trafficking into the host cell and provided a rationale for the use of CRN N-terminal sequences for genome wide searches, aimed at identifying and cataloguing candidate CRN effectors in pathogenic oomycete genomes.

A recent study shed a different light onto the supposed requirement of LXLFLAK motifs in CRN translocation (Zhang et al., 2016). Genome surveys spanning the eukaryote taxon uncovered CRN N-termini that lacked the LXLFLAK motif. Moreover, those that contained this motif were predicted to have an ubiquitin-like structure, similar to those found in the N-terminal region of SSK1/Mcs4 signalling proteins in fungi. In these analyses, the LXLFLAK motif was located in strand 2 and 3 of this ubiquitin-like domain, suggesting that structural features rather than sequence conservation underpin CRN translocation (Figure 1B) (Zhang et al., 2016). With a great number of “atypical” CRN N-termini identified, their contribution to translocation activity requires testing *in vivo*.

CRNs target host nuclear processes

In contrast to the RXLR effector class, all CRN effectors localised to date accumulate in the nucleus when expressed *in planta* (Stam et al., 2013b). As one would expect, nuclear localisation was found to be required for effector function in a number of cases, supporting the idea that CRN proteins target host nuclear processes. For example, the *P. infestans* CRN8 protein localises to the

nucleus and causes cell death, a phenotypic outcome thought to reflect virulence function (van Damme et al., 2012). Silencing of importin-α, a component of the nuclear pore complex required for active transport of proteins into the nucleus, led to altered PiCRN8 localisation and a reduction in cell death (Schornack et al., 2010). In addition, fusion of a nuclear exclusion signal (NES) to this effector drastically impeded nuclear accumulation and cell death occurrence (Schornack et al., 2010), supporting the idea of nuclear localisation requirements. Similar results were obtained for *P. sojae* and *P. capsici* CRNs PsCRN63 and PcCRN4 (also known as PcCRN83_152) respectively (Liu et al., 2011; Mafurah et al., 2015). However, PsCRN115, a CRN highly similar to PsCRN63 but without cell death inducing capacity, was shown to be able to supress cell death processes even when its nuclear localisation signal was mutated (Liu et al., 2011). Thus, while it looks like nuclear localisation is required for CRN cell death activity it remains unclear if suppression of plant defences requires accumulation in the nuclear compartment.

Unveiling CRN virulence functions

The fact that all CRN effectors accumulate in the host nucleus could indicate that they are targeting identical or a limited set of host processes. By extending the link between localisation and function however, this hypothesis is improbable as CRNs show different sub-nuclear localisation patterns (Stam et al., 2013a, 2013b). In addition, more detailed functional analyses have highlighted distinct cell death induction profiles and differential effects on PTI (Stam et al., 2013a), all supportive of diverse functions within this family. This observation is supported by recent work, aimed at understanding the virulence

targets and functions for a growing set of CRN proteins (Ramirez-Garcés et al., 2015; Song et al., 2015; Stam et al., 2013c; Zhang et al., 2015b).

Further studies have reinforced the notion of functional diversity whilst revealing phenomena that remain unexplained. Transient expression of two CRN effector domains in *N. benthamiana*, differing by only four amino acids, revealed opposing functions (Liu et al., 2011). PsCRN63 induced necrosis in plants while PsCRN115 was found to suppress cell death (Liu et al., 2011). Furthermore, over-expression of PsCRN115 was shown to enhance plant immunity whilst for PsCRN63 a decrease in resistance was observed (Li et al., 2016; Zhang et al., 2015a). Interestingly, both effectors were shown to directly interact with plant catalases and interfere with hydrogen peroxide (H_2O_2) accumulation. PsCRN63 was shown to increase H_2O_2 accumulation while PsCRN115 was shown to suppress this process. It was also suggested that PsCRN63 recruits plant catalases into the host nucleus leading to catalase destabilisation while PsCRN115 inhibits these events (Zhang et al., 2015b). Consistent with a role in infection, simultaneous silencing of both genes led to a reduced virulence phenotype on soybean (Liu et al., 2011), supporting the idea that these proteins are bona fide effectors. However, given that both genes were silenced, it remains unknown to what extent each effector contributes to virulence. Taken together, these results, though perhaps counterintuitive, provide some insights into the means by which this effector pair exhorts its function in plants. Nevertheless, whether these observations are extendible to the CRN protein family as a whole or if other CRN effector pairs exist, remains to be seen.

Importantly, despite being named after their ability to cause crinkling and cell death, cell death inducing activity is not a characteristic common to all CRN

effectors. Moreover, it appears that many CRNs that do not induce necrosis, supress host cell death processes. For instance, Shen et al. (2013) selected ten *P. sojae* CRN effectors and tested for their cell death inducing and suppression capacities respectively. Only one of these CRNs (PsCRN172-2) induced cell death when over-expressed in *N. benthamiana* leaves. The remaining 9 were able to suppress cell death caused by PsojNIP; 8 by PsCRN63; 5 by AVR3a+R3a; and 3 by Avh241 (Shen et al., 2013). Thus, it seems more likely that CRN effectors act as cell death regulators in host plants rather than inducers. However, in the absence of concrete evidence connecting cell death induction to virulence function, the significance of CRN induced necrosis remains a matter of speculation.

Interestingly, PsCRN63 was suggested to form homo-dimers and this dimerization was shown to be required for its ability to supress plant immunity processes and mediate host cell death (Li et al., 2016). Moreover, the authors suggested that PsCRN63 was able to form dimers with PsCRN115 and with unrelated PsCRN79 and PcCRN4. Thus, there is a possibility that PsCRN115 is increasing plant immunity by repressing PcCRN63 cell death in a dominant-negative manner. In addition, these authors suggest that the dimerization process could be widespread in CRN effectors, leading to the hypothesis that CRNs form complexes to enhance pathogen virulence (Li et al., 2016). If true, this hypothesis opens exciting research opportunities. However, it also raises new challenges on designing experiments and on drawing significant conclusions when studying individual CRN functions. In another major advance, it was demonstrated that the C-terminal half of PiCRN8 from *P. infestans* has kinase activity and is auto-phosphorylated when expressed in plant cells. In this work a kinase dead mutant of PiCRN8 was generated and was shown to have

dominant-negative effects on PiCRN8 cell death and to reduce *P. infestans* virulence when over-expressed *in planta*. Interestingly, and based on this work, PiCRN8 was also suggested to dimerize *in planta* (van Damme et al., 2012).

CRNs bind and modify host targets to promote virulence

Besides the interaction of PsCRN115 and PsCRN63 with plant catalases (Zhang et al., 2015b), there are few other examples of identified CRN host targets. A matrix yeast two hybrid screen identified *Arabidopsis* TCP14 as a major hub targeted by *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* effectors, including three *H. arabidopsidis* CRN effectors (*Arabidopsis* interactome Mapping Consortium, 2011; Mukhtar et al., 2011). Over-expression of a TCP14 from tomato, SlTCP14-2, was shown to enhance immunity against *P. capsici*. *P. capsici* CRN, CRN12_997, was shown to directly bind SlTCP14-2, abolishing the immunity increase mediated by SlTCP14-2. CRN12-997 is proposed to achieve this immunity increase abolishment by diminishing SlTCP14-2 association with DNA and by modifying SlTCP14-2 sub-nuclear localisation (Stam et al., 2013c).

More recently two CRN effectors were shown to achieve their virulence functions by interacting with host DNA. *P. sojae* PsCRN108 was shown to contain a putative DNA-binding helix-hairpin-helix (HhH) motif that inhibits the expression of Heat Shock protein (HSP) genes in *A. thaliana*, *N. benthamiana* and soybean. This is achieved by the binding of PsCRN108 to conserved promotor regions of HSP genes named heat shock elements (HSEs). HSEs are bound by heat shock transcription factors (Hsf's) leading to tight regulation of HSP expression. PsCRN108 was shown to be able to inhibit the binding of the

Hsf AtHsfA1a which induces HSP gene expression in response to stress (Song et al., 2015). Another study aimed to investigate the function of two related CRNs from the plant pathogenic oomycete *Aphanomyces euteiches* (AeCRN13) and from the amphibian pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (BdCRN13) also showed that both these effectors directly interact with DNA. These two cell death inducing effectors contain an HNH-like endonuclease motif that triggers plant DNA damage response (DDR). Mutation of key residues in the AeCRN13 HNH-like endonuclease motif abolished AeCRN13 capacity to interact with DNA, to induce DDR and to increase the susceptibility of *Nicotiana Benthamiana* to *P. capsici*. Thus the function of the HNH-like endonuclease motif on inducing DDR has been connected to AeCRN13 virulence function (Ramirez-Garcés et al., 2015).

From transposons to toxins: A role for CRN proteins in inter-organismal conflicts?

Recently a comprehensive study employed a combination of sequence analysis, structure prediction and comparison as well as comparative genomics to assess CRN occurrence across the Eukaryote taxon (Zhang et al., 2016). This study revealed that CRN effectors are not only widespread in parasitic organisms, but also occur in free living eukaryotes and land plants that are not known to have a pathogenic lifestyle (Zhang et al., 2016).The identification of CRN proteins in such a variety of organisms lead to their association with previously described proteins. Predicted proteins that resemble CRNs were found in trypanosomes where they are regarded as Retrotransposon Hot Spot

Proteins (RHSPs). RHSPs are expressed in the vicinity of genes required for pathogenesis and immune-invasion (Bringaud et al., 2002; Zhang et al., 2016). The association of CRNs with RHSPs lead authors to rename CRN proteins into CR (Crinkler-RHS-type) proteins (Zhang et al., 2016).

Making use of a vast collection of CR-proteins, Zhang and authors analysed and characterised CR domain structure by searching extensive databases of sequence profiles, including PFAM. Then CR proteins were compared to identify and delineate conserved domains that could be used for classification (using tools as PSIBLAST; HMM; JACKHMMER; and HHpred). Using this approach, a novel and comprehensive characterisation of CRN domain architecture was achieved (Figure 1B) (Zhang et al., 2016). In this analysis, the conventional division of CRN proteins into N-terminal domain (thought to be responsible for effector translocation) and C-terminal domains (though to be responsible for virulence effects) (Haas et al., 2009) remains unchanged. However our views on CRN domains are greatly challenged by this analysis and important possible insights gained on evolution and function. Firstly, the authors ruled out the presence of signal peptides, thought to be present at CRN N-termini. These N-terminal regions, defined as header domains, were predicted to form a ubiquitin-like (Ubl) fold in which predicted signal peptides as well as the conserved LXLFLAK motif are situated at conserved strands-1 and -3 respectively (Figure 1B). From this, the authors suggest that the LXLFLAK motif is important for translocation as they are important for Ubl domain structure. This Ubl N-terminal domain is significantly related to those found in fungal signalling proteins, namely SSK1/Mcs4. SSK1 orthologues play important roles in stress responses in various true fungi, and in some cases, are known to do so in a phosphorylation-dependent manner, employing an

interaction between their N-terminal domains and a MAPKKK heteromer (Calera et al., 2000; Calera and Calderone, 1999; Chauhan et al., 2006; Morigasaki and Shiozaki, 2013; Yu et al., 2016). From this, the authors suggest that CRN Ubl N-terminal domains could facilitate translocation inside the host and/or the host nucleus by analogous mechanisms (Zhang et al., 2016). To what extend these hypotheses ring true in oomycete-host interactions however, remains to be determined *in vivo*.

Besides the Ubl domain, CR N-termini feature various unrelated alpha-helical domains, somewhat conserved in a diverse set of organisms (Zhang et al., 2016). Header domains thus appear structurally distinct, suggesting a variety of mechanisms that govern translocation into the target cell. Despite this assumption, only one N-terminal domain, from *Angomonas*, shows a hydrophobic region implying possible membrane interactions and secretion. Moreover, CR proteins from diverse eudicot plants contain CR headers that contain helix-turn-helix (HTH) domains also found in the Myb transcription factor family. In Myb transcription factors, these domains are implicated in DNA-binding, suggesting that these eudicot proteins might not be secreted but target intracellular invasive DNA (Zhang et al., 2016).

As for CRN N-terminal domains, re-classification of CR C-termini have afforded new insights into CR(N) biology. In contrast to CRN N-termini and consistent with previous observations, C-terminal domains are highly diverse and often resemble enzymes (Figure 1C). Although high levels of diversity are known to be present, classification led to a limited set of domain configurations that were found to be prevalent. For example, CR C-termini containing a P-loop NTPase domain, combined with a nuclease domain of the restriction endonuclease

(REase) superfamily were found to account for slightly more than one-fourth of all CR C-termini. In addition, CR C-termini in which a REase superfamily domain is coupled to protein kinase domain was found to account for approximately one-sixth of the C-termini domains present in the dataset. In both cases, the toxicity function is believed to be specified by the REase domain, whilst the NTPase and Kinase domains would regulate REase activity or affinity towards nucleic acids (such as DNA) (Zhang et al., 2016). This view complies with studies on CRN8 in which disruption of kinase function did not abolish CRN cell death, but mutations in the newly annotated REase domain did (van Damme et al., 2012; Zhang et al., 2016). Moreover, these results indicate that targeting of nucleic acids such as DNA could be a defining feature, shared amongst CRN proteins (Figure 2). Indeed, this model is consistent with exclusive localisation of CRN proteins to the nucleus and importantly, two recent reports demonstrating binding of CRN effectors to DNA (Ramirez-Garcés et al., 2015; Song et al., 2015). Several other domains were identified as present in CR C-termini, including DNA binding domains (HNH nuclease and LK-nuclease), peptidase domains (trypsin, zincin-like metallopeptidase, and Ulp1- like peptidase), GTPase domains and non-enzymatic or transposon derived domains (Zhang et al., 2016) (Figure 1C). Thus, the prediction of enzymatic domains in CR or CRN proteins represents one important mean by which new hypotheses about CRN function can be constructed and subsequently tested (Zhang et al., 2016).

The structural analysis of CR proteins also unveiled similarities to proteins found in prokaryotes, allowing us to infer the evolutionary origin of CR proteins. NTPase coupled with REase domains are widespread in prokaryotes and linked with transposable elements. The role of transposable elements in the regulation

of gene transcription and regulation as well as chromatin structure has been well established and therefore these elements are considered motors that drive genome plasticity and adaptation in all kingdoms of life (Hua-Van et al., 2011). Consistent with this view, *P. infestans* CRN coding gene PITG_23144 was shown to have a gypsy retrotransposon inserted in its C-terminal domain (Haas et al., 2009). Even more striking was the discovery that *P. infestans* CRN coding genes, carrying the DC domain, are concentrated in genomic regions enriched for helitron transposons. Moreover, several CRN copies were found in a perfect tail-to-head conformation, mirroring arrangements seen for helitrons throughout the *P. infestans* genome (Haas et al., 2009). Given that helitrons are considered important factors that mediate gene duplication, exon shuffling and genome evolution (Kapitonov and Jurka, 2007), one hypothesis that has emerged is that CRN recombination and evolution is helitron mediated.

In contrast to CR C-terminal domains, there is no evidence for the presence of CR N-terminal domains in prokaryotes. CR C-terminal domains are therefore believed to have originated from prokaryotic proteins. This observation and the apparent activities of CR proteins towards nucleic acids, have led to the suggestion that CR-proteins originally evolved in prokaryotes in response to invasive intracellular DNA. Multiple lateral gene transfer events and subsequent coupling of CR proteins to a variety of header domains, allowed these toxins to be co-opted as effector proteins in eukaryotes. The observations and hypotheses emanating from work summarised here, provides a conceptual framework that in turn should lead to new experimental studies that inform on the biology of this ancient protein family in a range of eukaryote organisms (Zhang et al., 2016).

New approaches to study CRN biology and functions

With the increasing availability of pathogen genomes, understanding effector mode of action remains a major challenge and bottleneck. Despite recent efforts, new and more systematic ways are required to further understand CRN effector biology. Here we describe the areas where our knowledge on CRN effector biology remains poor or new opportunities have arisen for further exploration (summarised in Figure 2). Furthermore, we suggest new ways of tackling these areas, by taking advantage of our knowledge on CRN domain structures, plant-pathogen interactions and effector classes that are better understood.

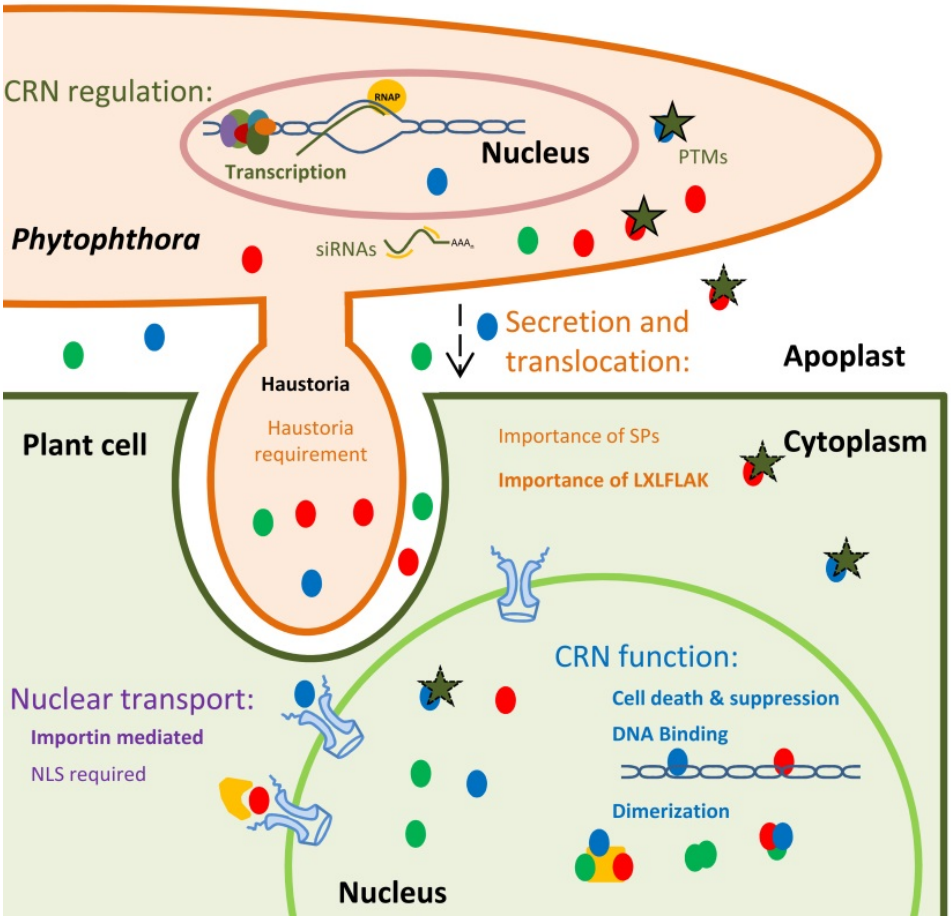


Figure 2 Schematic representation of current knowns and unknowns in CRN effector biology. CRNs (depicted as coloured circles) are highly expressed and regulated during infection, suggesting transcriptional control. It has been suggested that CRN could be regulated via siRNAs and PTMs, namely phosphorylation (green stars). A wide variety of CRNs have been identified as being phosphorylated in pathogen structures. However, the post-translational status inside plant cells or the apoplast (during transit) remains unknown. CRN secretion and translocation mechanisms remain widely uncharacterised. CRN N-termini were shown to be sufficient to mediate protein secretion and translocation into plant cells. The presence of the LXLFLAK motif was also shown to be required for this process. However, if CRN translocation is achieved in haustoria or if CRN predicted signal peptides are functional remains unclear. CRNs target host nuclear processes, but the mechanisms of trafficking into the nucleus, remain unknown. Importins mediate nuclear import by binding Nuclear Localisation signals (NLSs), present in most proteins destined for the nucleus. However import of CRNs without predicted NLSs has been observed. CRNs have been shown to mediate or suppress cell death processes. Besides proteinaceous nuclear host targets, CRNs have also been shown to target host DNA. Diverse CRNs were shown to form complexes in plant tissues. However, the nature of these dimers with regards to exact composition remains unclear.

The mechanisms required for CRN secretion and translocation into the host cell remain largely uncharacterised, due to the absence of tools that allow a comprehensive study on the translocation process. Whilst RXLRs are believed to be translocated in haustoria (Anderson et al., 2015), CRN proteins appear to

be present in pathogens that do not form haustoria, leading to the hypothesis that they might use distinct translocation mechanisms (Schornack et al., 2010; Zhang et al., 2016) (Figure 2). One way of confirming this notion is to create *Phytophthora* strains, unable to form haustoria, by disrupting factors required for their formation, such as Haustorial Membrane Protein 1 (HMP1) (Avrova et al., 2008). The successful application of CRISPR/CAS9 mediated gene editing in *Phytophthora* should allow the creation of such strains, provided they infect host plants to some degree, which in turn can be used for AVR3a based translocation assays on R3a plants. If feasible, this would tell us if CRNs require haustoria for their delivery and in addition, allow critical analogous experiments for the RXLR effector class. To gain further independent insights into translocation requirements, the identification and study of pathogen and host factors, able to interact with CRN N-terminal or CR-header domains would be of extreme use. Now that predicted structures for CR-header domains are available, rationalisation of candidate interactors in the context of translocation mode of action is ever more plausible.

CRN N-termini and CR-header domains have been divided into a diverse set of sub families raising the possibility that not all CR or CRN N-termini facilitate translocation (Zhang et al., 2016). To help resolve this important and biologically interesting observation, translocation experiments should be conducted using representatives of these different families. In such experiments, the presence or absence of predictable signal peptides should be taken into consideration as this may lead to discovery of new and unconventional secretion pathways or refinement of prediction software already available. Taken together, this information may unveil distinct translocation and

regulatory mechanisms, governing protein trafficking in diverse eukaryote systems.

As with the N-terminal domains, CRN C-terminal structure could be used to help us hypothesise on CRN function as proposed by Zhang and authors (Zhang et al., 2016). However, available experimental data demands some caution. In *P. infestans*, CRNs that share predicted effector (sub) domains, feature contrasting cell death inducing activities (Haas et al., 2009). Even more striking are the cases from *P. sojae* where only seven amino acid differences between PsCRN172-2 and PsCRN172-1 specify cell death inducing and suppressing activity respectively (Shen et al., 2013). Whilst these results mirror the PsCRN63 and PsCRN115 scenario, in which effectors only differ in four amino acids, exploration in other *Phytophthora* species will help determine whether these findings describe a general rule. Given that predicted structures now are available for CR and CRN proteins, mechanistic studies that aim to unravel the means by which CRN activity is regulated inside the host cell, will be of great value in our efforts to rationalise effector sequence-to-function relationships.

Despite the need for caution when over-interpreting sequence similarity, it would be of extreme value to be able to recognise which domains are present in each CRN, allowing inter-species and inter-article comparisons. For this we believe that it would be of extreme use to the field to agree on a CRN naming convention containing reference to the CRN N-terminal and C-terminal domain structure. The classification presented by Zhang and authors should be of use, especially when more structural data will be available in the future, allowing further refinement of sub family descriptions.

We already addressed the importance of clarifying the mechanisms used by CRN effectors to achieve translocation into the host cell. As all CRNs localise to the nuclear compartment, it would be interesting to understand the mechanisms used by CRNs to achieve nuclear translocation. It was shown that CRN nuclear localisation was mediated by the host machinery, namely by importin-α, as a cytoplasmic localisation shift of CRN C-terminal domains fused to GFP was observed in *N. benthamiana* plants silenced for importin-α homologs *NbImpα1* and *NbImpα2* (Schornack et al., 2010). Since importin-α has been shown to mediate nuclear import by binding nuclear localisation signals (NLS) in its cargo-substrates (Christie et al., 2015), it is not surprising that CRN proteins that carry NLS signals, travel to the nucleus in an importin-α dependent manner. Intriguingly, CRN proteins that lack a predictable NLS also can accumulate in the same way, suggesting that alternative mechanisms are at play (i.e. bound to another nuclear protein) or that the NLS prediction algorithms are not accurate, generating false negative results. Importin-α has been shown to interact with atypical NLS (Christie et al., 2015), so it is possible that the presence of a predictable NLS is not a strict requirement for transport to the host nucleus. The observation that CRN proteins can form dimers in plant cells, opens up the possibility of effector co-operation in trafficking.

Another significant question that remains unanswered in the field is the importance of CRN mediated cell death and its relevance to virulence. Cell death and virulence phenotypes coincide in several cases (Liu et al., 2011; Song et al., 2015; Stam et al., 2013b) suggesting that cell death represents a phenotype desired by the pathogen. An alternative view however is that cell death is an artefact associated with over-expression of an effector function. The observation that a small number of amino acid changes turn a cell death

inducing protein into a cell death suppressor suggests that cell death inducing activity may not be a critical function driving infection in the host. Furthermore, with many CRN proteins not inducing any cell death, it certainly is not a defining feature of this protein family. On the other hand, the suggestion that CR(N) proteins may have had toxin functions in a distant past would argue otherwise. For this reason, it would be of great interest to characterise the mechanisms underlying CRN mediated cell death and their connection with virulence activity. Given that some CRNs were shown to induce cell death at different rates (Stam et al., 2013a), it will be important to assess the levels of protein expression and experimental procedures to enable comparisons between cell death and non-cell death inducers.

With CRN effectors being highly expressed and having the ability to cause host cell death, it seems it would be necessary for CRNs to be tightly regulated during the infection process. As discussed above, post-transcriptional and post-translational control could be associated with this regulation. A better understanding on the control of CRN activity on both the transcript and protein level should allow key insights into effector as well as pathogen biology. As stated above siRNAs and PTMs, namely phosphorylation, could be responsible for CRN regulation. However, these two processes have not been connected with the control of CRN function to date. Understanding Post-translational modifications, in particular those that occur in *Phytophthora* and may not take place upon over-expression in plants, could help further (re)define the (cell death) activities of this protein family in more detail.

An important factor complicating the interpretation of cell death or virulence phenotypes, is the apparent ability of CRN proteins to form homo-dimers or

dimerize with other CRN effectors (Li et al., 2016). While it is not clear whether host proteins are part of these complexes, it is likely that diverse CRN effector complexes could modify host targets in distinct ways. This raises new challenges in experimental design, as a number of CRN effectors may be unable to achieve their true virulence functions alone. Although CRN co-expression could be attempted, the number of CRN combinations would render these experiments unfeasible, although a set of sensible criteria (gene expression, virulence functions, etc) could be implemented in a bid to reduce complexity. Systematic Yeast two Hybrid analyses or screens *in planta* should further rationalise intense future studies on CRN effector complex function in host-microbe systems.

Despite the valuable efforts aiming for the identification and characterisation of CRN virulence functions, CRN effector biology remains still largely uncharacterised. However, with our available knowledge on CRN distribution and structure, and with the ever improving techniques that enable an efficient study of plant-pathogen interactions we are on the verge of truly unveiling the role of CRN effectors and their biology. Indeed, CRN effector biology is emerging as a fertile research area where new and possibly game-changing concepts in effector biology may be discovered.

Chapter 3. Random mutagenesis screen provides new insights into PcCRN83_152 cell death and virulence function

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Introduction

Pathogen epidemics form one of the biggest constraints to crop growth and yield (Fisher et al., 2012; Oerke, 2006). Amongst the pathogens wreaking havoc on dicot crops, the oomycetes possibly represent one of the greatest threats to global food production (Kamoun et al., 2015; Lamour et al., 2007). Within the oomycetes, *Phytophthora* species form an extensive and diverse genus of plant pathogens that collectively affect virtually all dicot plants on earth (Kroon et al., 2012). For example, *Phytophthora infestans,* the causal agent of potato and tomato late blight (Fry et al., 2015), *P. sojae* (Tyler, 2007) and *P. capsici* (Lamour et al., 2012b) collectively cause billions of dollars' worth of losses on potato, tomato, soybean and pepper. In addition and more recently, the emergence of *Phytophthora* species such as *P. ramorum* (Brasier and Webber, 2010), *P. kernoviae* (Brasier et al., 2005) and *P. lateralis* (Green et al., 2013) that affect trees and shrubs, has meant that members of this genus have become a major threat to natural ecosystems. Given their importance, there is a

critical need to understand the biology of *Phytophthora,* their hosts and the infection process.

Plants are continuously bombarded by a diverse array of microbes that can cause disease. In most cases, infection is limited through the perception of Microbe or Pathogen Associated Molecular Patterns (MAMPs or PAMPs) by Pattern Recognition Receptors (PRRs). Recognition results in Pattern Triggered Immunity (PTI) and features a marked shift in cellular activity towards defence, defeating the vast majority of microbes (Boller and Felix, 2009; Jones and Dangl, 2006; Muthamilarasan and Prasad, 2013; Zhang and Zhou, 2010). In a select few cases and per definition, pathogens successfully infect plants of a given species. This suggests that host immune responses are suppressed or evaded, a pathogen characteristic that suggests an evolutionary basis for specialisation. Genome sequencing projects, combined with the development of computational pipelines and high throughput functional assays, have led to the identification of factors responsible for pathogen virulence (or pathogenicity) and therefore has revolutionised our thinking about plant pathogens. State of the art models emanating from functional genomics, biochemical and genetic studies describe pathogen molecules that are secreted from the pathogen and delivered into host tissues (effectors) where they subvert host immunity and trigger susceptibility (Hogenhout et al., 2009; Jones and Dangl, 2006; Kamoun, 2007; Win et al., 2012).

The availability and study of *Phytophthora* genome sequences have identified large and highly diverse candidate effector repertoires, with possible roles in infection (Bozkurt et al., 2012; Oliveira-Garcia and Valent, 2015; Schornack et al., 2009). Generally, these effectors can be categorised into two major classes,

defined by the host compartments in which these proteins and their respective molecular targets function. Apoplastic effectors accumulate in the host apoplast, where they target surface exposed or secreted host factors, often required or associated with defence (Doehlemann and Hemetsberger, 2013). Members of the cytoplasmic effector class however, are thought to traverse the host cell membrane and accumulate in distinct cellular compartments, where they act on their respective host target(s) (Hein et al., 2009; Kamoun, 2007). Efforts aimed at defining the effector repertoires in *Phytophthora* have led to the identification of two cytoplasmic effector subclasses, named the RXLRs and the Crinkling and necrosis (CRNs). These two groups are defined by the presence of conserved amino acid motifs (RXLR for RXLRs and LXLFLAK for CRNs) that are thought to be involved in the effector translocation process into the host cell (Haas et al., 2009; Rehmany et al., 2005; Schornack et al., 2010; Whisson et al., 2007). Whilst detailed functional studies have provided great insights into RXLR protein function (Anderson et al., 2015), the CRN protein family has thus far been understudied.

CRN proteins were originally discovered in a functional genomics study in which secreted proteins from *P. infestans* were expressed *in planta*. Ectopic expression of CRN1 and CRN2 led to a crinkling and necrosis (CRN) phenotype, which was presumed to reflect (an) effector function(s) after which these proteins were named (Torto et al., 2003). Subsequent studies then led to the identification of additional family members in other *Phytophthora* species as well as other distantly related oomycetes and organisms, suggesting that collectively, the CRNs form an ancient protein family, emerging before the RXLR effectors (Haas et al., 2009; Stam et al., 2013b; Zhang et al., 2016).

In addition to their discovery in a number of plant pathogens, CRN proteins have been implicated as virulence factors in a variety of plant-pathogen model systems. *P. sojae* lines in which PsCRN63 and PsCRN115 expression is reduced, exhibited a reduced virulence phenotype on soybean, suggestive of virulence function (Liu et al., 2011). Consistent with this observation, transgenic *Arabidopsis* plants that express PsCRN63 are more susceptible to *Pseudomonas syringae* and *P. capsici* (Li et al., 2016) indicating immune suppression that favours pathogen infection. Over-expression of another *P. sojae* CRN (PsCRN70) in *N. benthamiana* was also shown to enhance susceptibility of these plants against *P. parasitica* (Rajput et al., 2014). Moreover, over-expression of *P. sojae* PsCRN108 enhanced *Arabidopsis* and *N. benthamiana* susceptibility to *P. capsici* infections. In addition, silencing of PsCRN108 reduced *P. sojae* virulence on soybean (Song et al., 2015). Similarly, ectopic expression of the *P. infestans* effector PiCRN8 in *N. benthamiana* leaves led to an increase in susceptibility to *P. infestans* (van Damme et al., 2012). One CRN effector from *P. capsici*, PcCRN83_152 (also named PcCRN4) was also shown to be important for *P. capsici* virulence as transient over-expression of this effector in *N. benthamiana* leaves enhanced *P. capsici* growth while silenced *P. capsici* lines of this effector showed reduced growth in both *Arabidopsis* and *N. benthamiana* leaves (Mafurah et al., 2015; Stam et al., 2013b). In contrast to the RXLR family, members of the CRN protein family can be found in distinct oomycete lineages and in some cases, virulence functions have been demonstrated. AeCRN13, a CRN effector from the oomycete *Aphanomyces euteiches* was shown to enhance *P. capsici* virulence when transiently over-expressed in *N. benthamiana* leaves (Ramirez-Garcés et al., 2015).

Although CRNs are thought to aid in infection, the exact mechanisms by which these proteins function remain largely unknown. Only for a few CRN effectors the mechanisms of action have been unveiled. The *P. infestans* PiCRN8 C-terminal domain was reported to have similarity to plant serine/threonine kinases. Moreover it was confirmed by biochemical assays that the C-terminal domain of PiCRN8 had kinase activity (van Damme et al., 2012). However, the connection between PiCRN8 kinase activity and its virulence function remains unclear. More detailed CRN virulence function is known for *P. sojae* PsCRN108. This CRN was shown to be enhancing pathogen virulence by targeting the promotor regions of genes encoding for heat shock proteins (HSPs) and thereby reducing their expression (Song et al., 2015). The *P. sojae* effector PsCRN63 was also suggested to be increasing pathogen virulence by directly interacting and destabilising host catalases (Zhang et al., 2015b). Another study showed that over-expression of AeCRN13, a CRN effector from the oomycete *Aphanomyces euteiches*, enhances *N. benthamiana* susceptibility to *P. capsici* by binding host chromatin and triggering DNA damage responses (Ramirez-Garcés et al., 2015). In *P. capsici*, PcCRN12-997 was shown to bind to a tomato transcription factor, SlTCP14-2, inhibiting its association with DNA. Over-expression of SlTCP14-2 was shown to enhance immunity against *P. capsici*, a phenotype abolished by PcCRN12-997 over-expression (Stam et al., 2013c).

Although CRN functions have started to emerge, the biological relevance of cell death induced by some, but not all CRN proteins, is yet to be resolved. Several CRNs have been shown to work as suppressors of cell death and host defence responses (Rajput et al., 2014; Shen et al., 2013). Intriguingly, CRNs with high sequence similarity show opposite effects on cell death inducing activity, as is

the case for: PsCRN63 and PsCRN115 (Liu et al., 2011); and PsCRN171-1 and PsCRN171-2 (Shen et al., 2013). Thus, these CRN pairs could be useful tools to test the possible virulence function of CRN mediated cell death. While no virulence functions have been described for PsCRN171-1 and PsCRN171-2, studies on PsCRN63 and PsCRN115 have shown that the cell death inducing PsCRN63 enhances plant susceptibility while the cell death supressing PsCRN115 has the opposite effect (Zhang et al., 2015a, 2015b), suggesting a link between virulence function and CRN mediated cell death. However, PsCRN115 was shown to be a potent suppressor of PsCRN63 mediated cell death while having no effect on PsCRN63 virulence boost, suggesting that PsCRN63 mediated cell death and virulence boost consist of two independent mechanisms (Zhang et al., 2015b).

Attempting to gain new insights in the virulence function of CRN mediated cell death, PcCRN83_152 was used as a model. Over-expression of this *P. capsici* effector was shown to enhance *P. capsici* virulence and to induce plant cell death (Mafurah et al., 2015; Stam et al., 2013a, 2013b). Moreover, this CRN was also shown to mediate host chromatin re-localisation when over-expressed in *N. benthamiana* leaves (Stam et al., 2013a, 2013b). However, the host processes targeted by PcCRN83_152 connected to its virulence and cell death functions remain unknown.

In order to test if PcCRN83_152 mediated cell death is required for its virulence function(s), a PCR-based random mutagenesis screen was performed on PcCRN83_152 C-terminal domain (the domain responsible for both PcCRN83_152 virulence and cell death functions (Mafurah et al., 2015; Stam et al., 2013a, 2013b)). This screen allowed the identification of PcCRN83_152

variants that, despite not causing cell death, retain the capacity to enhance *P. capsici* virulence. Furthermore, no cell death (NCD) variants showed a capacity to repress PcCRN83_152 mediated cell death. These results suggest that PcCRN83_152 uses distinct mechanisms to achieve its virulence and cell death functions. CRN NCD variants were also shown to retain chromatin re-localisation capacities pointing to a virulence function of PcCRN83_152 mediated chromatin re-localisation.

Therefore, this work provides evidence suggesting that PcCRN83_152 induced cell death is not required for PcCRN83_152 mediated enhancement of *P. capsici* virulence. This discovery will hopefully shed a new light in our understanding of the virulence mechanisms used by CRN effector proteins. Moreover, this work generated well characterised PcCRN83_152 variants that can be used as extremely valuable tools to help us characterise the mechanisms underlying PcCRN83_152 virulence function.

Methods

Plant growth conditions

Nicotiana benthamiana plants were grown in a greenhouse under 16h of light and a temperature of approximately 25/22 °C (day/night). The plants were kept in these conditions during all the experiments unless stated otherwise.

PCR random mutagenesis screen

PcCRN83_152 C-terminal domain was PCR amplified using the primers: 83_flag_F (5’-

GACTACAAAGACGATGACGACAAGGAGGGGGTAGTTGGCTCA-3') and 83_Phus_R (5'-GGCGGTCGACGCGGCCGCTCACTTCTCGAACTGCGGGT-3'). The resulting PCR band was gel purified using MinElute Gel Extraction Kit (Qiagen) and used as a substrate for another round of PCR amplification using the primers: 83_Fus2_F (5'-CACCAGCTAGCATCGATGACTACAAAGACGATGACGACAA-3') and 83_Fus2_R (5'-GCCGCTCCAGGCGCGCCTCACTTCTCGAACTGCGG-3'). This amplicon was subsequently cloned into the viral vector pGR106 using the In-Fusion HD cloning kit (Clontech). This construct was then used as a substrate to create a library of mutated PcCRN83_152 variants using the Diversify PCR Random Mutagenesis Kit (Clontech) according to manufacturer's instructions. Two independent PCR reactions were performed aiming to obtain an average of 2 and 3.5 nucleotide mutations per 1000 base pairs and using the primers 83_Fus2_F and 83_Fus2_R (sequences above). The primers used do not add a start codon to PcCRN83_152 C-terminal sequence. Translation is initiated at the methionine at position 12 of PcCRN83_152 C-terminal domain. The mutagenized amplicons were transformed using the In-Fusion HD cloning kit (Clontech) into pGR106 and transformed into Stellar *E.coli* cells (Clontech). Transformed cells were grown overnight in a 37 °C shaking incubator and plasmids were extracted using the Quiaprep Miniprep kit (Qiagen). These plasmid mixes were then transformed in the *Agrobacterium tumefaciens* strain GV3101. Colonies resulting from these transformations were plated and PCR screened using vector specific primers: PVX2_F (5'-CAAACTAGATGCAGAAACCATAAG-3') and PVX2_R (5'-TTGACCCTATGGGCTGTGT-3'). Amplicons from these PCRs were sent for sequencing with the same primers (PVX2_F and PVX2_R). Positive colonies

were then toothpick inoculated into *Nicotiana benthamiana* leaves of plants with approximately three weeks old (as described in Torto et al., (2003). Cell death and mosaic viral symptoms were assessed between 7 to 10 days post inoculation. Cases where viral symptoms were not observed were excluded from further analysis. Differential cell death levels were not evaluated in this screen. Any level of cell death observed was counted as a positive cell death inducing event.

Sequence Analysis

Initial sequence analysis was performed using CodonCode aligner package version 4.2.3 (CodonCode Corporation). Using this program, sequence ends were trimmed maximising the region with an estimated error rate below 0.05%. Subsequently, variants with either forward or reverse sequences with less than 500 bases and a Phred quality score below 20 (estimated error rate at 1%) were removed from the analysis. Forward and reverse sequences for each variant were then aligned and consensus sequences were generated. Using custom made python scripts, consensus sequences were aligned and translated using MUSCLE (V3.8.31). This allowed the identification of nucleotide mutations and correspondent amino acid substitutions in our dataset. Sequences containing mutations leading to frameshifts and premature stop codons were removed from the analysis as were sequences containing nucleotide mutations with a Phred quality score of less than 30 (estimated error rate at 0.1%).

Re-cloning of NCD variants

Selected PcCRN83_152 variants were PCR amplified from GV301 cells using primers 83_cterm_F (5'-CACCGAGGGGGTAGTTGGCTCA-3') and 83_cterm_R (5'-TCACTTCTCGAACTGCGG-3'). They were then recombined into the entry vector pENTR/D-TOPO using the pENTR Directional TOPO cloning kit (Thermo Fisher Scientific) and sequence verified. Correct constructs were used for recombination into the binary vector pB7WGF2, with an N-terminal GFP-fusion and a 35S promotor element, using Gateway LR reactions (Invitrogen). Constructs were sequence verified and transformed into *Agrobacterium tumefaciens* strain AGL1. Wild type PcCRN83_152 cloned and tested in Stam et al. (2013b) was used in the same vector (pB7WGF2) and agrobacterium strain (AGL1). Avr3aKI and INF1 were used in pGRAB vector.

CRN cell death assays

All constructs were prepared for infiltration as described in Stam et al. (2013b). For cell death assays, cultures were mixed with *A. tumefaciens* AGL1 cells carrying the silencing suppressor p19 achieving final optical densities (ODs) of: 1.0 for CRN NCD variants; 1.0 for p19; 1.0 for EV; 1.0 for Avr3aKI; 0.25 for PcCRN83_152 wild type; and 0.25 for INF1. PcCRN83_152 wild type was used at a lower OD to ensure similar levels of protein expression as PcCRN83_152 variants showed less stability *in planta*. Scoring was performed between 2 to 7 days according to the experiment.

Cell death scoring was performed using a scale for 0 to 6 described in Stam et al. (2013b). For ion leakage measurements six leave discs from infiltrated leaves were collected, placed in 10 mL of Milli-Q water and shaken at 30 RPM

and at room temperature for two hours. Total dissolved solids (TDS) were then measured in solution using a Primo pocket TDS tester (Hanna instruments). For each point and treatment 6 measurements were taken. *P. capsici* culture filtrate (CF) and Pea broth (PB) were produced as described in Stam et al. (2013a).

Infection assays

P. capsici growth assays were done on leaves that had been infiltrated with appropriate *Agrobacterium* constructs using ODs as described above. Two days after infiltration, leaves were drop inoculated with 5µL of zoospore solution (50,000 spores per mL) from the *P. capsici* strain LT1534. Lesion diameters were measured three days post inoculation.

Western blotting

To test for the stability of PcCRN83_152 NCD variants, plant tissue, infiltrated with the respective constructs at the same conditions used for the cell death assays, was harvested two days post infiltration and frozen in liquid nitrogen. Protein extractions were done as in Stam et al. (2013a). Protein extracts were run on Biorad TGX gels before being transferred on PVDF membranes using Biorad Trans Blot Turbo Transfer System. Blots were blocked for 30 minutes with 5% milk in TBS-T (0.1 % Tween 20) and probed with GFP antibody (Santa Cruz) (1:2500) followed by anti-Mouse-HRP antibody (Santa Cruz) (1:20000). Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged on a Syngen GBox TX4 Imager. After imaging, for visualisation of total protein levels, membranes were

coomassie dyed using Imperial protein stain (Thermo Scientific) according to manufacturers' instructions.

Confocal Imaging

For confocal microscopy constructs were infiltrated as described above and ODs were adjusted to a final OD of 0.05 for all constructs without the presence of p19. For these assays, transgenic *Nicotiana Benthamiana* mRFP-H2B plants were used. Confocal imaging was performed 48 hours post infiltration on a Zeiss LSM 710 confocal microscope with a W Plan-Apochromat 40X /1.0 DIC M27 water dipping lens and using the settings: GFP (488 nm excitation and 400-600 nm emission); and mRFP (561nm excitation and 400–700 nm emission).

Results

Random mutagenesis screen generated a library of PcCRN83_152 variants with abolished cell death phenotype

PcCRN83_152 over-expression in *N. benthamiana* leaves induces plant cell death and promotes *P. capsici* virulence (Mafurah et al., 2015; Stam et al., 2013a, 2013b). To test if these two distinct phenotypes are connected, a random mutagenesis approach was taken. PcCRN83_152 C-terminal domain was amplified by error prone PCR reactions and cloned into a Potato virus X based vector (pGR106) and subsequently sequenced and screened phenotypically for the presence of cell death (Figure 1).

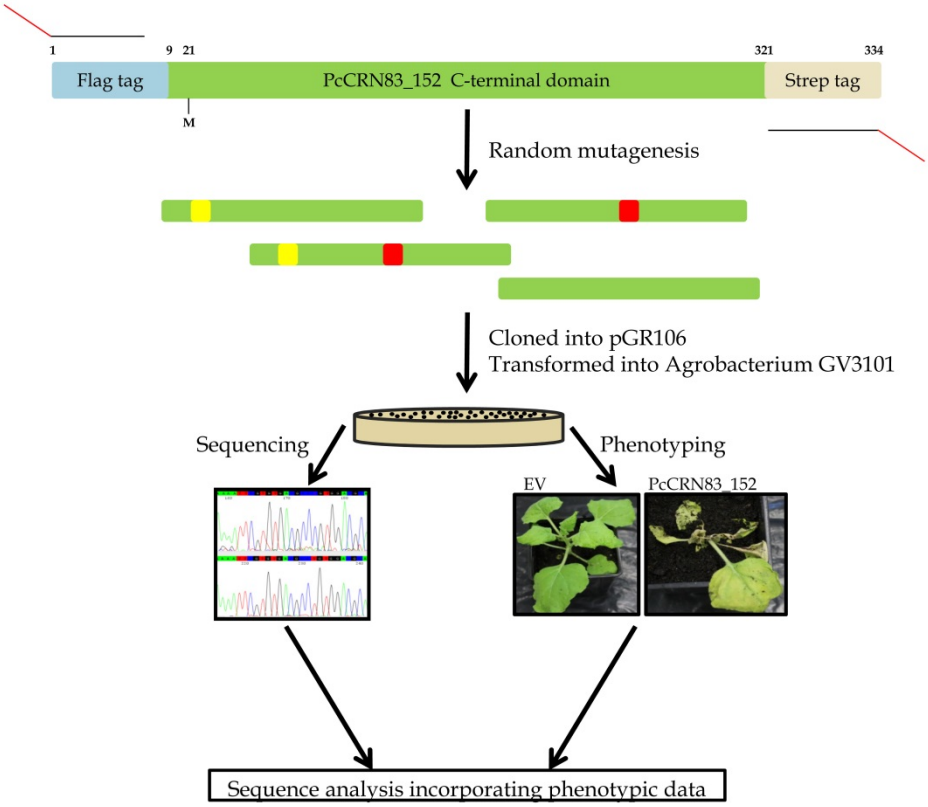

Figure 1 Random mutagenesis strategy. PcCRN83_152 C-terminal domain flanked with a Flag and Strep tag was used for error prone PCR reactions. Primers were designed to contain In-Fusion sites to allow direct cloning into pGR106 vector. The resulting PCR reactions were fused to pGR106 using In-Fusion cloning and transformed directly into *Agrobacterium* strain GV3101. Resulting *Agrobacterium* colonies were screened by PCR for the presence of the desired insert and positive colonies were screened phenotypically by toothpick inoculation of *N. benthamiana* leaves. Simultaneously, inserts from these colonies were amplified with vector specific primers and send for sequencing. PcCRN83_152 C-terminal domain first methionine (marked with an "M") was used as a translation start. Sequence and phenotypic analysis followed these procedures.

Sequence analysis using the CodonCode Aligner software package (V.4.2.3) generated a high quality library of sequences containing 506 PcCRN83_152 clones. Subsequent analyses reduced our library to 307 sequences from which we were confident of the mutational profile and were associated with phenotypic data (Figure 2).

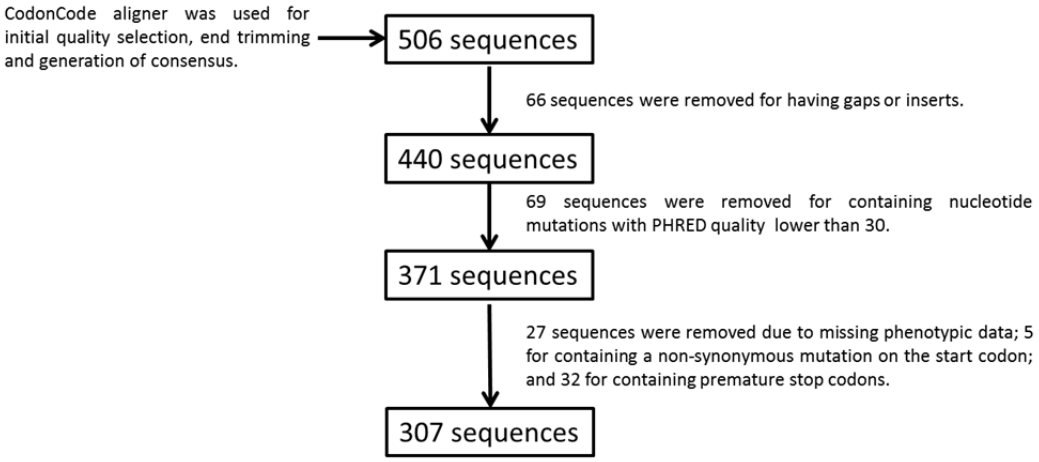

Figure 2 Sequence analysis pipeline. Using CodonCode Aligner software base calling and end trimming capacities, consensus sequences were generated for 506 PcCR83_152 variants. From these 506 variants, 66 contained gaps or inserts and were removed from the analysis. After this, another quality trimming step was performed in which all the sequences that contained nucleotide mutations in positions with Phred base calling quality of less than 30 were removed, leaving us with 371 sequences. The final 307 sequences were obtained by removing variants without conclusive phenotypic data, encoding premature stop codons or with amino acid substitutions in the start codon.

In order to characterise our library, we analysed how many nucleotide mutations and consequent amino acid substitutions were present in our library clones. Our analyses showed that, on average, our library clone has 2.3 nucleotide mutations and 1.7 amino acid substitutions. The library contained 107 clones with no amino acid substitutions which all showed cell death phenotype. Furthermore a shift to a non-cell death phenotype when increasing the number of sequence changes can be seen in our library (Figure 3).

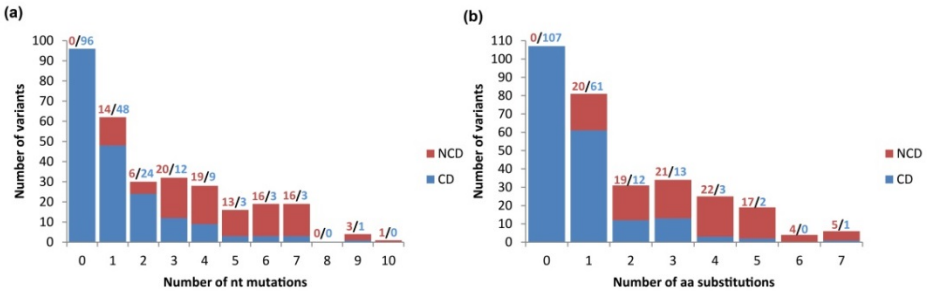

Figure 3 Characterisation of PcCRN83_152 library of variants. (a) Number of clones with either cell death (CD) or no cell death (NCD) phenotypes according to the number of nucleotide mutations they contain. (b) Number of sequences with either cell death (CD) or no cell death (NCD) phenotypes according to the number of amino acid (aa) substitutions they contain.

Our library contained amino acid substitutions in 62% of the targeted sequence. In order to analyse which regions of PcCRN83_152 could be associated with its cell death phenotypes, the amino acid substitutions unique for the NCD or CD set were plotted across PcCRN83_152 mutagenized sequence (Figure 4). Despite the presence of sequence regions where specific substitutions were only associated with lack of cell death, the coverage is not sufficient to take definite conclusions on the regions responsible for PcCRN83_152 cell death.

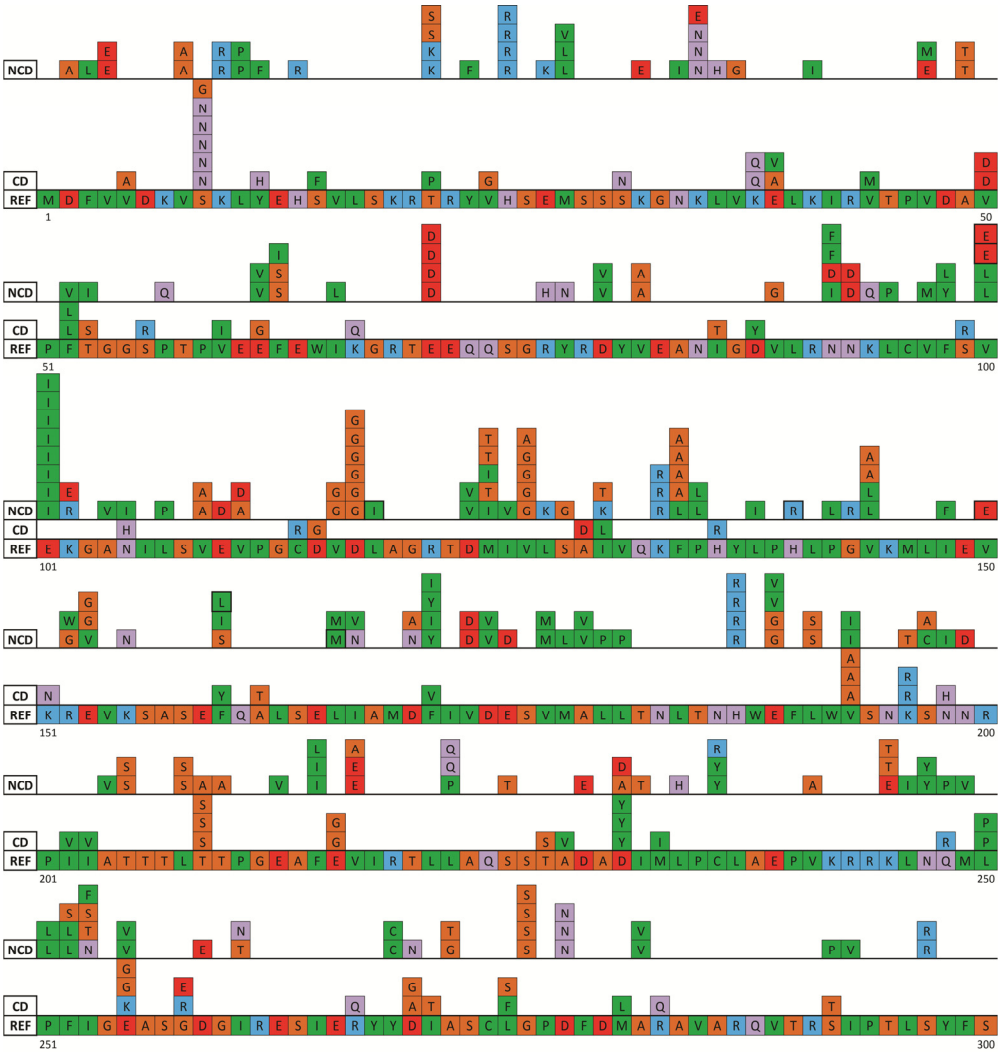

Figure 4 Distribution of amino acid substitutions across CRN83_152 C-terminal sequence. The amino acid substitutions present uniquely in the no cell death (NCD) or cell death (CD) set of the PcCRN83_152 library of variants were plotted against the wild type PcCRN83_152 amino acid sequence. Letters refer to amino acids and colours to amino acid characteristics according to the Lesk colour code. Single amino acid substitutions that are only present in the NCD set are signalised with a bold square.

PcCRN83_152 NCD variants are stably expressed *in planta*

The PCR based random mutagenesis screen described above allowed the identification of PcCRN83_152 C-terminal variants with abolished cell death phenotype. To test if the cell death abolishment was not due to lack of stability of these variants *in planta*, 14 of the no cell death (NCD) variants were cloned into a GFP N-terminal binary vector pB7WGF2 (Table 1). NCD variants with

single amino acid changes and with previous evidence of stability *in planta* using the viral system (data not shown) were the preferred candidates for cloning.

Table 1 Summary of the PcCRN83_152 variants described in this study

Variant	Amino acid changes	Cell death
2A10	L118I	+
2B5	L166M	-*
2B8	F63L	-
2F1	I130K	+
2F10	F160L	+
3H1	E27K; V131D	-
4A9	H140R	+
4B12	T21S; V82A	-
4C2	V150E	-
4D9	V100E	-
5E4	V97A	-
5H8	I66L; F160S; D170N; D259E	-
6D10	L191S	-
6E4	V89G	+

Notes: Presence or absence of cell death inducing capacities is indicated with a "+" and a "-" respectively. Lack of stability *in planta* is indicated with "*".

All the selected variants showed low expression levels when compared to the wild type protein (data not shown), so higher ODs were used to express these proteins. Nevertheless, even when expressed at similar levels of the wild type CRN, all the variants showed slower cell death phenotypes. Moreover, eight of

these variants had a completely abolished cell death phenotype and were picked for further analyses (Figure 5; Table 1).

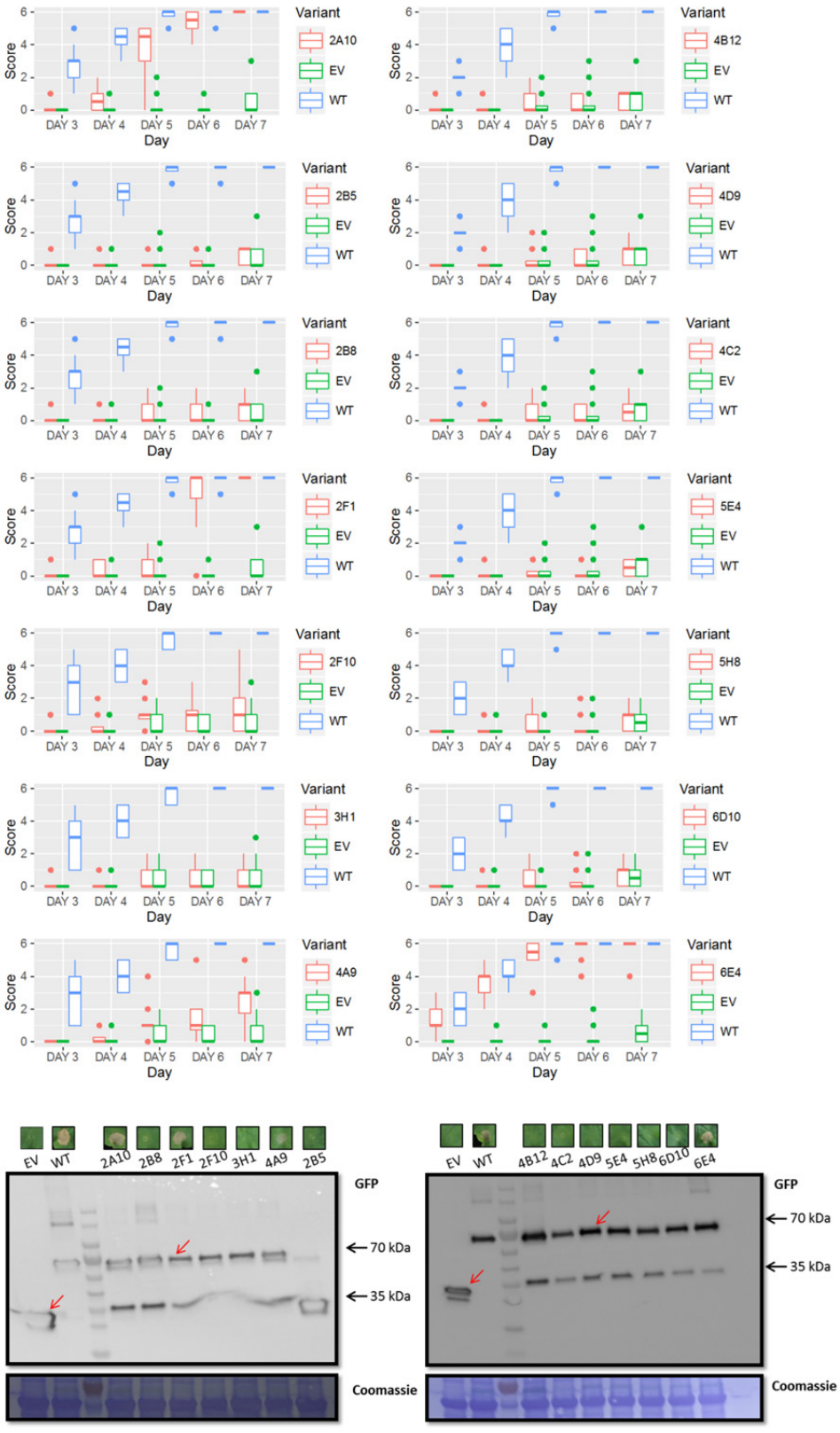

Figure 5 NCD variants show stability *in planta* and different rates of cell death inducing activity. NCD variants, wild type PcCRN83_152 and GFP were over-expressed in *N. benthamiana* leaves and cell death was scored using a scale from 0 to 6 where 0 stands for no cell death and 6 for complete dead plant tissue. Measurements were taken at 3, 4, 5, 6, and 7 days post infiltration. Representative leave images were taken at 7 days post infiltration. Samples for western blot were collected 3 days post-infiltration. Blots show that all the tested proteins, except 2B5, are expressed at levels similar to PcCRN83_152 wild type protein and a coomassie blue staining of the gels is shown as a loading control. Red arrows indicate the bands with sizes corresponding to PcCRN83_152 C-terminal construct fused to GFP (≈ 61 kDa) and GFP alone (≈ 27 kDa).

PcCRN83_152 NCD variants retain their ability to boost *P. capsici* infection

PcCRN83_152 was shown to boost *P. capsici* virulence when over-expressed in *N. benthamiana* leaves (Mafurah et al., 2015; Stam et al., 2013a, 2013b). To assess if NCD variants retained this ability, an infection assay was performed in *N. benthamiana* leaves over-expressing these variants. Four out of the eight NCD variants tested were shown to consistently boost *P. capsici* infection across three independent experiments (Figure 6), suggesting that PcCRN83_152 cell death is not a requisite for PcCRN83_152 mediated virulence enhancement.

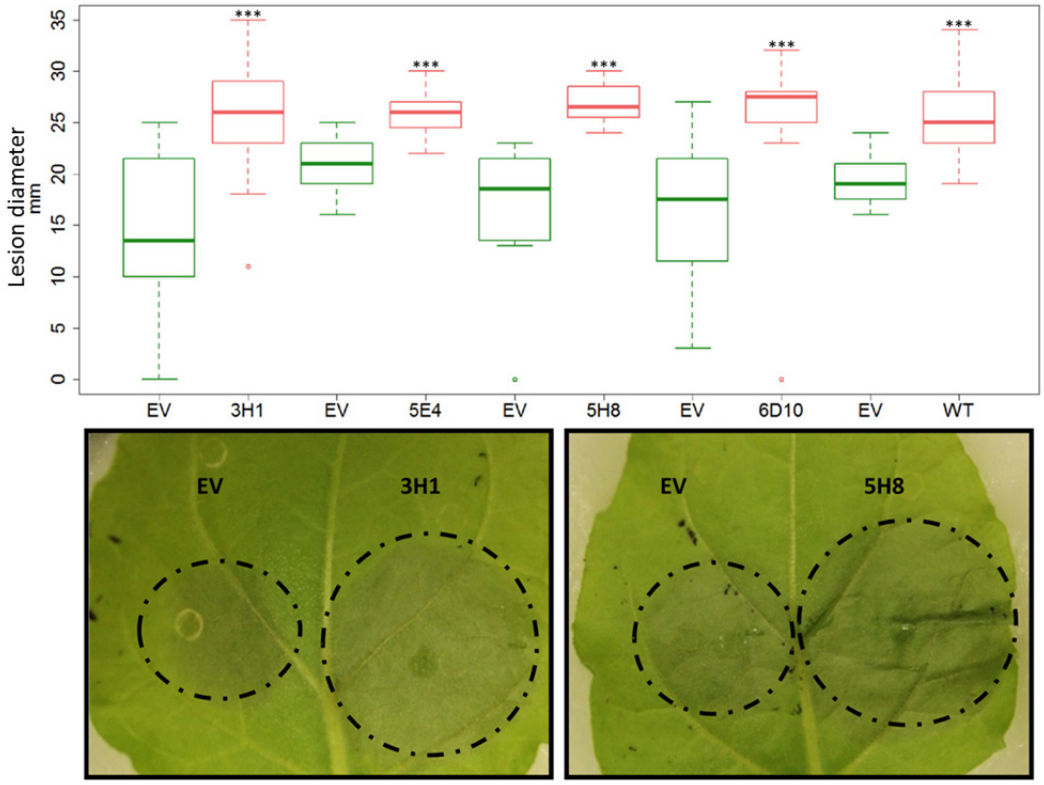

Figure 6 NCD variants boost *P. capsici* virulence. *N. benthamiana* leaves were infiltrated side by side with EV and PcCRN83_152 wild type or NCD variants. After two days leaves were inoculated with *P. capsici* strain LT1534. The graph shows data from one of three independent experiments with similar results. NCD variants with inconsistent boost results were excluded from the graph. Lesion diameters were measured three days after infection and are shown in millimetres (mm). Pictures show representative leaves for two of the NCD variants (3H1 and 5H8) three days after infection. "***" indicate a significant difference ($p<0.001$, t-test).

PcCRN83_152 NCD variants retain chromatin re-localisation capabilities

PcCRN83_152 was shown to localise unevenly in the host nucleus and to induce host chromatin re-localisation when over-expressed in *N. benthamiana* leaves (Stam et al., 2013a, 2013b). To assess if PcCRN83_152 mediated chromatin re-localisation is connected to its cell death or could be associated with its virulence phenotypes, the NCD variants were over-expressed in leaves of *N. benthamiana* transgenic mRFP-H2B plants and imaged by confocal microscopy (Figure 7). All PcCRN83_152 NCD variants localised in the plant nucleus, demonstrating that it is not nuclear exclusion that leads to the observed NCD phenotypes. Furthermore, all the tested variants conserved their

capacity of re-localising host chromatin. Individual NCD variants, while retaining chromatin re-localisation features, showed a variety of sub-nuclear distributions. Therefore, Figure 7 displays examples of observed sub-nuclear localisations of individual NCD variants without implying that different NCD variants possess distinct sub-nuclear localisation patterns. These results suggest that PcCRN83_152 mediated chromatin re-localisation is not a consequence of the cell death events mediated by this CRN effector. In addition, they point towards a virulence role of PcCRN83_152 mediated chromatin re-localisation.

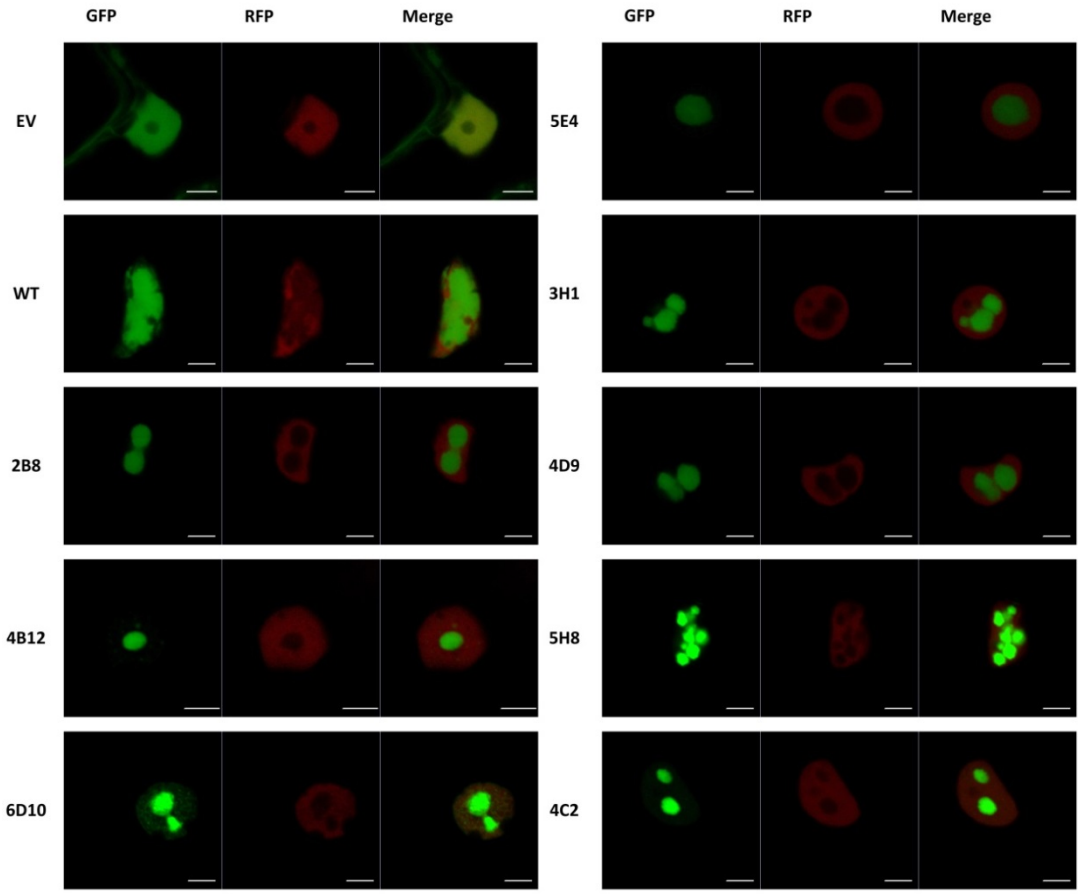

Figure 7 NCD variants retain chromatin re-localising capacity. Wild type PcCRN83_152 C-terminal domain (WT), NCD variants GFP tagged and GFP alone (EV) were expressed in transgenic mRFP-H2B *N. benthamiana* plants. Confocal microscopy was performed two days post-infiltration. Contrasting to EV-GFP, PcCRN83_152 NCD variants were shown to accumulate in sub-nuclear bodies varying in shape and number. Nevertheless, these variants retain the capacity of re-localising host chromatin, characteristic of PcCRN83_152 wild type protein. Scale bar indicates 5µm.

PcCRN83_152 NCD variants supress PcCRN83_152 mediated cell death

A kinase-inactive variant of the *P. infestans* effector PiCRN8 and the *P. sojae* effector PsCRN115 were shown to supress cell death induced by CRNs highly similar to them (kinase active PiCRN8 and PsCRN63 respectively) (van Damme et al., 2012; Zhang et al., 2015b). To test if PcCRN83_152 NCD variants supress PcCRN83_152 mediated cell death, PcCRN83_152 was co-expressed with individual NCD variants. PcCRN83_152 cell death was consistently diminished in the presence of five NCD variants across three independent experiments (Figure 8a). For two of the NCD variants this effect was reinforced using ion leakage measurements (Figure 8b). Interestingly, four NCD variants that consistently showed a virulence boost also consistently showed a suppressive effect on PcCRN83_152 cell death suggesting that cell death suppression and virulence boost can co-exist. This fact is further evidence suggesting that PcCRN83_152 cell death and virulence boost are two independent processes.

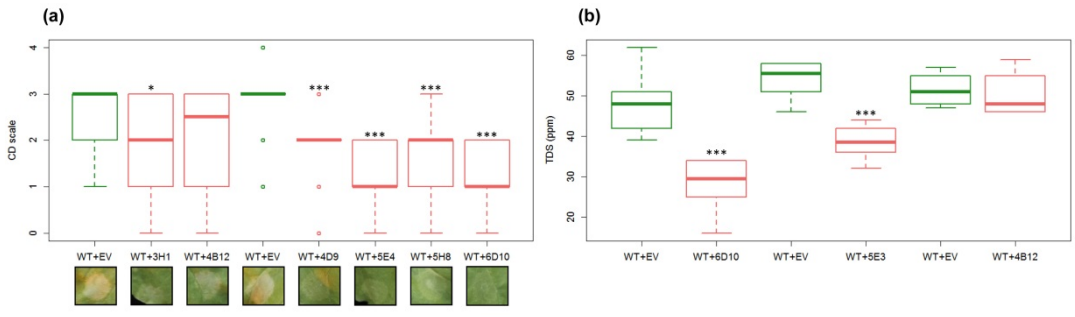

Figure 8 NCD variants supress PcCRN83_152 cell death phenotype. (a) PcCRN83_152 NCD variants or EV-GFP were co-expressed with the wild type version of PcCRN83_152 C-terminal domain (WT). Five NCD variants showed a suppressive effect on PcCRN83_152 mediated cell death over three independent experiments. Variants with inconsistent suppression results were excluded from the graph that shows data from one representative experiment. Cell death levels were assessed four days post infiltration using a cell death scale from 0 to 6 where 0 stands for no cell death and 6 for complete dead plant tissue. Pictures represent phenotypes four days post infiltration.(b) For three of these NCD variants, two that showed a suppression capacity and one that did not (4B12), the phenotypic scoring was complemented with ion

leakage assays confirming the phenotypic data. "***" indicate a significant difference ($p<0.001$, t-test). "*" indicate a significant difference ($p<0.05$, t-test).

CRN effectors have been shown to mediate the suppression of various cell death processes *in planta* (Rajput et al., 2014; Shen et al., 2013). In order to test if the PcCRN83_152 NCD variants could work as general cell death suppressors, we co-expressed two of these variants with *P. capsici* culture filtrate (CF) and with the *P. infestans* PAMP INF1 (Kamoun et al., 1997). The *P. infestans* effector Avr3aKI (Bos et al., 2006) was used as a positive control and, as expected, was capable of inhibiting CF and INF1 mediated cell death (Figure 9). However, the two tested NCD variants failed to supress both CF and INF1 mediated cell death (contrariwise one of them (5E4) is even shown to enhance CF mediated cell death) (Figure 9). These results raise the possibility of a dominant-negative effect of PcCRN83_152 NCD variants on PcCRN83_152 mediated cell death.

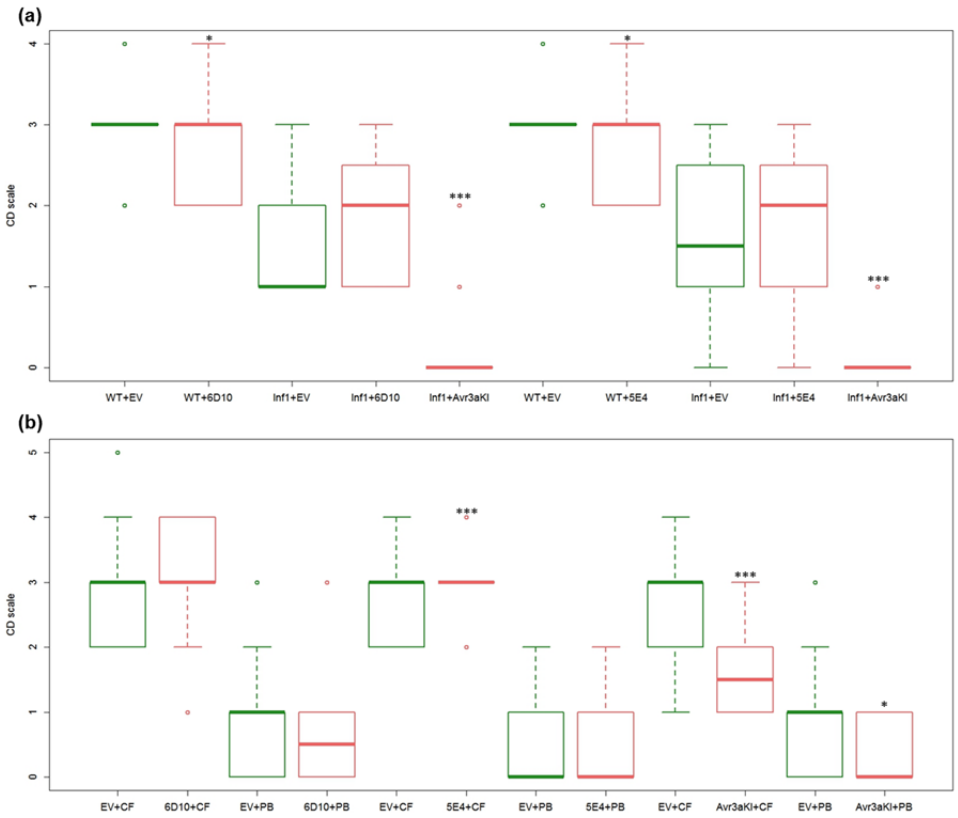

Figure 9 NCD variants do not supress INF1 and CF mediated cell death. (a) PcCRN83_152 NCD variants, EV-GFP and Avr3aKI were co-expressed with the wild type version of PcCRN83_152 C-terminal domain (WT) and INF1. Cell death levels were assessed four days post infiltration using a cell death scale from 0 to 6 where 0 stands for no cell death and 6 for complete dead plant tissue. (b) *N. benthamiana* leaves expressing both EV-GFP and either PcCRN83_152 NCD variants or Avr3aKI (each in one side of the leave) were infiltrated with either *P. capsici* culture filtrate (CF) or pea broth (PB). Cell death was scored 3 days after CF and PB infiltration. "***" indicate a significant difference ($p<0.001$, t-test). "*" indicate a significant difference ($p<0.05$, t-test).

Discussion

With the ever increasing availability of genome sequences for plant-microbes, a large repertoire of effectors has been identified. However, despite valuable efforts, the bottlenecks in the field reside on deciphering the functions of these numerous effector proteins. In relation to *Phytophthora* cytoplasmic effectors, numerous studies have succeeded in elucidating RXLR functions (Anderson et al., 2015), however CRN effectors have received considerably less attention. Recently, some studies provided new insights into CRN virulence functions

(Ramirez-Garcés et al., 2015; Song et al., 2015; Zhang et al., 2015a) but the virulence function(s) of CRN mediated cell death remain mostly unveiled.

In this study we made use of PcCRN83_152 features to address this question as this effector has a strong cell death phenotype and simultaneously enhances *P. capsici* growth. By means of a PCR based random mutagenesis screen, we generated a library of 307 PcCRN83_152 variants from which 108 lost their cell death inducing capacities. However our analysis did not succeed in identifying the amino acid region responsible for PcCRN83_152 cell death phenotype. Our library contained amino acid substitutions for 62% of PcCRN83_152 C-terminal amino acids. However, a higher coverage would be necessary to take definite conclusions about specific regions connected with cell death inducing activities. Moreover, it is important to note that we did not test for protein stability in our screen, so regions required for PcCRN83_152 stability will be picked up in our analysis even if they are not directly connected with PcCRN83_152 cell death phenotype.

Nonetheless, this screen generated PcCRN83_152 NCD variants that we could screen for virulence activities. From our library, 14 NCD variants were selected from which eight did not show any cell death inducing activity even when expressed at similar levels of PcCRN83_152 wild type protein (Figure 5). It is important to note though that all the selected NCD variants showed a more unstable nature when compared to the wild type protein, leading us to hypothesise that PcCRN83_152 may have important structural features that are affected in these variants.

These eight NCD variants with completely abolished cell death phenotype were tested for their capacity to enhance *P. capsici* virulence. Four out of these eight

variants were able to consistently boost *P. capsici* growth (Figure 5), suggesting that cell death is not required for PcCRN83_152 virulence functions. However, we cannot exclude that cell death, despite not being required, aids PcCRN83_152 virulence function as it is difficult to compare the levels of virulence boost between cell death and non-cell death inducing proteins when using different concentrations of *Agrobacterium* inoculum. Other hypothesis we cannot exclude is that the tested NCD variants are gaining a new virulence function unrelated to PcCRN83_152 wild type virulence function. Nevertheless, this hypothesis appears quite improbable as one would not expect that four out of the eight selected NCD variants would display newly acquired virulence functions. In addition, NCD variants retained nuclear localisation and the capacity of re-localising plant chromatin (Figure 7), further pointing to a conserved virulence function.

PcCRN83_152 NCD variants that are capable of enhancing *P. capsici* growth retain chromatin re-localisation capacities, suggesting a link between chromatin re-localisation and PcCRN83_152 virulence functions. However, while all tested NCD variants retained the capacity of chromatin re-localisation, only four of them were shown to consistently boost *P. capsici* virulence. Thus, it appears that chromatin re-localisation capacities are not sufficient per se for PcCRN83_152 virulence functions. Recently, two CRN effectors have been shown to directly bind DNA to achieve their virulence functions (Ramirez-Garcés et al., 2015; Song et al., 2015). In addition, the targeting of DNA-related processes has been predicted to be a conserved feature of CRN effectors (Zhang et al., 2016), turning further investigation into the mechanisms involved in PcCRN83_152 mediated chromatin re-localisation of upmost importance.

Despite their identification as cell death inducers (Torto et al., 2003), the induction of cell death is not a feature common to all CRN effectors (Haas et al., 2009; Shen et al., 2013; Stam et al., 2013b). On the contrary, several CRNs have been shown to work as general cell death suppressors (Shen et al., 2013). Even CRN effectors with high sequence similarity show opposite cell death phenotypes (Shen et al., 2013; Zhang et al., 2015b). Furthermore, a cell death- and kinase-inactive variant of the *P. infestans* effector PiCRN8 was shown to supress the cell death induced by the wild type version of PiCRN8 (van Damme et al., 2012). In this work we showed that five of the tested PcCRN83_152 NCD variants consistently supressed PcCRN83_152 mediated cell death (Figure 8). However, two of these NCD variants were not capable of supressing cell death mediated by INF1 (Figure 9a) and *P. capsici* CF (Figure 9b). These results point to a possible dominant-negative effect of the NCD variants on PcCRN83_152 mediated cell death. CRN effectors, including PcCRN83_152, have been suggested to form homo- and hetero- dimers in plant cells (van Damme et al., 2012; Li et al., 2016). Thus, one possible explanation for this cell death suppression by PcCRN83_152 NCD variants could be the destabilisation of functional CRN complexes where PcCRN83_152 is present. Nevertheless, regardless of the mechanisms implied in this suppression, the fact that four NCD variants which were shown to boost *P. capsici* infection also were shown to supress PcCRN83_152 mediated cell death, further points to a separation of PcCRN83_152 cell death and virulence functions.

In summary, in this work we addressed a question that has been overlooked in the CRN effector field, namely the virulence importance of CRN mediated cell death. A PCR based random mutagenesis screen enabled the identification of PcCRN83_152 variants that despite complete absence of cell death inducing

activity retained the capacity to boost *P. capsici* virulence. Thus, the results of this study point to a separation of PcCRN83_152 cell death and virulence functions. While it is not clear if these findings have parallel in other cell death inducing CRNs, this study provides new insights on CRN mediated cell death that need to be taken into account when trying to understand CRN virulence functions.

Chapter 4. Identification and characterisation of PcCRN83_152 host protein interactions

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Author Contributions

TA performed the majority of the experiments. AH and SR performed FLIM-FRET experiments. VMH helped with experiments that led to Figure 4, 9 and 11. TA and EH wrote this Chapter.

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Introduction

Plant pathogens continuously hamper crop production worldwide (Fisher et al., 2012; Oerke, 2006). In an evolutionary arms race, plants have evolved an immune system that allows them to fend off most would be pathogens (Jones and Dangl, 2006). Thus, by definition, successful pathogens need to overcome this plant immune system. An improved understanding on how the plant immune system works and how it is subverted by pathogens is crucial for improvement of crop production.

The Oomycota form a distinct lineage of water-dwelling Eukaryotic microbes that has an impact on crop production as well as on natural ecosystems (Fawke et al., 2015; Kamoun et al., 2015; Kroon et al., 2012; Lamour et al., 2007). For instance, *P. infestans* (Fry et al., 2015) and *P. sojae* (Tyler, 2007) constitute major worldwide threats to potato and soy beans, respectively. Moreover, *P. ramorum* and *P. cinnamomi* are examples of oomycetes that are globally

devastating natural forests and shrubs (Brasier and Webber, 2010; Burgess et al., 2016; Hardham, 2005; Rizzo et al., 2005).

The pathogenicity of microbes is thought to be mediated by the secretion of effectors, molecules that enhance pathogen fitness by targeting host processes (Hogenhout et al., 2009; Win et al., 2012). In gram-negative plant pathogenic bacteria, effectors are believed to be translocated by the action of a type III secretion system (T3SS) (Galan and Wolf-watz, 2006; Honour C. McCann and Guttman, 2008). The importance of bacterial effectors is noted by the fact that bacteria compromised in the T3SS have extremely reduced virulence capacities (Block et al., 2008; Jin et al., 2003). In oomycetes, several effectors have been shown to be crucial for virulence (Anderson et al., 2015). For instance, silencing of a single RXLR effector in *P. infestans* (Avr3a) did not influence *P. infestans in vitro* growth but led to a significant reduction of *P. infestans* virulence on potato (*Solanum tuberosum* cv Bintje) and *N. benthamiana*. This impairment of *P. infestans* virulence could be reverted by *in planta* over-expression of Avr3a (Bos et al., 2010). Effector virulence importance is also manifested by the presence in plants of highly specific Resistant (R) proteins that recognise effectors or their functions leading to the induction of plant immune responses (Dangl and Jones, 2001; Lee and Yeom, 2015; van Ooijen et al., 2007). R proteins capable of recognising oomycete effectors have been identified in plants. For instance, the resistance proteins R3A and R2 were identified in potato and were shown to be able to recognise *P. infestans* RXLR effectors Avr3a and Avr2 respectively (Armstrong et al., 2005; Gilroy et al., 2011; Huang et al., 2005).

Recent sequencing efforts of oomycete genomes revealed that these organisms contain large and complex effector repertoires that are usually divided into

apoplastic or cytoplasmic effectors according to the host compartments they target. Apoplastic effectors target extracellular plant proteins or plant surface receptors, while cytoplasmic effectors are translocated inside host cells where they are thought to perform their virulence functions (Asai and Shirasu, 2015; Bozkurt et al., 2012; Kamoun, 2006). The characterised functions of apoplastic effectors have been mostly connected to suppression of pathogen recognition events and with the inhibition of host extracellular proteases (Asai and Shirasu, 2015; Kamoun, 2006). For example, the *P. infestans* effectors, EPIC1 and EPIC2B, target a tomato secreted protease C14 that was shown to be involved in immunity processes as silencing of C14 in *N. benthamiana* turned these plants more susceptible to *P. infestans* infection (Kaschani et al., 2010).

Oomycete cytoplasmic effectors are divided into families according to the presence of conserved N-terminal motifs thought to be involved in their translocation mechanisms. The two major families of oomycete cytoplasmic effectors are the RXLRs and the CRNs (for Crinkling and Necrosis) defined by the conserved N-terminal motifs LXLFLAK for CRNs and RXLR for RXLRs (Schornack et al., 2010; Whisson et al., 2007). Besides the RXLRs and the CRNs, the existence of two other families of oomycete cytoplasmic effectors has been suggested. Genome sequencing of the necrotrophic *Pythium ultimum* revealed that this plant pathogen does not encode for RXLR effectors and is depleted in CRN effector numbers when compared to *Phytophthora* species (Lévesque et al., 2010). However, analyses of the *P. ultimum* predicted secretome revealed a new candidate family of effectors characterised by a YxSL[RK] motif in their N-terminal domain. Effectors from this family were also predicted to be encoded by other oomycete pathogens, namely *P. infestans*, *P. ramorum*, *P. sojae*, and *Aphanomyces euteiches* (Lévesque et al., 2010).

However, evidence supporting translocation or virulence functions for effectors of this family is still lacking. Another family of oomycete cytoplasmic effectors was identified by the analysis of the genome of the obligate biotroph *Albugo laibachii* (Kemen et al., 2011). This CHXC family of effectors is characterised by the presence of a CHXC motif in their N-terminal domain, which has been shown to mediate effector translocation into the host cells. Moreover, *Pseudomonas syringae* strains expressing two of these CHXC effectors (CHXC2 and CHXC7) were more virulent in *Arabidopsis*, further indicating CHXC as new family of oomycete effectors (Kemen et al., 2011).

Despite CRN and RXLR effectors having been greatly implicated in mediating pathogen virulence (Anderson et al., 2015; Chapter 2), the molecular mechanisms and host targets responsible for these virulence phenotypes only now start to be unveiled. The characterisation of these mechanisms and targets could lead to the discovery of new plant immunity processes. Moreover, plant resistance based on the modification of effector targets have been proposed to constitute a durable source of resistance (Gawehns et al., 2013). Thus, great research efforts have been directed at the identification of effector host targets, with a range of methods being employed. An example of the approaches being used is the combination of experimental data with a yeast-two hybrid-based pipeline to identify interactions between *Arabidopsis* proteins with effectors from two of its pathogens (*Pseudomonas syringae* and *Hyaloperonospora arabidopsidis*) through network analysis (Mukhtar et al., 2011). This work allowed the identification of 165 effector targets in *Arabidopsis* and, importantly, it also allowed the identification of common proteins or immunity processes that are targeted by effectors from two evolutionary distant pathogens. This suggests that unrelated pathogen effectors are evolving to target similar plant

immunity processes (Mukhtar et al., 2011), hinting towards the existence of key immunity hubs in plants. Thus in identifying the targets of effector proteins, we may identify these important hubs as one can hypothesise that those targets can be shared by a number of other unrelated pathogen effectors in a variety of plant-pathogen systems.

In this work we aimed to identify the host proteins targeted by the *P. capsici* effector PcCRN83_152 that was shown to cause cell death when over-expressed in *N. benthamiana* leaves and to enhance *P. capsici* virulence in the same system (Mafurah et al., 2015; Stam et al., 2013a, 2013b). In chapter 3 we aimed to assess if these two phenotypes were connected and showed that PcCRN83_152 mediated cell death is not required for its virulence boosting capacities. Regardless of this distinction, the mechanisms and plant processes that PcCRN83_152 targets in order to achieve these phenotypes are unknown. Thus, in order to identify PcCRN83_152 host targets, we performed an Y2H screen with this effector against a *N. benthamiana* derived cDNA library. Using this assay we identified the interaction of PcCRN83_152 with one *N. benthamiana* protein, NbSLX1, and a tomato protein, SlSIZ1Δ867. The interaction of PcCRN83_152 with NbSIZ1, NbSLX1, and SlSIZ1Δ867 was also confirmed *in planta* by FLIM-FRET experiments.

SIZ1 (for containing both SAP and MIZ domains) proteins encode for E3 SUMO (small ubiquitin-like modifier) ligases. SUMOs were identified for their capacity to bind to a Ras-like GTPase, a protein involved in nuclear pore complex (NPC) function, and mediate its sub-cellular re-localisation (Matunis et al., 1996). SUMOs were named for their small but significant homology to ubiquitin (around 18 % of sequence identity) and their small size (around 11 kDa) (Mahajan et al.,

1997; Matunis et al., 1996). Similarly to ubiquitin, SUMO attachment to its substrates depends on three families of proteins (E1 activating enzymes, E2 conjugating enzymes, and E3 ligases). While E1 and E2 enzymes are sufficient to perform SUMOylation in some cases, E3 protein ligases are thought to facilitate this process by either promoting complex formation between the E2 enzyme and the SUMO substrate or by stimulating the ability of the E2 enzymes to discharge SUMO into its target substrates (Gareau and Lima, 2010).

Since their discovery, SUMOs have been identified in a huge range of eukaryotic organisms, and connected with a diverse range of biological functions (Geiss-friedlander and Melchior, 2007; Johnson, 2004) but mostly with nuclear processes related to transcription regulation (Chymkowitch et al., 2015), genome stability (Nie and Boddy, 2016) and cellular stress responses (Enserink, 2015). A proteomics study aimed at identifying SUMO targets in *Arabidopsis* showed that from the identified SUMO targets with known sub-cellular localisation, 76% were predicted to localise to the nucleus. Consistent with predicted nuclear localisation, the majority of *Arabidopsis* SUMO targets were connected with nuclear functions such as transcription, chromatin modification, RNA-related processes, DNA maintenance and repair, and nuclear pore assembly (Miller et al., 2010).

SUMOylation has been highly connected with plant responses to biotic stresses (van den Burg et al., 2010; Park et al., 2011). In fact, one of the first studies on plant SUMOylation showed that ethylene-inducing xylanase (EIX) from the fungus *Trichoderma viride*, was being SUMOylated in a process that regulated the capacity of EIX to induce cell death on tobacco leaves. Another fact that demonstrates the importance of SUMOs in plant immunity related processes is

the existence of two *Xanthomonas campestris* effectors (XopD and AvrXv4) that mimic the function of plant SUMO proteases and mediate the removal of SUMOs from its substrates *in planta* (Kim et al., 2008; Roden et al., 2004).

Endonucleases, enzymes responsible for hydrolysis of nucleic acids, are involved in many cellular processes such as DNA replication, repair and recombination (Aleksandrushkina and Vanyushin, 2012). Importantly, endonucleases have also been connected with plant immunity. An *Arabidopsis* genetic screen, aimed to characterise mutants with compromised recognition of turnip crinkle virus (TCV) by the *Arabidopsis* R protein (HRT), identified the protein CRT1 (compromised for recognition of Turnip Crinkle Virus) (Kang et al., 2008). Besides interacting with HRT, CRT1 was shown to interact with three other R proteins SSI4, RPS2 and Rx, and to be an important mediator of defence responses triggered by these R proteins (Kang et al., 2008). Subsequently, CRT1 was identified as a nuclear translocated functional endonuclease that has roles not only in effector triggered immunity (ETI) but also in pathogen-associated molecular pattern (PAMP)-triggered immunity, basal resistance, non-host resistance and systemic acquired resistance (Kang et al., 2012).

SLX1 (for Synthetic Lethal of unknown function) proteins were identified in a yeast genetic screen for absence of cell viability in yeast cells lacking Sgs1, a helicase required for genome stability (Mullen et al., 2001). Since then SLX1 functions have been well characterised in yeast and mammal systems (Rouse, 2009). SLX1 was shown to bind SLX4, a multi-domain protein that regulates various proteins involved in genome maintenance and stability, and to be involved in regulating mechanisms such as homologous recombination,

replication fork restart, DNA inter-strand crosslink repair and telomere length control (Nowotny and Gaur, 2016; Rouse, 2009; Schwartz and Heyer, 2011).

Interestingly, both SLX and SIZ1 proteins have been described to work together on regulating genome stability. In yeast cells, SLX5 was shown to interact with SIZ1, leading to SIZ1 ubiquitination and consequent degradation (Westerbeck et al., 2014). Furthermore, SLX1 and SLX4 were shown to be to SUMOylated and this SUMOylation was shown to be required for SLX1 and SLX4 functions (Lian et al., 2016; Ouyang et al., 2015; Sarangi et al., 2014). The connection between SLX and SUMO functions is also demonstrated by the suggestion that SLX4 complexes could act as SUMO E3 ligases (Guervilly et al., 2015). Human SLX4 was shown to interact with a SUMO charged E2 conjugating enzyme (UBC9) and to promote the SUMOylation of SLX4 itself and of the XPF-ERCC1 endonuclease involved in DNA repair and recombination (Guervilly et al., 2015).

In this work we show that PcCRN83_152 interacts with NbSIZ1, SlSIZ1Δ867, and NbSLX1 in plant nuclei. Moreover we show that NbSIZ1 and SlSIZ1Δ867 have roles on *P. capsici* virulence. Further work focused on characterising the mechanisms of PcCRN83_152 interaction with its targets, and taking advantage of the available PcCRN83_152 NCD variants described in Chapter 3 of this thesis, could unveil novel mechanisms of plant immunity and provide novel understanding of the strategies used by *P. capsici* to subvert plant defences.

Methods

Y2H assays

Y2H screening was performed according to manufacturer's instructions using the Dualsystems Y2H system (Biotech). PcCRN83_152 C-terminal domain amplified as described in Stam et al. (2013b) was cloned into the bait vector pLexAN (with the LexA DNA binding domain) and transformed into the yeast strain NMY51. Yeast cells expressing PcCRN83_152 C-terminal domain were then transformed with an available cDNA library (obtained from a mixed library of *N. benthamiana* leaves infected with *P. capsici, P. infestans* and aphids) in the vector pGAD-HA (with the GAL4 activation domain). Transformants were selected on dropout media lacking leucine, histidine and thymine, and complemented with 2.5 mM of the competitive inhibitor 3-amino-1,2,4-triazole (3-AT), to supress possible leaky expression of the HIS3 reporter gene. Yeast colonies growing on this selective media were then subjected to a galactosidase assay to assay for LacZ gene activation according to manufacturer's instructions (Dualsystemsbiotech). Positive bait constructs were recovered and transformed into *Escherichia coli* (*E. coli*) Mach1 cells and sequenced using a vector specific forward primer GAL4AD (5'- AATACCACTACAATGGAT-3'). Two full length putative targets (SlSIZ1Δ867 and NbSLX1) were cloned (as described below) into pGAD-HA and transformed along with the control protein Lamin–C (also in pGAD-HA) into NMY51 yeast strains expressing PcCRN83_152 C-terminal domain. Transformants were subsequently tested for growth in the selective media and for LacZ gene activation as described above.

Identification and cloning of PcCRN83_152 candidate targets

Sequencing data for PcCRN83_152 putative targets was analysed using Codoncode aligner software package version 4.2.3 (CodonCode Corporation). Putative targets were identified by performing a BLASTN of the sequencing contigs against the *N. benthamiana* genome v1.0.1 predicted cDNAs (https://solgenomics.net/organism/Nicotiana_benthamiana/genome) and against the *Phytophthora capsici* v11 finished cDNAs dataset (http://genome.jgi.doe.gov/Phyca11/Phyca11.home.html). When more than one predicted gene was recovered from the BLAST analysis the candidate with highest BLAST score was selected (Table 1). For comparison with *Arabidopsis* versions, BLASTP was performed (https://www.arabidopsis.org/Blast/). Protein domains were described by searching the Pfam database (version 30.0) (http://pfam.xfam.org/).

SlSIZ1Δ867 (Solyc11g069160.1.1Δ867) was amplified from *Solanum lycopersicum* cDNA using the primers SUMOF (5'-CACCATGGATTTGGTTGCTA-3') and SUMOR (5'-CTTGGTCTTACAGAACGACGTTGA-3'). NbSLX1 (Niben101Scf17482g00013.1) and NbSIZ1 (Niben101Scf04549g09015.1) were amplified from *Nicotiana benthamiana* cDNA using the primers: ENDOF (5'-CACCATGGGGAAACGGAAG-3') and ENDOR (5'-TTACATCAGACAAAAATAGGTGTCTG-3'); and SOL_F (5'-CACCATGGATTTGGTTGCTAGTTGC-3') and TGAC_R (5'-CTATCCAGAATCCGAATCAATACTT-3') respectively. All the forward primers were designed to contain the four base pair sequence (CACC) at the 5' end to allow directional cloning using the Gateway cloning system (Invitrogen). These amplified fragments were then recombined into the pENTR D-TOPO vector

(Invitrogen). pENTR D-TOPO constructs were sequence verified and used for recombination into appropriate destination vectors via Gateway LR reactions (Invitrogen). Constructs in destination vectors were again sequence verified and transformed into the *Agrobacterium tumefaciens* strain AGL1.

Plant growth conditions

Nicotiana benthamiana plants were grown in a greenhouse under 16 hours of light and a temperature of approximately 25/22 °C (day/night). *Arabidopsis thaliana* plants were grown in a growth cabinet at 22°C, 16 hours of light and 70% of humidity. The plants were kept in these conditions during all experiments unless stated otherwise.

***Agrobacterium* growth and infiltration conditions**

All constructs were prepared for infiltration as described in Stam et al. (2013b). In brief, *A. tumefaciens* AGL1 cells carrying the appropriate constructs were grown in liquid Lysogeny broth (LB) supplemented with appropriate antibiotics at 28 ^{o}C (shaking at 225 revolutions per minute (RPMs)) until mid-log phase. Optical Densities (ODs) were measured at 600 nm and *Agrobacterium* cells were pelleted and re-suspended in infiltration media (10 mM MgCl2 and 150 µM acetosyringone) to achieve the desired ODs. Subsequently, these re-suspensions were infiltrated into four to five week old *N. benthamiana* leaves.

BiFC assays

For BiFC assays, constructs were cloned into the BiFC vectors pCL112 (PcCRN79_188; PcCRN20_624; PcCRN12_997; PcCRN83_152) and pCL113 (NbSLX1, SlSIZ1Δ867, SlTCP14-2) (Bos et al., 2010). Control proteins (PcCRN79_188; PcCRN20_624; PcCRN12_997; and SlTCP14-2) were cloned as previously described (Stam et al., 2013b, 2013c). Final ODs of 0.05 were used for all constructs and confocal imaging was performed 48 hours post infiltration on a Zeiss LSM 710 confocal microscope with a W Plan-Apochromat 40X /1.0 DIC M27 water dipping lens and with an excitation wavelength required for YFP detection of 514 nm.

FLIM-FRET assays

For FLIM-FRET assays, SlSIZ1Δ867 and NbSIZ1 were used in an RFP vector (pK7WGR2); PcCRN83_152 and PiRXLR04145 (Zheng et al., 2014) were used in a GFP vector (pB7WGF2); and NbSLX1 was used in both described GFP and RFP vectors. All the constructs were used at an *Agrobacterium* OD of 0.2, except for PcCRN83_152-GFP that was also used at an OD of 0.05 due to high levels of GFP signal. Plant growth conditions and FLIM-FRET measurements were performed as described in Le Roux et al. (2015).

Co-localisation assays

For co-localisation assays, constructs were infiltrated at a final OD of 0.1. NbSLX1, NbSIZ1 and SlSIZ1Δ867 were used in an RFP vector (pK7WGR2). PcCRN83_152 was used in a GFP vector (pB7WGF2). Images were collected 48 post infiltration on a Zeiss LSM 710 confocal microscope with a W Plan-

Apochromat 40X /1.0 DIC M27 water dipping lens using the following settings: 488 nm excitation and 400-600 nm emission for GFP; and 561nm excitation and 600–700 nm emission for RFP.

TRV-based VIGS in *N. benthamiana*

Virus-induced gene silencing (VIGS) fragments were amplified from NbSIZ1 and NbSLX1 clones using the primers: Sumo_Vigs_Phusion_Frag3_F (5'-AAGGTTACCGAATTCTCTAGACAGCAAACCGGAAGACCAG-3') and Sumo_Vigs_Phusion_Frag3_R (5'-GGGACATGCCCGGGCCTCGAGTTCAGCATCACCTGCTGGTA-3') for NbSIZ1; and Endo_frag1_F (5'-AAGGTTACCGAATTCTCTAGAGAAGGATCTGCAGGATCCAC-3'), Endo_frag1_R (5'-GGGACATGCCCGGGCCTCGAGTAAATGCTAGGGGATTATCAACTAAA-3'), Endo_frag2_F (5'-AAGGTTACCGAATTCTCTAGATACAACCAATGATTGGGACAA-3'), Endo_frag2_R (5'-GGGACATGCCCGGGCCTCGAGTACCATGTAGCATGGAGGAGT-3'), Endo_frag3_F (5'-AAGGTTACCGAATTCTCTAGACAGAAAGCAGATTCTTCGCC-3') and Endo_frag3_R (5'-GGGACATGCCCGGGCCTCGAGATCATTGGTTGTATACTCATTCTTGTC-3') for the three NbSLX1 fragments respectively. For negative control a GFP fragment was amplified using the primers eGFP_Fw (5'-TACCGAATTCTCTAGATGACCCTGAAGTTCATCTGC-3') and eGFP_Rv (5'-

ATGCCCGGGCCTCGAGGAAGAAGTCGTGCTGCTTC-3'). Amplified fragments were cloned into the Tobacco Rattle Virus vector (pTRV2) (Ratcliff et al., 2001) using the In-Fusion HD cloning kit (Clontech). *Agrobacterium strains* containing desired pTRV2 constructs were mixed 1:1 with strains containing pTRV1 vector and re-suspended to a final OD of 0.5 in infiltration media (10 mM 2-(*N*-Morpholino) ethane sulfonic acid (MES), 10 mM $MgCl_2$ and 250 µM acetosyringone). Cultures were then incubated for 3 hours in the dark at room temperature followed by infiltration into 4-leaf-stage *N. benthamiana* plants. Two weeks post-infiltration, leaves were detached, analysed by quantitative reverse-transcriptase PCR (qRT-PCR), and used for *P. capsici* infection assays (described below). qRT-PCR analyses to assess silencing levels of NbSIZ1 were performed using Universal ProbeLibrary System Technology (Roche) in a StepOnePlus machine (Applied Biosystems). The primers used were NbSiz1_qPCR_F (5'-ACTTACTCGCGTTCGTCGTT-3') and NbSiz1_qPCR_R (5'-ATCACTATCGGCATTTTCAGTG-3') for NbSIZ1 expression. Expression levels of NbSIZ1 were compared to levels of the constitutive gene tubulin using the primers designed against tomato tubulin Tomato_Tub_F (5'-CATGGCTTGCTGTCTCATGT-3') and Tomato_Tub_R (5'-CCACAGCAGCATTAACATCC-3').

P. capsici growth and infection assays

Phytophthora capsici strains LT1534 and LT263 were grown on V8 agar plates at 24 °C in the dark for 3 days. These plates were then moved to a continuous light incubator at 24 °C and incubated for further 3 days in order to stimulate sporangia formation. Zoospores were obtained by flooding *P. capsici* plates

with ice-cold water to dislodge sporangia that was subsequently collected and incubated in light for between 20 to 60 minutes to stimulate zoospore release.

To assess the effect of PcCRN83_152 targets over-expression in *P. capsici* virulence, NbSIZ1 was used in the RFP vector (pK7WGR2) and compared against free RFP (empty pK7WGR2). SlSIZ1∆867 and NbSLX1 were used in the RFP vector (pGWB461) and compared to free RFP (empty pGWB461). All constructs were mixed 1:1 with *A. tumefaciens* AGL1 cells carrying the silencing suppressor p19 and re-suspended to a final OD of 0.5 for NbSIZ1 and free RFP (pK7WGR2), and of 0.25 for NbSLX1, SlSIZ1∆867 and free RFP (pGWB461). Two days after infiltration, leaves were drop inoculated with either 5 µL (NbSLX1, SlSIZ1∆867 and free RFP (pGWB461)) or 10µL (NbSIZ1 and free RFP (pK7WGR2)) of zoospore suspension (250,000 spores per mL) of the strain LT1534. Lesion diameters were measured 3 days post inoculation.

For VIGS, leaves were collected two weeks post-infiltration and drop-inoculated with 5µL of zoospore suspension (50,000 spores per mL) of the strain LT1534. Lesion diameters were measured 2 and 3 days post inoculation.

For *Arabidopsis* infection, four weeks old plants were spray inoculated with a spore suspension of 100,000 spores/ml of the *P. capsici* strain LT263. Infection was assessed eight days after spray inoculation.

Infection of *N. benthamiana* transgenic plants was achieved by spray inoculation of whole plants with a spore suspension of 50,000 spores/ml of the *P. capsici* strain LT1534. Infection was assessed three days after spray inoculation.

Cell death assays

For cell death assays SlSIZ1Δ867, NbSIZ1 and NbSLX1 and free RFP (EV) were used in the RFP vector pK7WGR2. PcCRN83_152 C-terminal domain and free GFP (EV) were used in the GFP vector (pB7WGF2). The described *Agrobacterium* cultures were mixed with *A. tumefaciens* AGL1 cells carrying the silencing suppressor p19 and re-suspended to a final OD of 1 for p19, SlSIZ1Δ867, NbSIZ1, NbSLX1 and free RFP, and 0.25 for PcCRN83_152 and free GFP. Cell death levels were scored three, four and five days post infiltration using a CD scale described in Stam et al. (2013b). Tissues for Western blot analysis were collected two days post-infiltration.

Protein stability assays

For protein stability assays, NbSLX1, SlSIZ1Δ867 and SlTCP14-2 were used coupled with an N-terminal FLAG tag using the vector pGWB12. PcCRN83_152, PiRXLR04145 and free GFP (EV) were used in a GFP vector (pB7WGF2). All constructs were mixed with *A. tumefaciens* AGL1 cells carrying the silencing suppressor p19 and re-suspended to a final OD of 0.5 for p19, SlSIZ1Δ867, NbSLX1 and free RFP, and 0.25 for PcCRN83_152 and free GFP. Leaf samples were collected two, three and four days post-infiltration.

Western Blotting

Protein extractions were done as in Stam et al. (2013a). Protein extracts were run on Biorad TGX gels before being transferred on PVDF membranes using Biorad Trans Blot Turbo Transfer System. Blots were blocked for 30 minutes with 5% milk in TBS-T (0.1 % Tween 20) and probed with primary antibodies

against GFP (Santa Cruz) (1:2500), FLAG (Santa Cruz) (1:2500), or RFP (1:1000) (Chromoteck). Secondary antibodies used were anti-Mouse-HRP antibody (Santa Cruz) (1:20000) or anti-RAT-HRP antibody (Santa Cruz) (1:20000). Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged on a Syngen GBox TX4 Imager. After imaging, for visualisation of total protein levels, membranes were treated with Imperial protein stain (Thermo Scientific) according to manufacturers' instructions.

Generation of *N. benthamiana* transgenic plants

N. benthamiana transgenic plants were generated via an *Agrobacterium* mediated transformation protocol adapted from Horsch et al. (1985). In summary, *N. benthamiana* leaves were infiltrated with *Agrobacterium* Agl1 cells carrying the desired constructs (NbSIZ1 and RFP) in the vector pK7WG2 and with ODs varying between 0.4 and 0.8. Leaf discs were subsequently cut and transformants were selected in plates containing Kanamycin (100 mg/ml). To assess levels of NbSIZ1 expression qRT-PCR analyses were performed with the conditions and primers described above.

Results

Y2H screen identified two putative host targets of PcCRN83_152

PcCRN83_152 was shown to cause a strong cell death phenotype when expressed in *N. benthamiana* leaves and to enhance *P. capsici* virulence in the

same system (Stam et al., 2013b). Silencing of this CRN in *P. capsici* was also shown to reduce pathogen virulence in *N. benthamiana* and *Arabidopsis*, a phenotype recovered by ectopic expression of PcCRN83_152 (Mafurah et al., 2015). Moreover, PcCRN83_152 shows an uneven sub-nuclear localisation pattern and is able mediate host chromatin re-localisation in plant nuclei (Stam et al., 2013a, 2013b).

Despite these interesting features, the mechanisms underlying PcCRN83_152 virulence and cell death phenotypes have not yet been described. As a first step to unveil these mechanisms, an Y2H screen was performed using a library of candidate targets generated from *N. benthamiana* leaves challenged with *P. capsici, P. infestans* and aphids. With this screen, nine different candidate target proteins of PcCRN83_152 were identified (Table 1).

Table 1 Summary of putative interactors of PcCRN83_152 in yeast

Protein candidate Targets	Hits	Pfam domains
E3 SUMO-protein ligase SIZ1 (Niben101Scf04549g09015.1)	3	zf-MIZ (pfam02891); SAP (pfam02037); PHD (pfam00628)
Structure-specific endonuclease subunit SLX1 (Niben101Scf01771g01024.1)	2	GIY-YIG (pfam01541)
Dynein light chain 2 (Niben101Scf06556g00002.1)	2	Dynein_light (pfam01221)
Zinc finger protein (Niben101Scf02509g06003.1)	2	zf-H2C2_2 (pfam13465)
4-hydroxy-3-methylbut-2-enyl diphosphate reductase (Niben101Scf33689g00006.1)	2	LytB (pfam02401)
Inactive poly [ADP-ribose] polymerase	1	RST

(Niben101Scf02375g00013.1)		(pfam12174)
Uncharacterised protein (jgi|Phyca11|103964|e_gw1.8.750.1)	1	
14-3-3-like protein GF14 (Niben101Scf00477g00017.1)	1	14-3-3 (pfam00244)
General regulatory factor 12 (Niben101Scf09559g02008.1)	1	14-3-3 (pfam00244)

From these candidate targets, two proteins, a tomato E3 SUMO ligase ((*Sl*SIZ1Δ867) (Solyc11g069160.1.1Δ867)) and a structure-specific endonuclease subunit SLX1 ((*Nb*SLX1) (Niben101Scf17482g00013.1)) were able to activate all three reporter genes (*HIS3*, *ADE2* and *LacZ*) in yeast cells co-expressing PcCRN83_152 (Figure 1). This allowed growth of the yeast in media lacking histidine and adenine, and expression of β-galactosidase, resulting in a blue colouration of the yeast colonies subjected to a β-galactosidase assay. The activation of all three reporter genes suggests genuine interaction between PcCRN83_152 and these two candidate interactors in yeast. Activation of the reporter genes did not occur when PcCRN83_152 targets were co-expressed with the human control protein Lamin C (Figure 1).

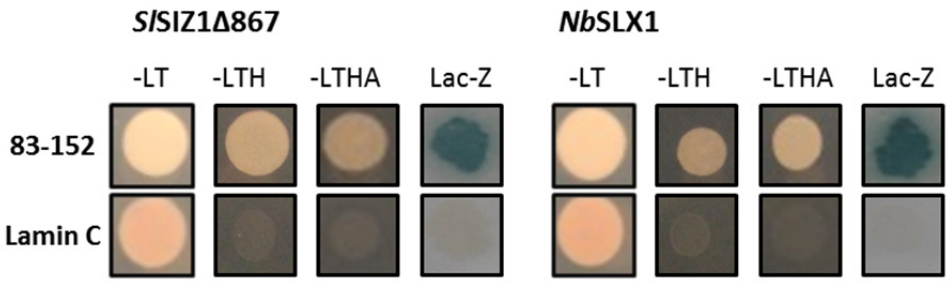


Figure 1 SlSIZ1∆867 and NbSLX1 specifically interact with PcCRN83_152 in yeast. Yeast colonies co-expressing the targets (SlSIZ1∆867 and NbSLX1) and PcCRN83_152 grew on dropout media lacking leucine, thymine and histidine supplemented with 1mM of 3-AT (-LTH) and media lacking leucine, thymine, histidine and adenine (-LTHA) and showed blue colour under β-galactosidase assay. Conversely, yeast colonies co-expressing the same targets and the control protein Lamin C did not grow on selective media and showed no blue colouration under the β-galactosidase assay.

Sequencing of the yeast clones recovered from the Y2H screen allowed the identification of the genes encoded by these clones via a BLASTN analysis against the *P. capsici* and *N. benthamiana* genomes.

Initially, due to difficulties in amplifying the *N. benthamiana* NbSIZ1 gene, a tomato SlSIZ1 (Solyc11g069160.1.1) gene was cloned instead. This tomato gene was selected for cloning as it showed the best identity to the NbSIZ1 gene recovered from the Y2H screen (Niben101Scf04549g09015.1). However, due to an error on primer design the last 10 amino acids of this tomato version are missing and are substituted by vector sequence (Solyc11g069160.1.1∆867). In subsequent trials, an NbSIZ1 gene highly similar to the gene picked up in the Y2H clones (Niben101Scf04549g09015.1) was cloned. To assess if the cloned NbSIZ1 was the closest homolog to the well described *Arabidopsis* AtSIZ1 gene a BLASTP analysis was performed with the NbSIZ1 gene against the *Arabidopsis* predicted proteome (https://www.arabidopsis.org/Blast/) showing that the best BLAST hit on the *Arabidopsis* proteome corresponded to the well characterised AtSIZ1 (AT5G60410.2). However, when BLASTing AtSIZ1 back

to the *N. benthamiana* genome, the best BLAST hit did not correspond to our cloned sequence but rather to other NbSIZ1 version (Niben101Scf15836g01010.1). Thus, our cloned version of NbSIZ1 and the characterised AtSIZ1 are not reciprocal best BLAST hits. This may be due to incorrect *N. benthamiana* genome annotations as there are five genes that have similarity to AtSIZ1 and are annotated to be SIZ1 proteins in the *N. benthamiana* genome, while in *Arabidopsis* only one gene is annotated as SIZ1. For clarity, a protein alignment of AtSIZ1, NbSIZ1 and SlSIZ1Δ867 is available in Supplementary figure 1. Also, an alignment of the five annotated SIZ1 proteins annotated in the *N. benthamiana* genome is available in Supplementary figure 2. Nonetheless, Pfam searches showed that the cloned NbSIZ1 contains the two domains that are characteristic of SIZ1 proteins (SAP and MIZ domains). Further functional assays are required to test if the cloned NbSIZ1 is a functional E3 SUMO ligase with biological roles equivalent to AtSIZ1.

In relation to the NbSLX1 gene, primers were designed to amplify it as identified by BLASTing of the yeast clone sequences (Niben101Scf01771g01024.1). However the gene amplified when re-BLASTed to the *N. benthamiana* genome is more similar to another NbSLX1 variant (Niben101Scf17482g00013.1). A protein alignment of the cloned NbSLX1 and the two variants predicted to be encoded by the *N. benthamiana* genome can be seen in supplementary figure 3. Again to assess if the cloned NbSLX1 is a functional endonuclease with roles similar to other SLX1 proteins described in yeast and mammal systems, further experiments are required. However, Pfam searches find an endonuclease domain (GIY-YIG) in the cloned NbSLX1 protein.

SlSIZ1∆867, NbSIZ1 and NbSLX1 interact with PcCRN83_152 in planta:

SlSIZ1∆867 and NbSLX1 were shown to interact with PcCRN83_152 in yeast. However, Y2H screens are prone to the identification of false positive interactions due to, among other reasons, possible incorrect folding in yeast of the proteins involved in the tested interactions (Brückner et al., 2009). Thus, an alternative method to confirm these interactions *in planta* is required.

One of the most commonly used methods aimed at confirming protein-protein interactions *in vivo* is Co-immunoprecipitation (Co-IP) (Phizicky and Fields, 1995). Multiple Co-IP experiments were performed to confirm the interaction of PcCRN83_152 and its putative targets (data not shown). However, a clear interaction between this CRN effector and its putative targets was never observed using this method, raising the possibility that the tested interactions are not taking place *in planta* or are taking place in a weak and/or transient manner.

Besides co-IPs, other methods to assess protein-protein interactions were used to test PcCRN83_152 interactions *in planta*. The first of these was Bimolecular fluorescence complementation (BiFC) (Bhat et al., 2006; Kerppola, 2006), also called Split YFP assay. This method is based on fusing the two proteins for which the interaction is to be tested to the N- and C-terminal fragments of yellow fluorescent protein (YFP). Individually, the YFP fragments are non-functional, so close proximity of the two tested proteins is required for YFP reconstitution and fluorescence (Bhat et al., 2006).

In our experiments we saw reconstitution of YFP fluorescence in the combination of PcCRN83_152 and its putative targets (SlSIZ1∆867 and

NbSLX1). However, this reconstitution was not specific for PcCRN83_152, as three other CRNs (PcCRN79_188; PcCRN20_624; and PcCRN12_997), were also capable of reconstituting fluorescence. Furthermore, PcCRN83_152 co-expressed with a transcription factor SlTCP14-2 also showed reconstituted fluorescence. To assess if these re-constitutions were more specific to the putative interactions in a quantitative manner, nuclei showing fluorescence were counted (Figure 2). Despite differences observed in the number of fluorescent nuclei present across different protein combinations, these appear to be more construct dependent and do not point to any specific interaction *in planta*.

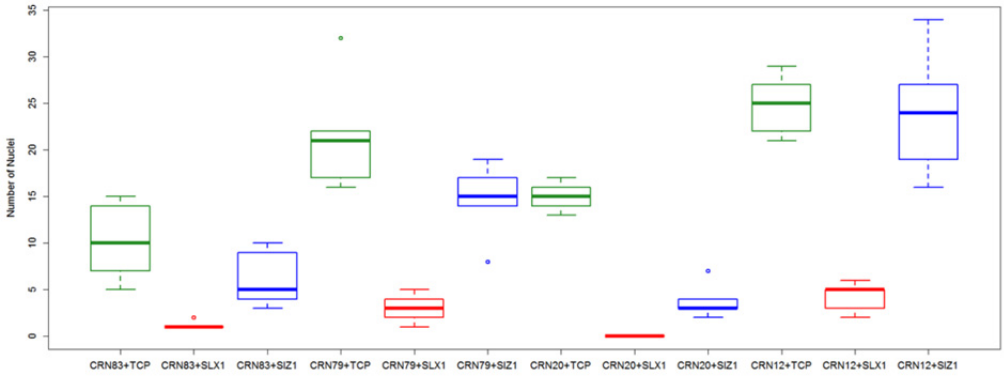


Figure 2 BiFC assays are inconclusive regarding PcCRN83_152 interactions *in planta*. PcCRN83_152 (CRN83) was co-expressed with two of its putative candidate targets SlSIZ1Δ867 (SIZ1) and NbSLX1 (SLX1) and with a tomato transcription factor SlTCP14-2 (TCP). As controls PcCRN79_188 (CRN79), PcCRN20_624 (CRN20) and PcCRN12_997 (CRN12) were co-expressed with the same plant proteins. Number of nuclei showing reconstituted YFP fluorescence were counted in five randomly picked confocal planes for each of the protein combinations tested.

Fluorescence lifetime imaging (FLIM) can be used to measure Förster resonance energy transfer (FRET) allowing the study of protein-protein interactions in a method named FLIM-FRET (Sun et al., 2011b). This method is based on two fluorophores, one donor and one acceptor. When the two fluorophores are in close proximity (distances smaller than 10 nm), energy is transferred from the donor to the acceptor causing a measurable decrease on

the donor fluorescence lifetime (Bücherl et al., 2014). Using FLIM-FRET we showed that PcCRN83_152 interacts with NbSLX1 (Figure 3a), NbSIZ1 (Figure 3b) and SlSIZ1Δ867 (Figure 3c) *in planta* (Table 2).

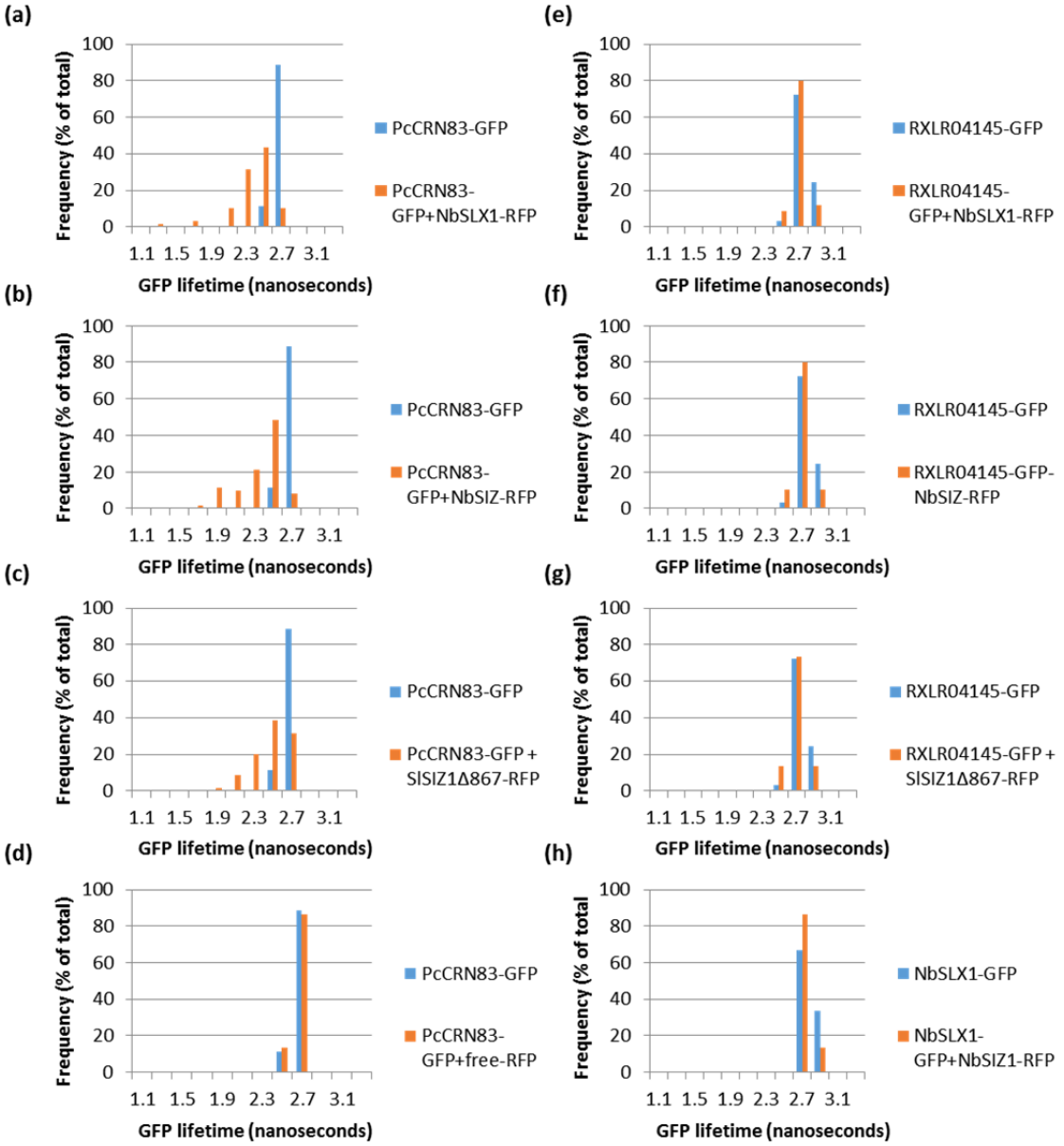


Figure 3 FLIM-FRET analysis shows that PcCRN83_152 interacts with NbSLX1, NbSIZ1 and SlSIZ1Δ867 *in planta*. PcCRN83_152 and PiRXLR04145 were expressed alone or in combination with NbSLX1, NbSIZ1, SlSIZ1Δ867 and free RFP in *N. benthamiana* leaves. NbSLX1 was also expressed alone or with NbSIZ1 in *N. benthamiana* leaves (h). Histograms show the distribution of nuclei (%) according to GFP lifetime.

These interactions did not occur between PcCRN83_152 targets and a nuclear localised and cell death inducer RXLR effector (PiRXLR04145) (Figure 3e, f and g), and between PcCRN83_152 and free RFP (Figure 3d), suggesting specificity of PcCRN83_152 interactions. Moreover, we assessed if the two PcCRN83_152 *N. benthamiana* targets could be interacting with each other and this was not the case (Figure 3h).

Table 2 FRET-FLIM analysis shows that PcCRN83-152 physically interacts with NbSLX1, NbSlZ1 and SlSlZ1Δ867 in nuclei of *N. benthamiana*

Donor	**Acceptor**	**t [(a)]**	**SD[(b)]**	**Dt[(c)]**	**N[(d)]**	**E[(e)]**	**p-value [(f)]**
PcCRN83-152-GFP	-	2.555	0.057	-	160	-	-
PcCRN83-152-GFP	NbSLX1-RFP	2.285	0.249	270	60	10.57	1.5×10^{-28}
PcCRN83-152-GFP	NbSlZ1- RFP	2.258	0.219	297	62	11.65	3.6×10^{-38}
PcCRN83-152-GFP	SlSlZ1Δ867- RFP	2.366	0.194	208	60	8	4×10^{-19}
CRN83-152-GFP	Free RFP	2.566	0.064	-11	60	-	0.24
PiRXLR04145-GFP	-	2.651	0.064	-	90	-	-
PiRXLR04145-GFP	NbSLX1-RFP	2.607	0.083	44	60	1.66	3.9×10^{-4}
PiRXLR04145-GFP	NbSlZ1- RFP	2.593	0.076	58	40	2.21	1.5×10^{-5}
PiRXLR04145-GFP	SlSlZ1Δ867- RFP	2.603	0.08	77	60	2.8	4×10^{-6}
NbSLX1- GFP	-	2.693	0.053	-	30	-	-
NbSLX1- GFP	NbSlZ1- RFP	2.626	0.064	67	30	2.5	4×10^{-5}

[a]Mean lifetime in nanoseconds. For each nucleus, average fluorescence decay profiles were plotted and fitted with exponential functions using a nonlinear square estimation procedure. Mean lifetime was calculated according to $t = S\ a_i t_i^2 / S\ a_i t_i$ with $I(t) = S\ a_i\ e^{-t/ti.}$
[b]Standard deviation
[c]Dt= tD-tDa, where tD is the lifetime in the absence of the acceptor and tDA is the lifetime of the donor in the presence of the acceptor
[d]Total number of measured nuclei
[e]Percentage of FRET efficiency (E = 1 - tDA/tD)
[f]*P* value of the difference between the donor lifetimes in the presence and in the absence of the acceptor (Student's *t*-test)

PcCRN83_152 co-localises with its targets in the host nucleus

PcCRN83_152 localises in the host nucleus where it mediates chromatin re-localisation (Stam et al., 2013a). To assess the location at which the interactions of PcCRN83_152 with its confirmed proteinaceous targets were taking place, confocal microscopy experiments were performed. These experiments revealed that all PcCRN83_152 protein targets localised to the host nuclei (Figure 4). Importantly, PcCRN83_152 was shown to co-localise with its targets inside the plant nucleus, further pointing to an interaction of these host proteins with PcCRN83_152 occurring in the host nucleus. SlSIZ1Δ867 and NbSIZ1 showed identical sub-nuclear localisation patterns (in the nucleoplasm with sporadic detection of higher signal spots). Interestingly in some nuclei and in the presence of PcCRN83_152, both SlSIZ1Δ867 and NbSIZ1 showed re-localisation into the sub-nuclear areas where PcCR83_152 is absent. In this phenotype, PcCRN83_152 targets are re-localising identically to DNA in the presence of PcCRN83_152, indicating a possible binding of PcCRN83_152 targets to DNA and suggesting a possible role of PcCRN83_152

in controlling the binding of their host targets to DNA.

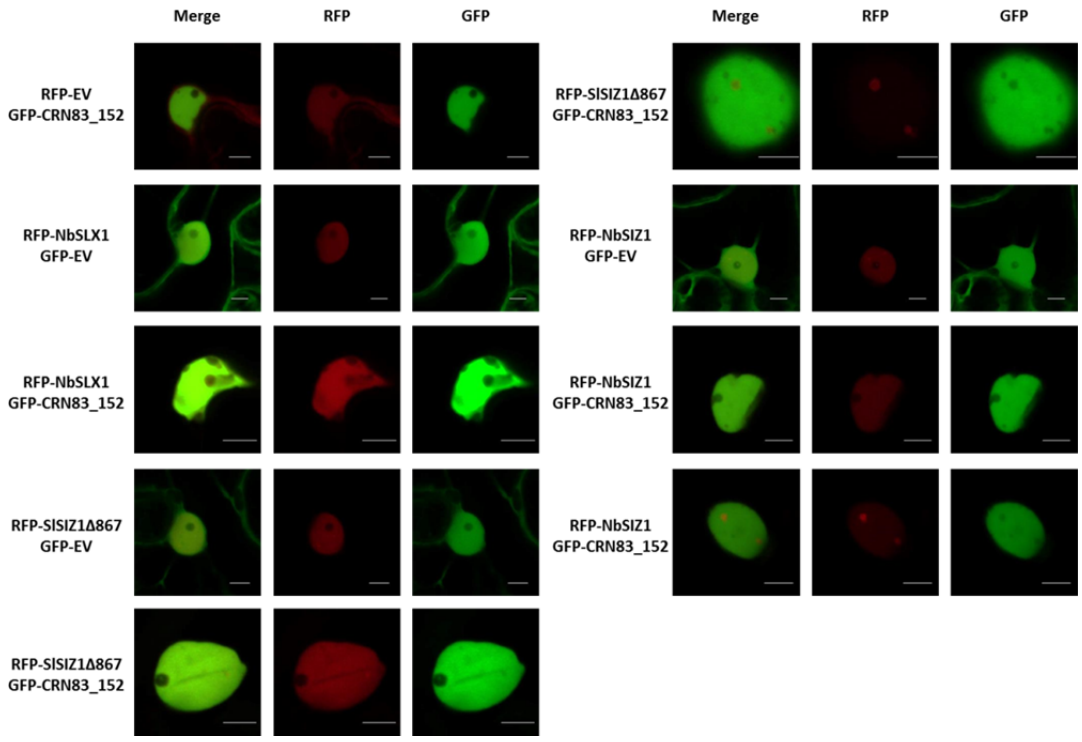


Figure 4 PcCRN83_152 co- or re-localises its plant targets. PcCRN83_152 and free GFP were co-expressed with PcCRN83_152 plant targets or free RFP in *N. benthamiana* leaves. Images were collected by confocal microscopy 48 hours post-infiltration. Scale bar indicates 5 µm.

SlSLX1 is down regulated during *P. capsici* infection

Microarray analyses of the *P. capsici*–tomato interaction were previously conducted in our lab (Jupe et al., 2013), and were used here to investigate the expression of PcCRN83_152 putative targets *in planta*. Interrogation of this microarray data lead to the observation that the expression of both PcCRN83_152 putative targets SlSIZ1 and SlSLX1 (the best BLAST hit from NbSLX1 in the tomato genome) seem to be slightly downregulated at the later stages of infection (Figure 5).

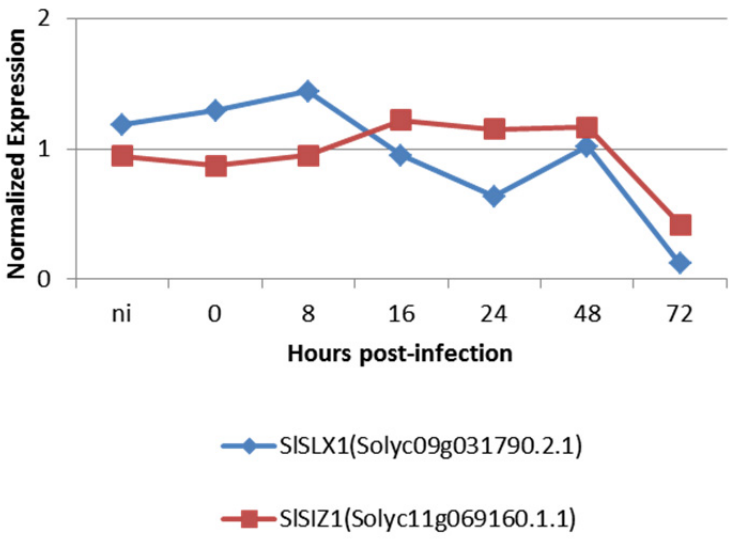


Figure 5 Expression profile of PcCRN83_152 targets over a *P. capsici* infection time course on tomato leaves. Expression was obtained by interrogating an available dataset (Jupe et al., 2013). Expression levels showed are normalized against the expression of the constitutive control tomato gene β-tubulin.

However, one-way ANOVA analysis (using Benjamini and Hochberg multiple testing correction, P≤0.005) (Jupe et al., 2013)), showed that only SlSLX1 was statistically significantly downregulated during *P. capsici* infection and only from 48 to 72 hours post-infection.

NbSIZ1 and SlSIZ1Δ867 have an impact on *P. capsici* virulence.

To study the importance of PcCRN83_152 targets on *P. capsici* virulence, we over-expressed these targets in *N. benthamiana* leaves that subsequently were drop-inoculated with a suspension of *P. capsici* spores. Measuring the diameters of visible *P. capsici* growth enabled us to conclude that NbSIZ1 and SlSIZ1Δ867 over-expression reduced the size of *P. capsici* lesions (Figure 6). NbSLX1 over-expression appears to also impair *P. capsici* virulence, but this phenotype lacks consistency across different experiments.

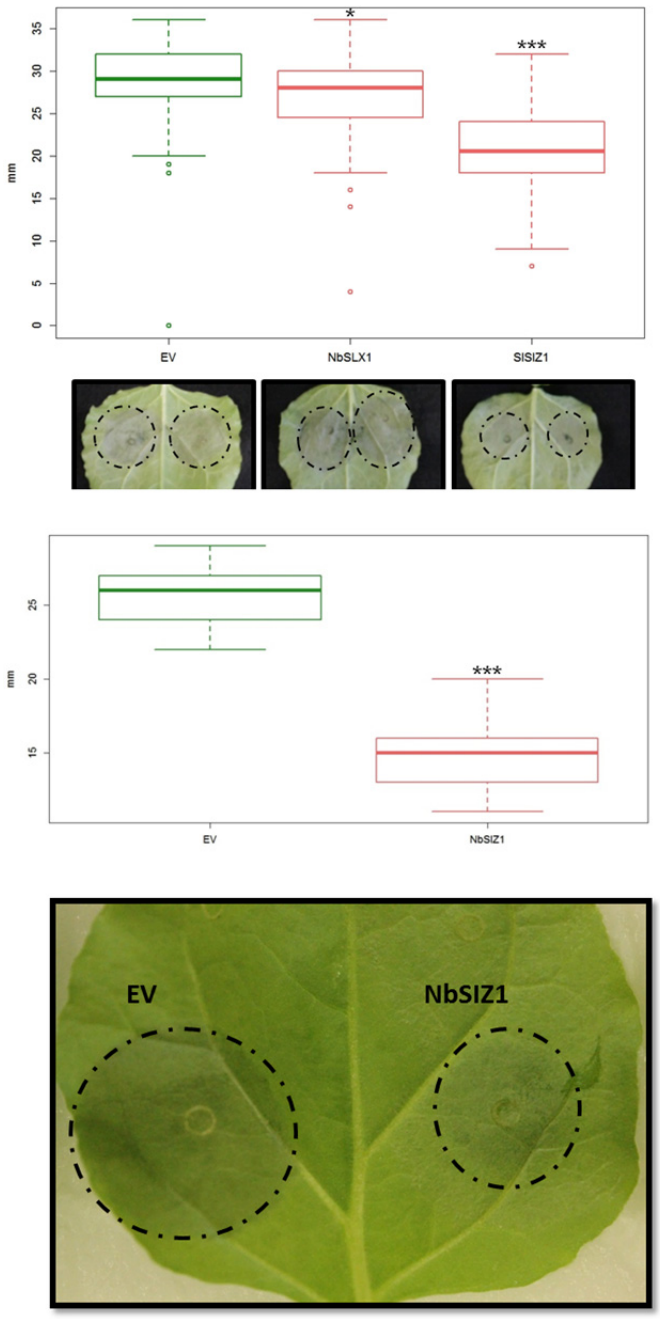


Figure 6 Impact of PcCRN83_152 targets on *P. capsici* virulence. Leaves infiltrated with constructs encoding NbSIZ1, SlSIZ1Δ867 and NbSLX1 RFP-tagged or free RFP (EV) were drop-inoculated with a solution of *P. capsici* zoospores two days post-infiltration. Lesion diameters were measured three days post-infection. Photos show representative leaves three days post-infection. "***" indicate a significant difference ($p<0.001$, t-test). "*" indicate a significant difference ($p<0.05$, t-test).

To further assess possible functions of PcCRN83_152 targets in plant immunity against *P. capsici,* we silenced NbSIZ1 in *N. benthamiana* using virus-induced gene silencing (VIGS) (Lu et al., 2003). We analysed silencing levels using qRT-PCR and observed that silencing was taking place (Figure 7a). We also observed that silencing of NbSIZ1 reduced the virulence of *P. capsici* in *N. benthamiana* leaves (Figure 7b).

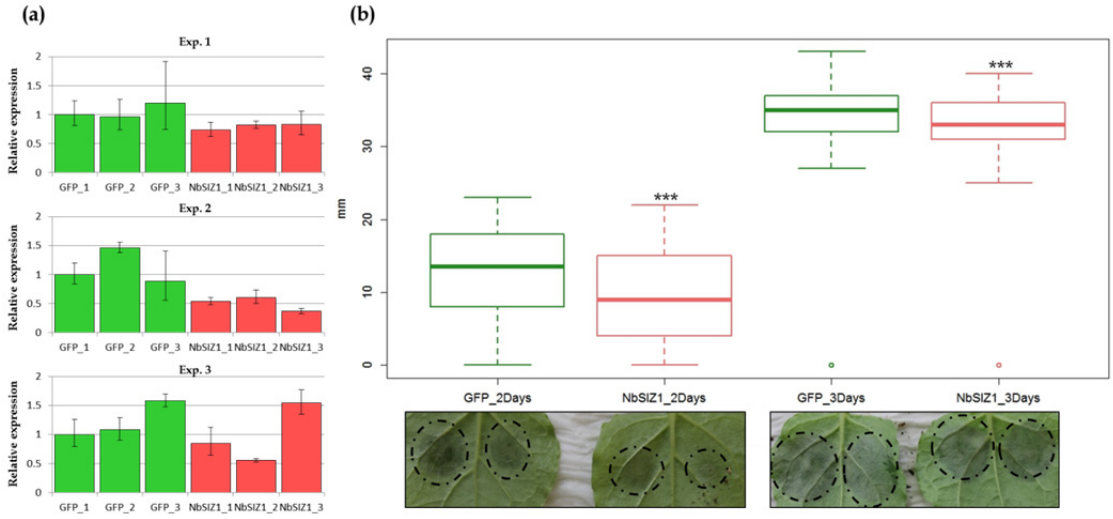


Figure 7 Impact of NbSIZ1 silencing on *P. capsici* virulence. (a) Three independent VIGS experiments were performed aiming for NbSIZ1 silencing using a GFP fragment as a negative control. cDNA obtained from three independent leaves was used for qRT-PCR analyses. Histogram shows normalised expression against the expression of tubulin and compared with one of the leaves from the negative control. (b) Leaves that were not collected for qRT-PCR analysis were drop-inoculated with a solution of *P. capsici* zoospores and lesion diameters were measured two and three days post-infection. Photos show representative leaves two and three days post-infection. "***" indicate a significant difference ($p<0.001$, t-test).

The same experiment was performed for NbSLX1. However, for this protein silencing levels were not assessed. Thus, despite the absence of virulence differences on *N. benthamiana* leaves putatively silenced for NbSLX1 using three independent constructs, we cannot take definite conclusions on the virulence impact of NbSLX1 silencing (Figure 8).

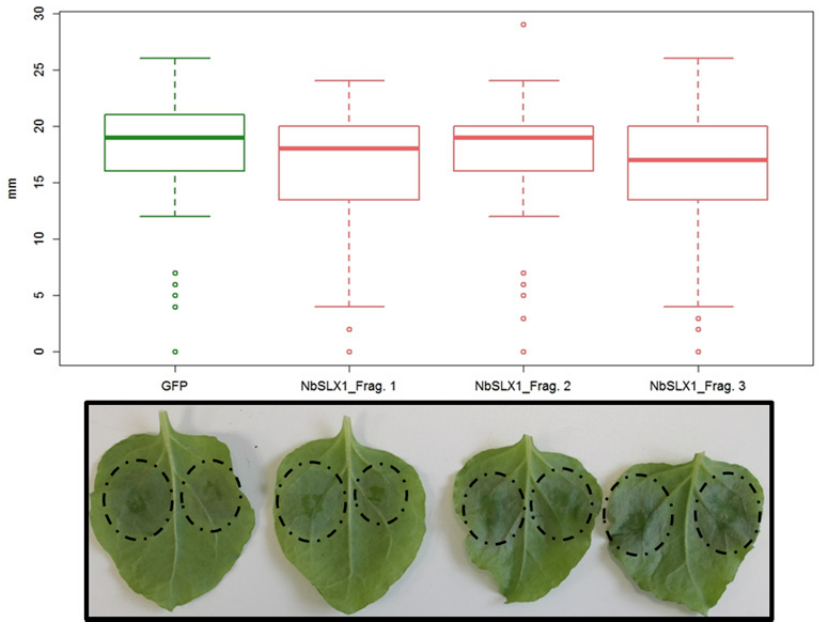


Figure 8 Impact of putative NbSLX1 silencing on *P. capsici* virulence. (a) VIGS assays using three independent constructs aiming for silencing of NbSLX1 and one negative control construct aiming to silence GFP were performed in *N. benthamiana* plants. Leafs were collected and were drop-inoculated with a solution of *P. capsici* zoospores and lesion diameters were measured two days post-infection. Photos show representative leaves two days post-infection.

We show above that transient over-expression of NbSIZ1 in *N. benthamiana* leaves increased the resistance of these leaves against *P. capsici* infection (Figure 6). To assess if constitutive over-expression of NbSIZ1 could also mediate increased resistance to *P. capsici*, transgenic *N. benthamiana* plants that constitutively over-express NbSIZ1 were generated. A preliminary assay, using only two plants from a single line over-expressing NbSIZ1 (NbSIZ1_5), suggests that NbSIZ1 constitutive over-expression enhances resistance to *P. capsici* infection when compared to wild type plants or plants over-expressing RFP (Figure 9a). It is also important to note that in this assay the plants were used with the same age, being the smaller size of plants from NbSIZ1_5 line a phenotype possibly caused by NbSIZ1 over-expression. The over-expression of NbSIZ1 gene in plants from the NbSIZ1_5 line was confirmed by qRT-PCR analysis (Figure 9b).

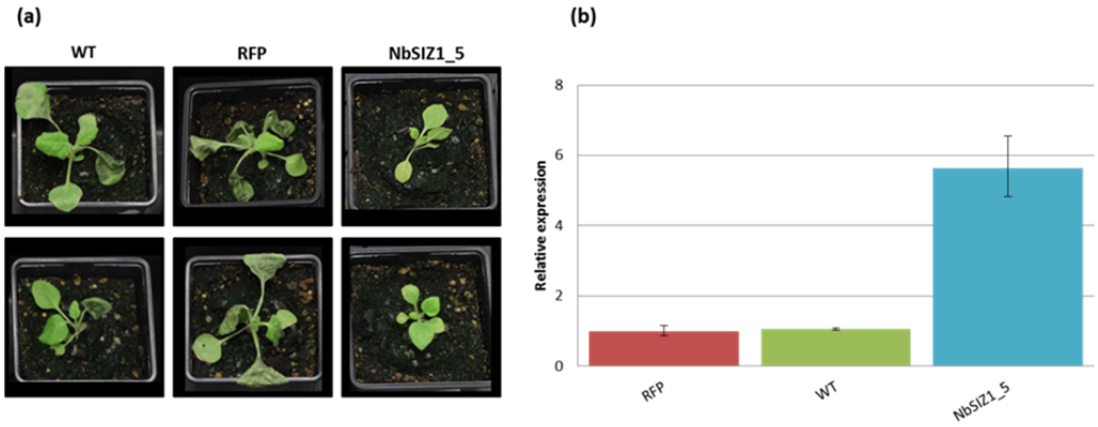


Figure 9 *N. benthamiana* plants constitutively over-expressing NbSIZ1 show increased resistance to *P. capsici* infection. (a) Wild type (WT) and transgenic *N. benthamiana* plants over-expressing RFP and NbSIZ1 (NbSIZ1_5) were spray inoculated with a suspension of *P. capsici* zoospores. Images were taken three days post-infection. (b) qRT-PCR analysis was performed using cDNA obtained *N. benthamiana* transgenic plants from the line NbSIZ1_5, a line putatively over-expressing RFP and wild type (WT) *N. benthamiana* plants.

Arabidopsis AtSIZ1 is also connected to *P. capsici* virulence

P. capsici has been reported as able to infect *Arabidopsis* plants (Wang et al., 2013). Moreover, *Arabidopsis* AtSIZ1 is well characterised and *Arabidopsis* knockouts for this protein have been generated and characterised (Jin et al., 2008; Miura et al., 2005). Thus, we used these knockout plants (*siz1-2*) to assess the importance of AtSIZ1 in *P. capsici* virulence.

Siz1-2 plants show a dwarf growth phenotype due to elevated salicylic acid (SA) levels that can be partially reverted by the over-expression of a bacterial gene encoding a salicylate hydroxylase (nahG) (Lee et al., 2006). As elevated SA levels have been connected to increased resistance to a variety of biotic stresses, including *P. capsici* infections, (Robert-Seilaniantz et al., 2011; Wang et al., 2013) we used *nahG siz1-2* plants in our experiments to exclude the putative impact of *siz1-2* described phenotypes (elevated SA levels and dwarfism) in *P. capsici* virulence. For infection of *Arabidopsis* a suspension of *P. capsici* zoospores was sprayed in *Arabidopsis* plants and levels of *P. capsici*

infection were measured by the percentage of infected leaves and the number of infected leaves per plant (Figure 9). As expected due to the elevated SA levels in these plants, *siz1-2* plants showed enhanced resistance to *P. capsici* when compared to wild type (Col-0) *Arabidopsis* plants. Interestingly, *nahG siz1-2* plants showed increased susceptibility to *P. capsici* infection when compared to *nahG* plants, further pointing to a role of AtSIZ1 in plant immunity (Figure 10).

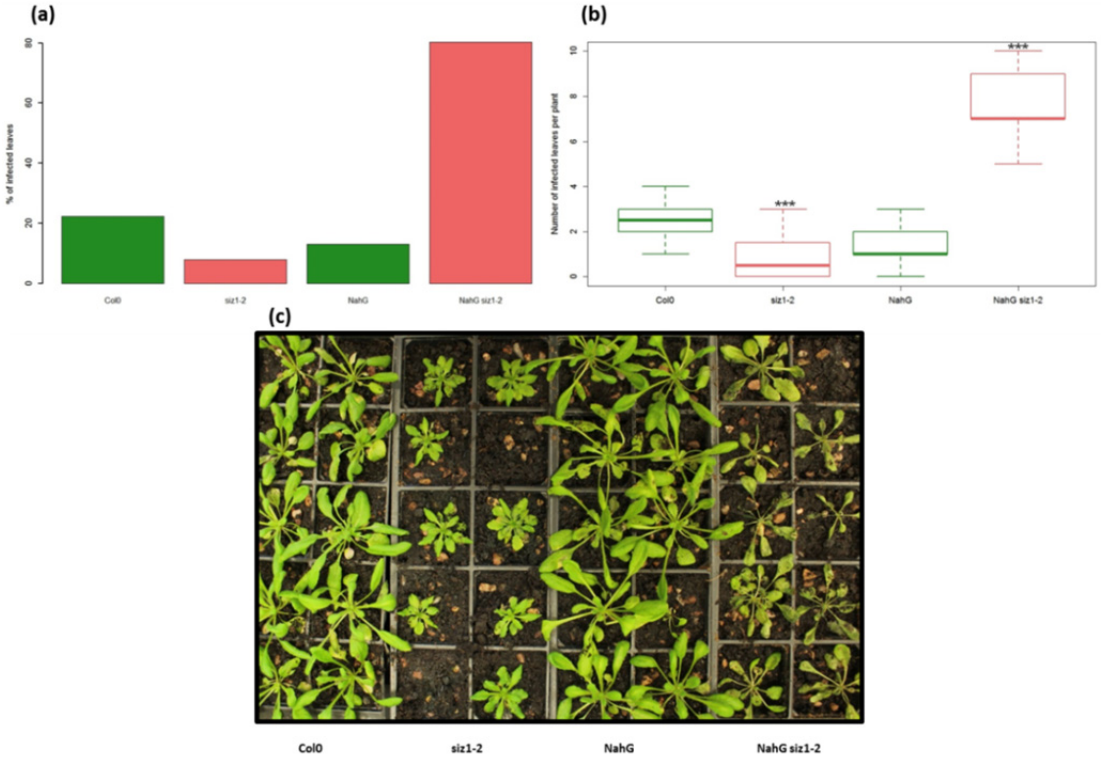
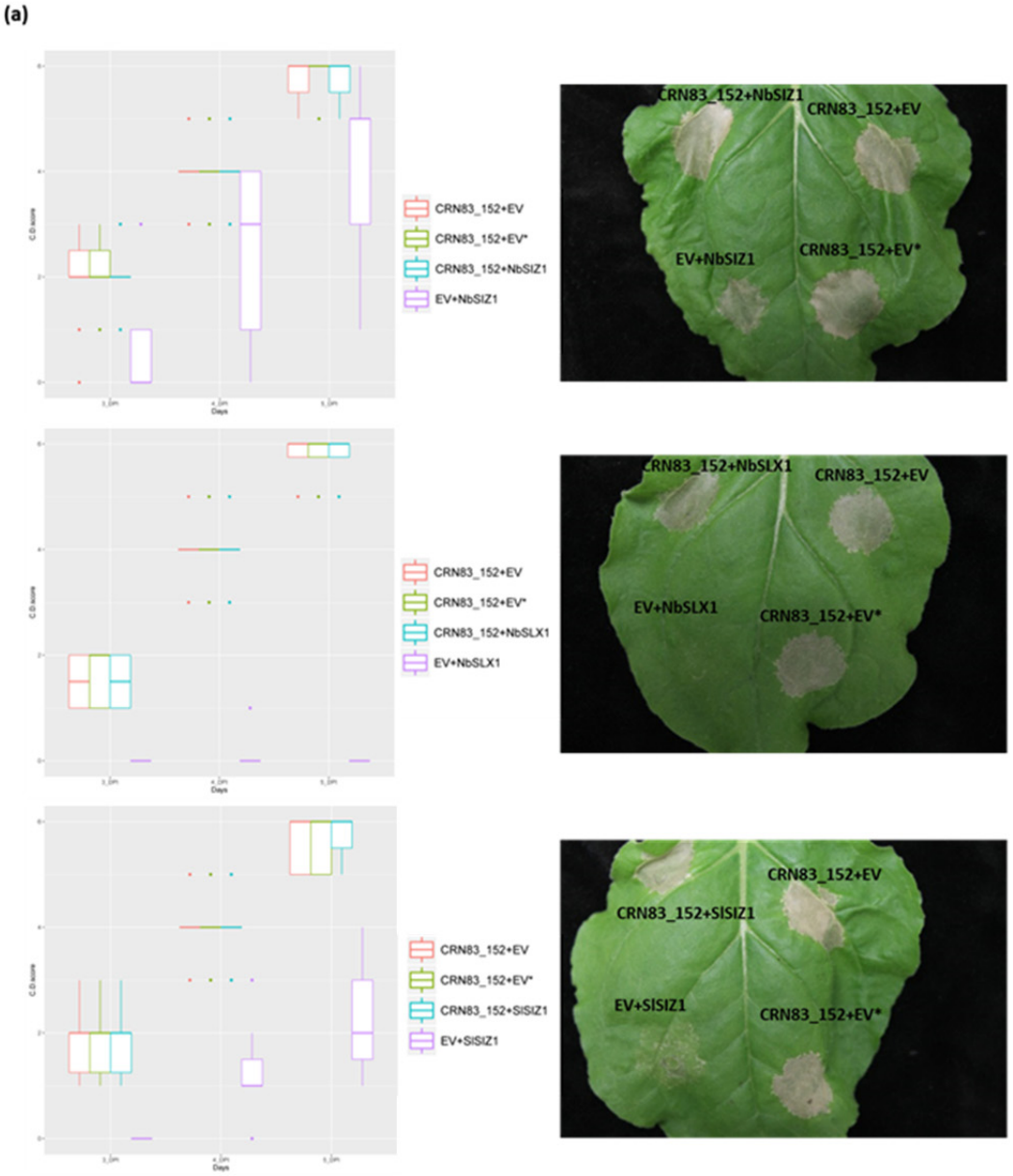


Figure 10 AtSIZ1 roles on mediating immunity against *P. capsici*. *Arabidopsis* plants were spray inoculated with a suspension of *P. capsici* zoospores. Eight days after inoculation, *P. capsici* infections were measured by assessing (a) the percentage of leaves with visible lesions and (b) the average number of these leaves per plant. (c) Picture was taken eight days post-infection and encompasses all plants used in the assay. "***" indicate a significant difference ($p<0.001$, t-test).

NbSIZ1, NbSLX1 and SlSIZ1∆867 do not affect PcCRN83_152 mediated cell death phenotype.

In order to assess the influence of PcCRN83_152 targets in PcCRN83_152 mediated cell death, we co-expressed NbSIZ1, NbSLX1 and SlSIZ1∆867 with PcCRN83_152 in *N. benthamiana* leaves and subsequently visually assessed for the presence of plant cell death. In this experiment we observed that none of the PcCRN83_152 targets influenced PcCRN83_152 mediated cell death (Figure 11a). Interestingly, cell death was observed in *N. benthamiana* leaf areas over-expressing NbSIZ1 and SlSIZ1∆867, opening the possibility that SIZ1 proteins could be inducing cell death using mechanisms similar to the ones used by PcCRN83_152. Expression of all constructs in this experiment was confirmed by Western blot (Figure 11b)


(a)
CRN83_152+EV
CRN83_152+EV*
CRN83_152+NbSIZ1
EV+NbSIZ1
CRN83_152+NbSIZ1
CRN83_152+EV
EV+NbSIZ1
CRN83_152+EV*
CRN83_152+EV
CRN83_152+EV*
CRN83_152+NbSLX1
EV+NbSLX1
CRN83_152+NbSLX1
CRN83_152+EV
EV+NbSLX1
CRN83_152+EV*
CRN83_152+EV
CRN83_152+EV*
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CRN83_152+EV
EV+SlSIZ1
CRN83_152+EV*

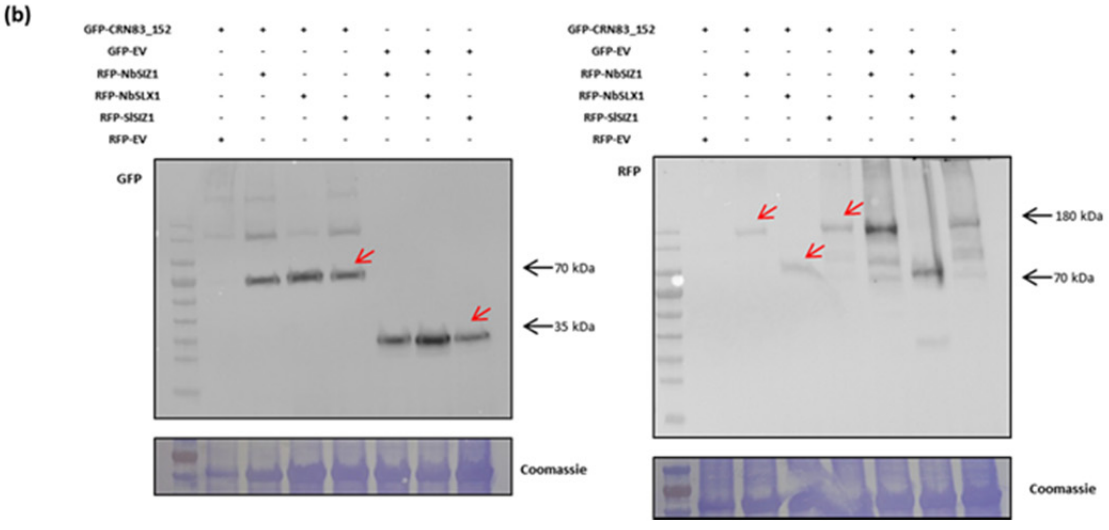

(b)
GFP-CRN83_152
GFP-EV
RFP-NbSIZ1
RFP-NbSLX1
RFP-SlSIZ1
RFP-EV
GFP
70 kDa
35 kDa
Coomassie
RFP
180 kDa
70 kDa
Coomassie

Figure 11 PcCRN83_152 target impact on PcCRN83_152 mediated cell death. NbSIZ1, NbSLX1 and SlSIZ1∆867 were co-expressed with PcCRN83_152 in *N. benthamiana* leaves. (a) Cell death was visually assessed three, four and five days post-infiltration using a cell death scale from 0 to 6 where 0 stands for no cell death and 6 for complete dead plant tissue. Leaf images were taken five days post-infiltration. (b) Western blotting was performed with protein samples collected two days post-infiltration. Coomassie was used as a loading control (showing band that correspond to Rubisco size). Red arrows indicate expected protein sizes: GFP-CRN83_152 ≈ 65.5 kDa ; GFP-EV ≈ 28 kDa; RFP-NbSIZ1 ≈ 132.5 kDa ; RFP-NbSLX1 ≈ 69.3 kDa; RFP- SlSIZ1∆867 ≈ 131 kDa ; RFP-EV ≈ 27 kDa.

PcCRN83_152 mediated cell death destabilises NbSLX1 and SlSIZ1∆867 *in planta*

PcCRN83_152 interactions were shown to occur *in planta*. Nevertheless, the effects of PcCRN83_152 on its targets remain unknown. Here we show that in the presence of PcCRN83_152 the stability of NbSLX1 and SlSIZ1∆867 is diminished (Figure 12). This decrease in stability did not occur in SlTCP14-2 in the presence of PcCRN83_152. However, NbSLX1 and SlSIZ1∆867 stability was equally impaired in the presence of the cell death inducing effector PiRXLR04145, indicating that either NbSLX1 and SlSIZ1∆867 protein stability is impaired during general cell death processes, or that PcCRN83_152 and PiRXLR04145 are inducing cell death via related mechanisms.

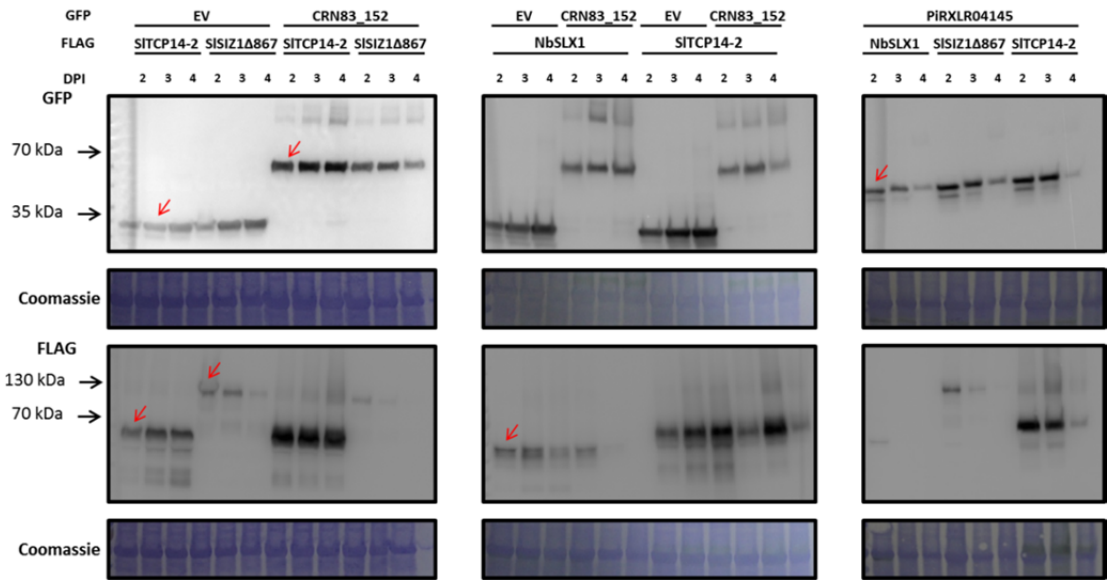


Figure 12 PcCRN83_152 destabilises NbSLX1 and SlSIZ1Δ867 *in planta*. Western blotting was performed with protein samples collected two, three and four days post-infiltration (DPI). Coomassie used as a loading control (showing bands that correspond to Rubisco size). Expected sizes: GFP-CRN83_152 ≈ 65.5 kDa ; GFP-EV ≈ 28 kDa; GFP-PiRXLR04145 ≈ 35 kDa ; FLAG-NbSLX1 ≈ 43.3 kDa; FLAG- SlSIZ1Δ867 ≈ 105 kDa ; FLAG-SlTCP14-2 ≈ 51 kDa.

Discussion

Plant pathogens continuously hamper crop production worldwide (Oerke, 2006). In order to achieve pathogenicity, these microbes evolved large effector repertoires, which target host processes to enhance pathogen fitness (Hogenhout et al., 2009; Kamoun, 2007; Win et al., 2012). One of these pathogens is *P. capsici*, causing huge loses in Solanaceous crops worldwide (Kamoun et al., 2015; Lamour et al., 2012b). As other members of the *Phytophthora* family, *P. capsici* encodes large numbers of putative effector proteins including members from the CRN effector family (Stam et al., 2013b). However, the host processes targeted by this family of effectors remain mostly uncharacterised.

Here we aimed to unveil the host processes targeted by the *P. capsici* CRN effector PcCRN83_152. This CRN effector was shown to cause cell death and enhance *P. capsici* virulence when over-expressed in *N. benthamiana* leaves (Stam et al., 2013b). Furthermore, *P. capsici* strains silenced for PcCRN83_152 showed reduced virulence phenotypes in *N. benthamiana* and *Arabidopsis* plants (Mafurah et al., 2015). Nonetheless, with the exception of PcCRN83_152 capacity to mediate host chromatin re-localisation (Stam et al., 2013a), the functions of PcCRN83_152 *in planta* remain undescribed.

With this work we successfully identified two plant targets for PcCRN83_152 (SlSIZ1Δ867 and NbSLX1) via an Y2H screen (Figure 1 and Table 1). Subsequently, by FLIM-FRET analysis, we confirmed interactions *in planta* between PcCRN83_152 and three plant proteins NbSIZ1, NbSLX1, and SlSIZ1Δ867 (Figure 3 and Table 2). These newly identified PcCRN83_152 interactions appear to occur in the host nuclear compartment, as PcCRN83_152 was shown to co-localise with its targets in *N. benthamiana* nuclei (Figure 4).

While no consistent immunity functions were observed for NbSLX1 in our assays, over-expression of SlSIZ1Δ867 and NbSIZ1, and silencing of NbSIZ1 in *N. benthamiana* were shown to impair *P. capsici* virulence (Figures 6, 7 and 8). Moreover, *N. benthamiana* transgenic plants over-expressing NbSIZ1 appear to be more resistance to *P. capsici* infection (Figure 9), pointing to an important role of SIZ1 proteins in processes mediating plant immunity against *P. capsici* infections. In relation to NbSLX1, as in the VIGS assay the silencing levels of NbSLX1 were not assessed, we cannot assume silencing efficiency and exclude a virulence effect of NbSLX1 silencing in *N. benthamiana* plants.

Furthermore, structural data suggested that SLX1 exists in its free form as an autoinhibited homodimer which is disrupted by the action of SLX4 leading to SLX1 activation (Nowotny and Gaur, 2016). Thus, it is possible that the absence of immunity phenotypes when over-expressing NbSLX1 could be due to the absence of high levels of the *N. benthamiana* version of SLX4. However, BLAST analyses with *S. cerevisiae* and human SLX4 failed to identify a putative SLX4 ortholog in *Arabidopsis* (Bauknecht and Kobbe, 2014), raising the possibility that in plants SLX1 does not require activation or that other SLX4 unrelated protein is activating SLX1.

The *Arabidopsis* AtSIZ1 has been connected to plant responses against biotic stresses (van den Burg et al., 2010; Lee et al., 2006). *Arabidopsis siz1-2* plants were shown to have enhanced resistance against *Pseudomonas syringae* infection, a phenotype that was abolished in *siz1-2 nahG* plants, connecting enhanced plant resistance with the elevated SA levels characteristic of *siz1-2* plants (Lee et al., 2006). Nevertheless, enhanced resistance against infections from the necrotrophic plant pathogenic fungus *Botrytis cinerea* was not observed in *siz1-2 Arabidopsis* plants, suggesting that SA levels do not impact *Botrytis cinerea* pathogenicity (Lee et al., 2006). Our data points to an influence of *siz1* knockout on the resistance of *Arabidopsis* plants against *P. capsici* infections, as *siz1-2* plants were shown to be more resistant than wild-type (Col-0) plants (Figure 10). Our results go in accordance with the described role of SA on resistance of *Arabidopsis* plants against *P. capsici* as *Arabidopsis* mutants with defects in SA signalling pathways were shown to display severely compromised resistance to *P. capsici* (Wang et al., 2013). Interestingly, we show that *siz1-2 nahG* plants do not only reverse *siz1-2* mediated increase of resistance, as was shown for *P. syringae* (Lee et al., 2006), but show increased

susceptibility to *P. capsici* when compared to wild type *Arabidopsis* plants, suggesting that AtSIZ1 has a role in enhancing immunity against *P. capsici*. These results in *Arabidopsis* agree with the results obtained in *N. benthamiana* where it was observed that over-expression of NbSIZ1 increased plant resistance to *P. capsici* infection, but do not agree with the observed decrease in virulence in *N. benthamiana* plants silenced for NbSIZ1..

SIZ1 and SLX1 proteins have been implicated in DNA repair mechanisms on mammalian and yeast systems (Rouse, 2009; Strunnikov et al., 2001; Westerbeck et al., 2014), connecting PcCRN83_152 targets with PcCRN83_152 mediated chromatin re-localisation phenotypes. Nevertheless, besides this connection, the virulence roles of PcCRN83_152 interaction with its targets remain uncharacterised. In this study, we showed that over-expression of PcCRN83_152 targets does not impair the capacity of PcCRN83_152 to induce plant cell death. However, as the cell death inducing activity of PcCRN83_152 could be independent of its virulence functions (Chapter 3), it is not clear if over-expression of PcCRN83_152 targets does not impair PcCRN83_152 mediated boost of *P. capsici* growth. While virulence experiments with a cell death inducing protein are challenging (due to difficulties in timing infection to occur in live tissues and in measuring *P. capsici* growth diameters in dead tissue), the newly available NCD PcCRN83_152 variants that retain virulence functions could be used to test this hypothesis. PcCRN83_152 targets were also shown, in this work, to be destabilised by the presence of PcCRN83_152. However, this destabilisation also occurs in the presence of the cell death inducer *P. infestans* effector PiRXLR04145, indicating that PcCRN83_152 mediated target destabilisation could be connected with general cell death processes. Again, the available NCD PcCRN83_152 variants that

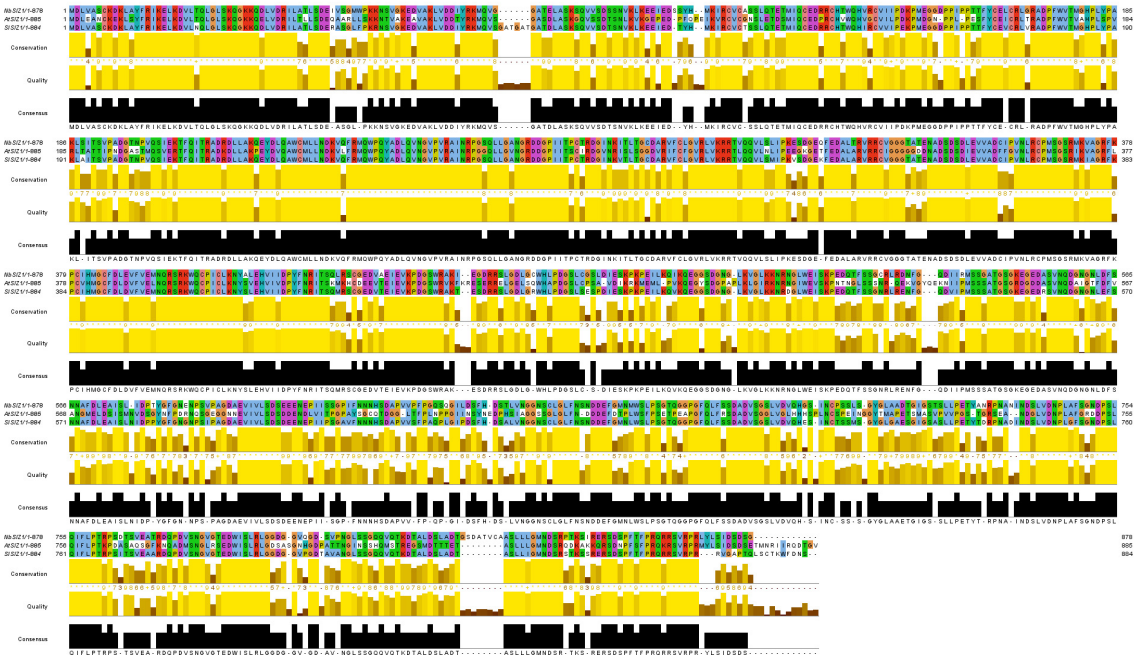
retain the capacity to enhance *P. capsici* virulence (Chapter 3) constitute excellent tools to test the cell death independent influence of PcCRN83_152 on the destabilisation of its targets.

The described functions of PcCRN83_152 targets in yeast and mammal systems and the PcCRN83_152 capacity to re-localise host chromatin, even in the absence of cell death, lead to the hypothesises that PcCRN83_152 could be destabilising its targets to induce DNA damage in plants. Recently, CRN effectors from *Aphanomyces euteiches* (AeCRN13) and from the amphibian pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (BdCRN13) were shown to cause cell death, to interact with nuclear DNA and to have a virulence function connected to the triggering of DNA damage responses (DDRs) in plants (Ramirez-Garcés et al., 2015). AeCRN13 and BdCRN13 were also shown to possess an HNH-like endonuclease motif that is required for the capacity of these CRNs to bind DNA, trigger plant DDRs, and in the case of AeCRN13 to enhance *N. benthamiana* susceptibility against *P. capsici* infections (Ramirez-Garcés et al., 2015). Thus, DDR induction has been shown to be a strategy used for cell death inducing CRN effectors to enhance plant susceptibility towards *P. capsici* infections. Testing if PcCRN83_152 triggers DDRs, similarly to BdCRN13 and AeCRN13, either by direct DNA binding or by interacting with its proteinaceous targets could give us more insights in the plausibility of this hypothesis.

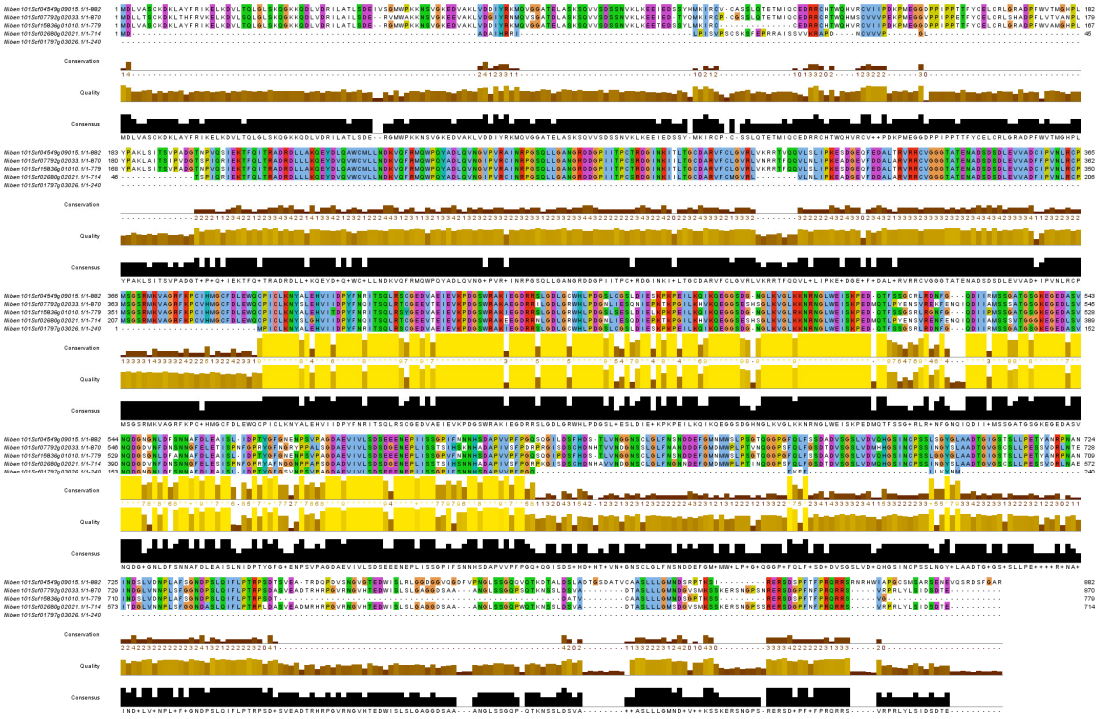
In summary, we have identified and confirmed three plant proteins (SlSIZ1Δ867, NbSIZ1 and NbSLX1) as interactors of the *P. capsici* CRN effector PcCRN83_152. Given the *P. capsici* virulence and cell death phenotypes, the availability of NCD PcCRN83_152 variants, and the connections of SIZ1 and

SLX1 proteins with immunity and DNA damage responses, further studies on this effector could lead to great advances in our current understanding on CRN virulence and cell death mechanisms, and new insights in the roles of SIZ1 and SLX1 proteins in plant immunity processes.

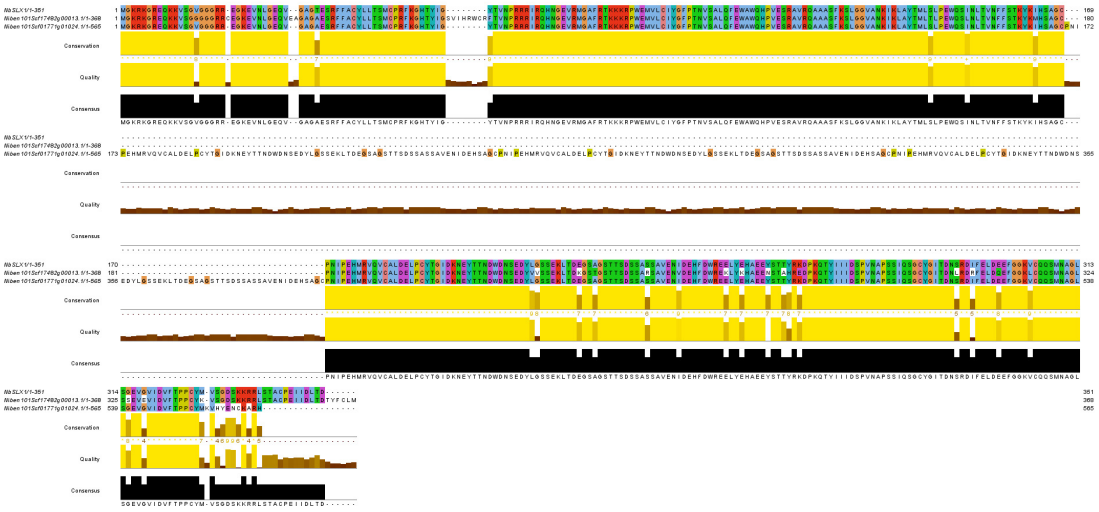
Supplementary files



Supplementary Figure 1 Alignment of SIZ1 proteins used in this study. The amino acid sequences of the proteins used in this study (NbSIZ1 and SlSIZ1Δ867) were aligned with the amino sequence of AtSIZ1. Alignment was performed using Jalview (V.2.8.2) and using ClustalX colours.



Supplementary Figure 2 Alignment of SIZ1 proteins encoded in the *N. benthamiana* genome. The amino acid sequences of the SIZ1 proteins predicted to be encoded by the *N. benthamiana* genome were aligned. Alignment was performed using Jalview (V.2.8.2) and using ClustalX colours.



Supplementary Figure 3 Alignment of NbSLX1 proteins. The amino acid sequence of the cloned NbSLX1 protein was aligned with the two amino acid sequences of the NbSLX1 proteins predicted to be encoded in the *N. benthamiana* genome. Alignment was performed using Jalview (V.2.8.2) and using ClustalX colours.

Chapter 5. Proteomics-based identification of candidate effectors from *Phytophthora capsici*

T. M. M. M. Amaro, H. J. C. Cornelisse, E. Huitema. Unpublished work.

Author Contributions

TA performed all the experiments. HC helped with data analyses. TA and EH wrote this Chapter.

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Introduction

Plant-microbe interactions are complex and feature dynamic biological processes that greatly influence plant fitness either positively (mutualistic microbes) or negatively (pathogenic microbes) (Farrar et al., 2014; Mine et al., 2014). Accordingly, plants and microbes have co-evolved mechanisms to modulate the outcomes of their interactions (Chisholm et al., 2006; Jones and Dangl, 2006; Oldroyd, 2013). Molecular genetic approaches have allowed significant progress on characterising the mechanisms underpinning host-microbe interactions. However, plant-microbe interactions involve complex signalling pathways with connections with other cellular processes, including those regulating growth and responses to abiotic factors. Therefore, global and systematic approaches are required to paint an accurate picture of the processes that underpin plant-microbe interactions, before those deemed essential can be identified (Kissoudis et al., 2014; Kumar et al., 2016; Mine et al., 2014; Pritchard and Birch, 2011).

Recent genome sequencing efforts have greatly contributed to an increased understanding of the virulence mechanisms deployed by oomycetes, which form an important class of plant pathogenic eukaryotic microbes (Bozkurt et al., 2012; Fawke et al., 2015; Thines and Kamoun, 2010). Analyses of available genome sequences for a range of plant pathogenic oomycetes, including *Albugo* (Kemen et al., 2011), *Hyaloperonospora* (Baxter et al., 2010), *Pythium* (Lévesque et al., 2010) and several *Phytophthora* species (as for example *P sojae* (Tyler et al., 2006), *P. infestans* (Haas et al., 2009) and *P. capsici* (Lamour et al., 2012a)) have shown that these microbes encode large repertoires of predicted secreted proteins that are believed to aid pathogen virulence (effectors) (Bozkurt et al., 2012). The pipelines used for effector identification are usually dependent on the prediction of protein secretion, based mainly on the presence of signal peptides and absence of trans-membrane domains (Sonah et al., 2016). However, recent findings have shown that the presence of a signal peptide is not a requirement for protein secretion.

Signal peptides are thought to be required for protein secretion using the endoplasmic reticulum - Golgi apparatus pathway (Delic et al., 2013; Novick et al., 1981; Sakaguchi, 1997). However, unconventional secretion pathways have been widely described involving direct translocation across the plasma membrane, ABC-transporter based secretion, secretion by exosomes, and secretion by membranes shedding/blebbing (Ding et al., 2012; Nickel and Seedorf, 2008; Rabouille et al., 2012; Robinson et al., 2016). While non-conventional secretion mechanisms remain uncharacterised in oomycetes, a *P. sojae* effector (PsIsc1) with virulence roles was suggested to be secreted via unconventional secretion pathways. In this study, it was shown that this protein could mediate the translocation of the Avr1 C-terminal domain to soybean

plants carrying the *Rps1b* gene and condition resistance phenotypes (Liu et al., 2014). In sum, these findings highlight the importance of alternative approaches to identify plant pathogen effectors that are not identifiable through signal peptide predictions.

Proteomic approaches have been successful on identifying secreted proteins from plant pathogenic microbes (Delaunois et al., 2014; Gupta et al., 2015). For example, a proteomic approach combining two-dimensional gel electrophoresis and chromatography-tandem mass spectrometry (LC-MS/MS) analyses, allowed the identification of proteins thought to be secreted by the broad host range plant pathogenic fungi *Botrytis cinerea* during early infection stages (Espino et al., 2010). These putative fungal secreted proteins include a large number of predicted enzymes thought to facilitate the degradation of plant defensive barriers as well as proteins with no predicted function, forming a valuable set of candidate effectors (Espino et al., 2010). A similar proteomic approach was performed in order to characterise the *Phytophthora infestans* secretome (Meijer et al., 2014). This approach allowed the identification of putative secreted *P. infestans* proteins with gene ontology terms connected with pathogenesis, cell wall modifications, defence responses and proteolytic processes. Previously known effectors, from the CRN and RXLR families, were also identified as secreted in this analysis, providing a good indication of the success of a proteomics approach to identify pathogen effectors. Importantly, in this study several putative secreted proteins with no predicted signal peptides were identified, pointing to the importance of using proteomic approaches as a complement to genomic and transcriptomic studies when aiming for a complete view of *Phytophthora* effector complements (Meijer et al., 2014).

In this study we aimed for a proteomics-based characterisation of the *P. capsici* secretome. Due to the challenging nature of isolating apoplastic fluids from infected tissues (Alexandersson et al., 2013), we analysed the secretome of *P. capsici* grown on V8 and Pea broth (PB) media. This study allowed the identification of 93 putative secreted proteins from *P. capsici*. From these 93 proteins, 45 were shown to be differentially expressed during *P. capsici* infection of tomato leaves (Jupe et al., 2013), suggesting a role of these proteins as *P. capsici* effectors. Thus, this study has helped identify the first experimentally defined subset of the *P. capsici* secretome. This dataset is expected to lead to the identification of new *P. capsici* effectors, complementing previous genomic, transcriptomic and functional analyses in *P. capsici* (Stam et al., 2013b).

Methods

P. capsici growth conditions and sampling

A transgenic *P. capsici* LT1534 strain, expressing eGFP, was grown on V8 plates at 24 °C for 4 days (2 days in dark and 2 days in light conditions). Sporangia were collected by flooding the plates with ice-cold water and gently scraping the mycelial mats. Sporangia suspensions were then incubated for 30 minutes in a 24 °C light incubator to allow zoospore release. After, 10 mL of zoospore suspension (100000 zoospores/mL) were incubated at 24 °C in petri dishes sealed with parafilm for 3 hours. After these 3 hours, water was decanted and 20 mL of either clarified V8 or PB were added to the plates. After 2 days of incubation in the dark at 24 °C, media (secreted fraction) was

collected by decanting, after which 1X protease inhibitor cocktail (Thermo Scientific) and 1X phosSTOP (Sigma) were added. 12 mL of each secreted fraction were concentrated into approximately 500 µL (24 times concentrated) using Amicon Ultra 15 mL Centrifugal Filters (Merck).

Mycelial samples were collected from the plates and dried in paper. 400 mg of each dried mycelial mat were then frozen in liquid nitrogen and proteins were extracted in 500 µL of GTEN buffer (10% Glycerol, 25 mM Tris, 1 mM EDTA, 150 mM NaCl) supplemented with 10 mM DTT, 1X protease inhibitor cocktail (Thermo Scientific) and 1X phosSTOP (Sigma).

Mass spectrometry and data analysis

Protein samples were reduced and alkylated in solution with 45 mM DTT and 100 mM iodoacetamide respectively. Proteins were then subject to in-solution digestion with sequencing grade trypsin (Roche) overnight at 37 °C. The resulting peptides were cleaned using a C18 (POROS R2, Applied Biosystems) column. Elution was performed using 2 x 40 µl of 50 % acetonitrile, 0.1 % trifluoro acetic acid (TFA). Eluted peptide samples were then dried down to approximately 10 µl via vacuum centrifugation before being re-adjusted to a final volume of 12 µl with 0.1 % TFA.

Peptide samples were analysed on an Orbitrap Velos (Thermo Scientific) mass spectrometer in a 240 minutes run. Protein identification and quantification was carried out using MaxQuant software version 1.5.5.1 (Cox et al., 2009; Cox and Mann, 2008). Raw mass spec data files were searched against a combined *S. lycopersicum* (Sato et al., 2012) and *P. capsici* (Lamour et al., 2012a) proteome file. False discovery rate was set to < 1% and peptides mapping to more than

one protein were removed from the analysis. Venn diagrams were designed using VENNY2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Signal peptide predictions were performed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP) using default settings (Petersen et al., 2011). Expression profiles and domain annotations were obtained from Jupe et al. (2013).

Western blotting and Krypton staining

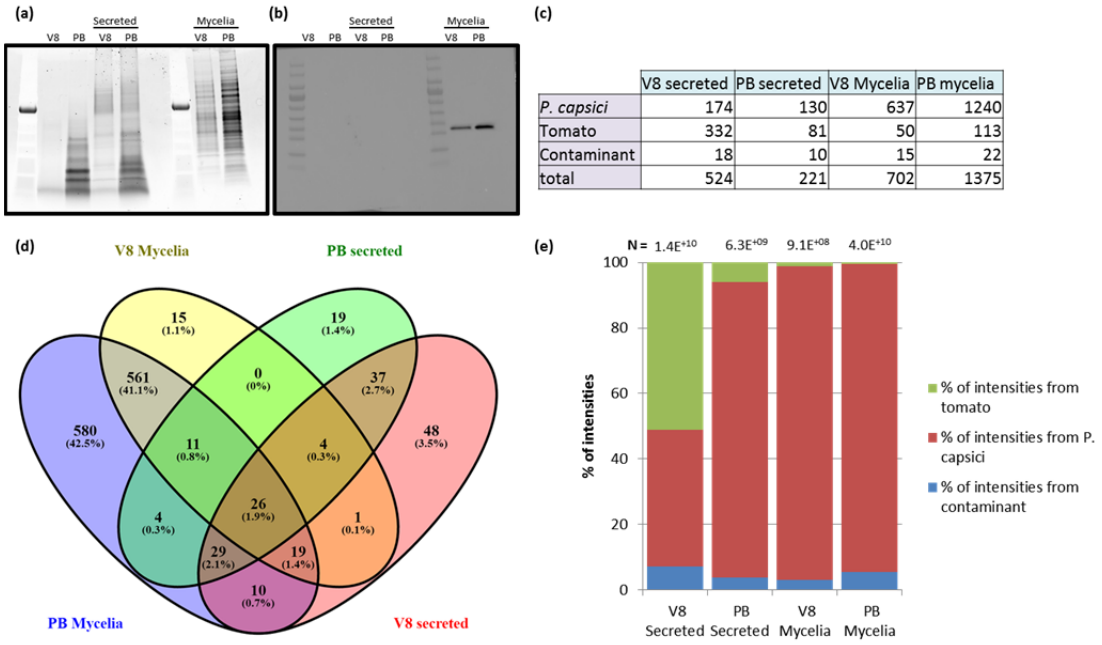
For Western blotting, protein samples were run on Biorad TGX gels before being transferred on PVDF membranes using the Biorad Trans Blot Turbo Transfer System. Blots were blocked for 30 minutes with 5% milk in TBS-T (0.1 % Tween 20) and probed with primary antibodies against GFP (Santa Cruz) (1:2500) and secondary anti-Mouse-HRP antibodies (Santa Cruz) (1:20000). Blots were incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged on a Syngen GBox TX4 Imager.

For Krypton staining protein samples were run in a Biorad TGX gel and this gel was then treated with Krypton Fluorescent Protein Stain (Thermo Scientific) following manufacturer's instructions. Gel imaging was conducted on a Typhoon FLA 7000 machine (GE Healthcare Lifesciences).

Results

Proteomic approach aims to characterise the *P. capsici* secretome

In order to obtain *P. capsici* secreted fractions, a GFP expressing strain of *P. capsici* was initially grown in V8 and pea broth (PB) after which the mycelial mat and the media (supernatant) were separated and collected. The overall protein composition of the samples was assessed via protein krypton staining (Figure 1a). Samples consisting of either V8 or PB media after *P. capsici* growth (secreted samples) contained a greater range of distinct protein bands when compared to media alone, suggesting the presence of secreted proteins from *P. capsici* in the supernatant (Figure 1a). To exclude the possibility of significant contamination from lysed mycelia, we performed a western blot to detect cytoplasmic GFP. These analyses revealed a strong GFP signal from both mycelial samples (V8 and PB) whereas no evidence of contamination was found in our supernatant samples. Although low levels of contamination cannot be excluded, these results gave us more confidence on the separation method used (Figure 1b).



	V8 secreted	PB secreted	V8 Mycelia	PB mycelia
P. capsici	174	130	637	1240
Tomato	332	81	50	113
Contaminant	18	10	15	22
total	524	221	702	1375

Figure 1 Overview analysis of proteomics approach to identify *P. capsici* secreted proteins. (a) Overall protein composition of samples analysed by LC-MS/MS was visualised

using krypton staining. Loaded protein amounts were not equalized. (b) These same samples were also analysed by western blot for detection of GFP. Bands observed in mycelia samples correspond to predicted GFP size (≈ 28 kDa). (c) Table with the number of proteins identifyed by LC-MS/MS analysis by sample and by origin. (d) Venn diagram comparing *P. capsici* protein presence in the four samples. (e) Analysis of the origin of label-free quantification (LFQ) intensities in our samples. Percentages of total intensities for each sample are shown. Total intensities per sample (N) are indicated.

To identify the proteins present in each sample, the mycelial and secreted samples were analysed by LC-MS/MS and peptides were searched against the *P. capsici* and tomato predicted proteomes using the Maxquant software package (Cox et al., 2009). This analysis allowed the identification of *P. capsici* proteins in both mycelial and secreted samples (Figure 1c). A comparison of the proteins present in our samples suggests growth media dependent differential secretion or detection of *P. capsici* proteins, as we observed that 19 and 48 proteins were identified specifically in secreted fractions from PB and V8 respectively (Figure 1d). However, more repeats of this experiment are required to verify this putative differential secretion.

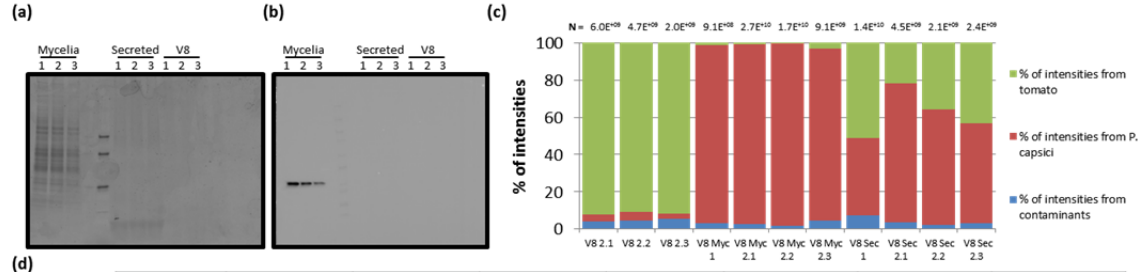
The presence of proteins in our media only controls may hamper the identification of low-abundant and secreted proteins from *P. capsici*. In order to assess the levels of contamination in our samples with non *P. capsici* proteins, we analysed the distribution of label-free-quantification (LFQ) intensities across proteins with *P. capsici* and tomato origin or that belong to a group of known and commonly encountered contaminants of proteomic analysis (Cox et al., 2009; Hodge et al., 2013). As expected, the percentage of tomato protein contamination was higher in secreted samples (51.1% for V8 secreted and 5.9% for PB secreted) when compared to mycelial samples (1.2% for V8 mycelia and 0.5% for PB mycelia) (Figure 1e). The higher percentage of the tomato protein intensities in V8 secreted samples in comparison with PB

secreted samples is possibly due to the data being searched against the tomato proteome (tomato being the main constituent of V8 media) and not the pea proteome (pea being the main constituent of PB). The presence of usual contaminant proteins of proteomic studies (as for example human keratin and trypsin (Hodge et al., 2013)) was relatively consistent across experiments (7.2% for V8 secreted, 3.8% for PB secreted, 3.1% for V8 mycelia and 5.6% for PB mycelia respectively).

Initial data analysis showed that more *P. capsici* secreted proteins were identified in V8 media samples (174) when compared to those derived from PB (130) (Figure 1c). Moreover, PB media showed a higher number of proteins identified in the mycelial samples, which could become contaminants in our experiments (Figure 1c). Therefore, V8 media was selected for further analysis and three additional biological repetitions of this experiment were performed using V8 media and samples were analysed by LC-MS/MS. Control samples of V8 media not exposed to *P. capsici,* were also analysed to prevent possible false identification of *P. capsici* proteins by media derived peptides.

The overall protein composition of the samples from three additional repetitions was also visualised using krypton staining of protein gels (Figure 2a). Levels of GFP contamination were also assessed in these samples, showing absence of GFP detection in secreted and V8 media only samples and strong signal in the mycelium extracts (Figure 2b). In accordance with the results previously described, an analysis of the intensities, generated in subsequent LC-MS/MS analyses on these samples, showed that supernatant samples have a high proportion of overall intensity originating from tomato proteins (51.1%, 22.0%, 35.7% and 43.2%), contrasting mycelia samples (1.2%, 0.7%, 0.5% and 3.2%).

Furthermore, and as expected, V8 media only samples have even higher numbers of intensities originating from tomato proteins (92.0%, 90.8% and 91.8%) (Figure 2c). Given the high level of background signal emanating from proteins present in the V8 media, *P. capsici* proteins identified by V8 derived peptides were subtracted from the analyses as they are likely to represent false positive in our *P. capsici* proteome searches. Therefore, numbers presented in subsequent analyses exclude this false positive set and are shown in Figure 2d.



(d)

	V8 Mycelia 1	V8 Mycelia 2.1	V8 Mycelia 2.2	V8 Mycelia 2.3	V8 Secreted 1	V8 Secreted 2.1	V8 Secreted 2.2	V8 Secreted 2.3
P. capsici	605	1037	1016	778	134	113	112	124
Tomato	3	33	29	18	36	31	34	27
Contaminant	1	2	1	4	2	1	0	0
Total	609	1072	1046	800	172	145	146	151

Figure 2 Proteomics approach identifies *P. capsici* proteins. (a) Overall protein composition of samples analysed by LC-MS/MS was visualised using krypton staining. Loaded protein amounts were not equalized. (b) These same samples were also analysed by western blot for detection of GFP. Bands observed in mycelia samples correspond to predicted GFP size (≈ 28 kDa). (c) Analysis of the origin of label-free quantification (LFQ) intensities in our samples. Percentages of total intensities for each sample are shown. Total intensities per sample (N) are indicated. (d) Table with the number of proteins identifyed by LC-MS/MS analysis by sample and by origin. Proteins identified in V8 media only samples are removed from this table. Column headers identify the samples and row headers identify protein origin (mapping to *P. capsici* proteome, to tomato proteome or to known contaminants of proteomic experiments).

Highly abundant proteins may hamper the detection of low abundant *P. capsici* proteins

The numbers of *P. capsici* proteins identified in this study were relatively low, particularly in the secreted samples (Figure 2d). Despite the fact that the levels and number of secreted *P. capsici* proteins are bound to be low, we aimed to

pinpoint possible reasons explaining our low *P. capsici* protein identification rate.

In plant green tissue, the protein RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) can constitute up to 40% of the total plant protein content, thereby hampering the identification of low abundant proteins in proteomic approaches (Bindschedler and Cramer, 2011). Therefore we tried to assess if, as in plants, highly abundant proteins could be present in our dataset that hamper the identification of low abundant *P. capsici* proteins. Therefore, we divided the identified proteins into ten different intensity categories, using total LFQ values measured across the entirety of our samples. Interestingly, we observed that around 2% of the proteins detected (59) contributed with more than 50% of the total intensity values associated with our dataset (Figure 3a). The majority of these 59 proteins were from *P. capsici* (48), suggesting that highly abundant proteins in *P. capsici* could be hampering the identification of low abundant proteins in our experiments. In addition, tomato proteins were also present in this high presence list (6), highlighting the impact of the V8 media in our analysis. Finally, usual contaminants of proteomic studies (as Keratin and trypsin) (Hodge et al., 2013) are also present in this list (5), highlighting the importance of careful sample handling when performing proteomic experiments (Figure 3b).

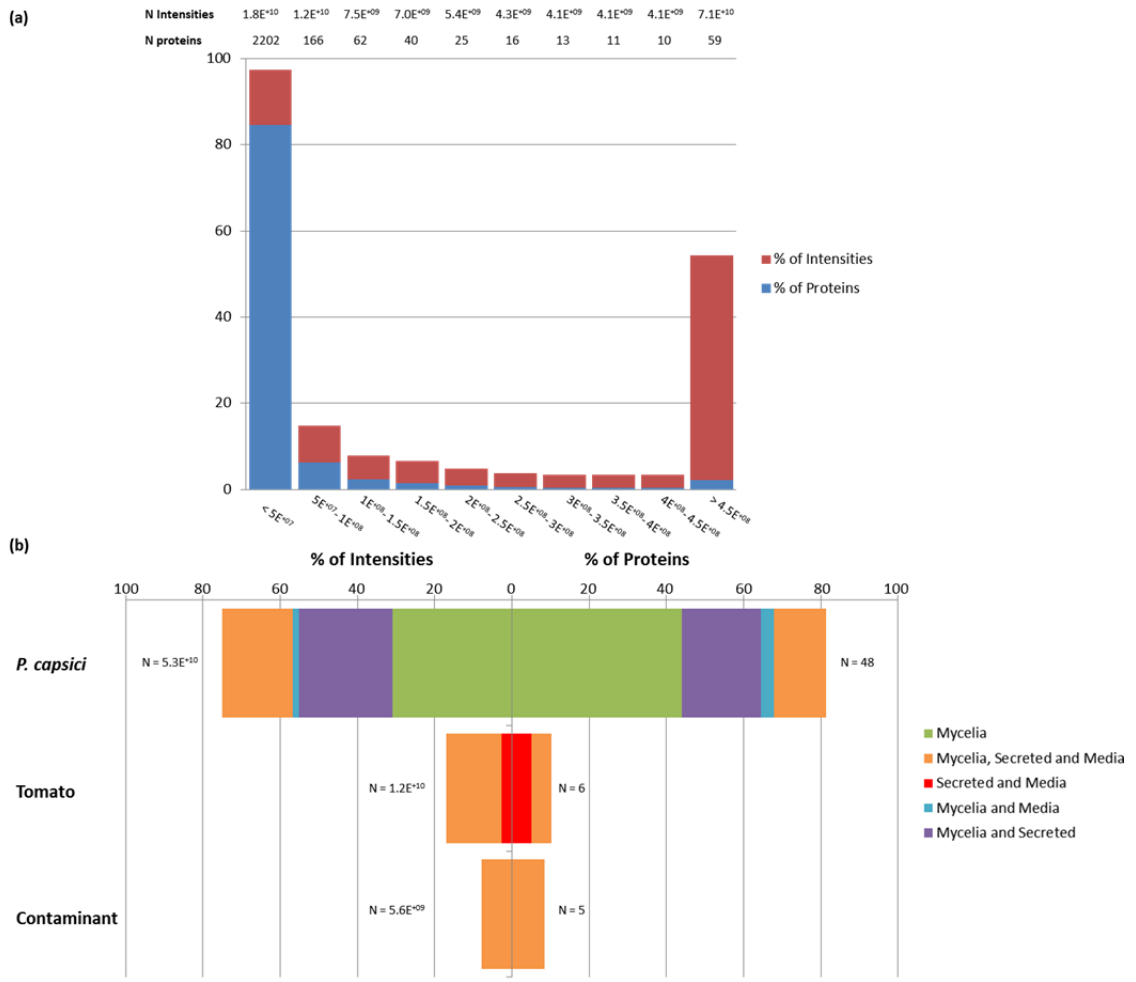


Figure 3 Analysis of highly abundant proteins presence in our dataset. (a) Proteins identified in the entirety of our samples were dividied in ten categories according the the sum of their LFQ intensities across the different samples. Graph shows percentage of proteins and correspondent percentage of intensities for every category. Number of proteins and intensities for every category are also shown. (b) Analysis of the 59 highly abundant proteins. These proteins were divided by origin and by type of sample where they were present. Numbers of proteins and intensities are shown.

Interestingly the presence of these highly abundant proteins varied across sample type. While contaminants were present in all samples (mycelia, secreted and media samples), tomato highly abundant proteins were present only in mycelia and secreted samples and *P. capsici* highly abundant proteins were mostly present in mycelial samples (26 proteins). Highly abundant *P. capsici* proteins that appear to be present in all samples (including V8 media alone) are most likely false matches to the *P. capsici* proteome. Nevertheless,

the presence of *P. capsici* highly abundant proteins unique to samples where *P. capsici* is expected (mycelia and secreted samples) provides an indication that highly abundant proteins from *P. capsici* are hampering and could possibly hamper future proteomic studies aiming to identify low abundance *P. capsici* proteins. A list of these *P. capsici* proteins is shown in Table 1.

Table 1 Description of highly abundant *P. capsici* proteins

Gene number	iprDesc
Phyca11_511842	Carbohydrate kinase, FGGY
Phyca11_19592	Chaperonin Cpn60
Phyca11_507000*	Glycoside hydrolase, family 17
Phyca11_505882	Malate dehydrogenase
Phyca11_505507	Annexin
Phyca11_538407	Aspartate/other aminotransferase
Phyca11_502682	Pyridoxal phosphate-dependent enzyme, beta subunit
Phyca11_563829	
Phyca11_559116	Aconitase/3-isopropylmalate dehydratase large subunit, alpha/beta/alpha
Phyca11_105777	ATP-citrate lyase/succinyl-CoA ligase
Phyca11_504332	D-isomer specific 2-hydroxyacid dehydrogenase, catalytic region
Phyca11_503983	Phosphoenolpyruvate carboxykinase, N-terminal
Phyca11_509552	Glutamine synthetase, catalytic region
Phyca11_505468*	TonB box, conserved site
Phyca11_502609	Electron transfer flavoprotein, alpha subunit, C-terminal
Phyca11_511385	Alpha-D-phosphohexomutase, N-terminal
Phyca11_506671*	Triosephosphate isomerase
Phyca11_529073*	Elicitin
Phyca11_505958	Transaldolase
Phyca11_507728*	Ketose-bisphosphate aldolase, class-II
Phyca11_504296*	Thaumatin, pathogenesis-related
Phyca11_509774	Methionine synthase, vitamin-B12 independent
Phyca11_511907	Protein synthesis factor, GTP-binding
Phyca11_503545	PEP-utilizing enzyme
Phyca11_503568*	Enolase
Phyca11_509045*	SCP-like extracellular

Note: * indicates proteins identified in the secreted as well as in the mycelia set.

Proteomic approach allows the identification of *P. capsici* putative secreted proteins

In order to analyse the consistency of our data across biological repeats, we compared the lists of *P. capsici* proteins identified in each repetition. This allowed us to establish that 42% of proteins were identified in all four mycelial samples (Figure 4a) whereas 33% of proteins were identified in all secreted samples (Figure 4b).

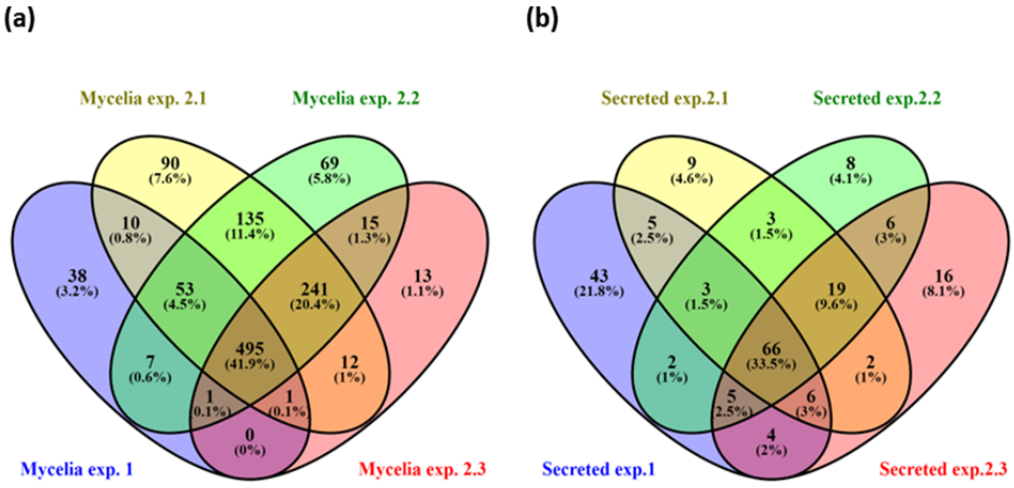


Figure 4 Comparisson of *P. capsici* proteins present in four repeats.(a) Venn diagram comparing *P. capsici* proteins identified in V8 mycelia samples and not present in V8 media only samples. (b) Venn diagram comparing *P. capsici* proteins identified in V8 secreted samples and not present in V8 media only samples.

For this study we created two distinct datasets: one containing proteins that are identified in at least three biological repetitions of mycelia samples (mycelia set); and another set of candidate secreted proteins, identified in at least three biological repetitions of media supernatant (secreted set). We then asked to what extent these sets overlap and whether any overlap could inform us on levels of cellular contamination. These analyses revealed that our mycelial and

secreted sets are distinct, as only 21 proteins were found to be common in both sets (Figure 5a).

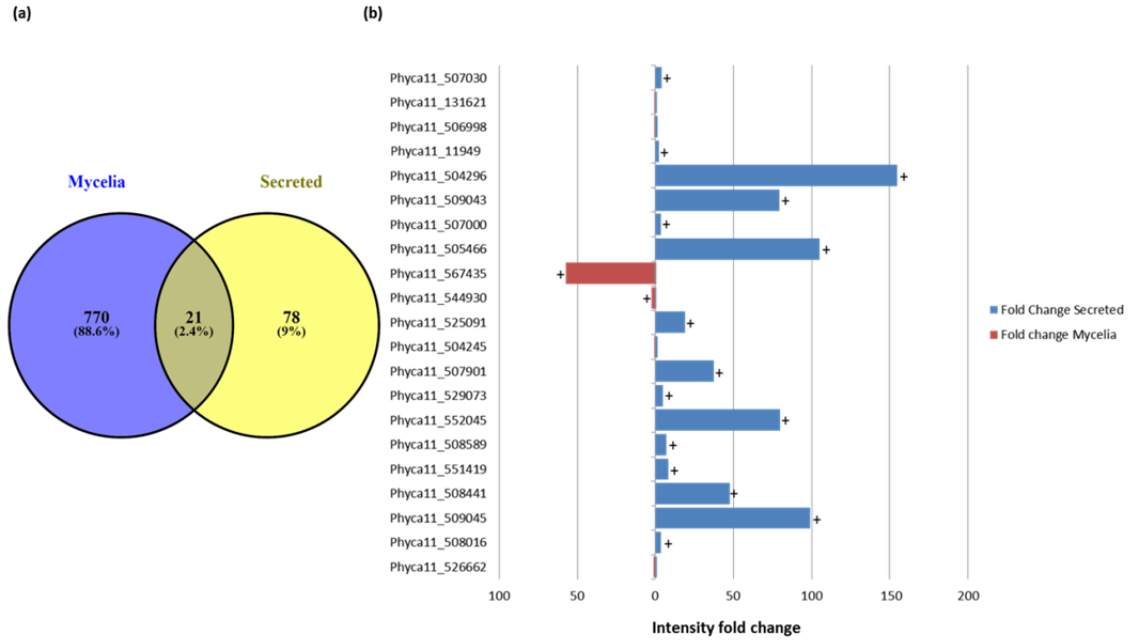


Figure 5 Comparisson of mycelial and secreted sets. (a) Venn diagram comparing *P. capsici* proteins that were identified in at least three V8 mycelia samples with proteins that were identified in at least three V8 secreted samples. (b) Fold change over the intensity average was performed for each the 21 proteins shared in the mycelia and secreted sets. + indicates a fold change equal or superior to two.

An analysis of the average intensities of these 21 shared proteins in the two distinct data sets showed that 15 of these proteins were at least two times more abundant in the supernatant derived set and were therefore more likely to be secreted proteins. In contrast, we found two other proteins that appeared to be more abundant in the mycelial samples and were therefore considered cellular proteins (Figure 5b). This analysis generated our two final sets that from now on will be designed as the mycelia set (772 proteins) and the secreted set (93 proteins).

As the vast majority of secreted proteins in *Phytophthora* feature a predictable signal peptide sequence, we set to investigate to what extent, our secreted protein set feature a predictable secretory leader sequence. Using the SignalP 4.1 server (Petersen et al., 2011), we observed that 68% (63 proteins) of the proteins in our secreted set contain a predicted signal peptide (Figure 6). This result contrasted with results found in the mycelia derived set where less than 3% were predicted to be secreted (figure 6). The enrichment of signal peptide presence in our secreted set points to an efficient identification of *P. capsici* secreted proteins using our method.

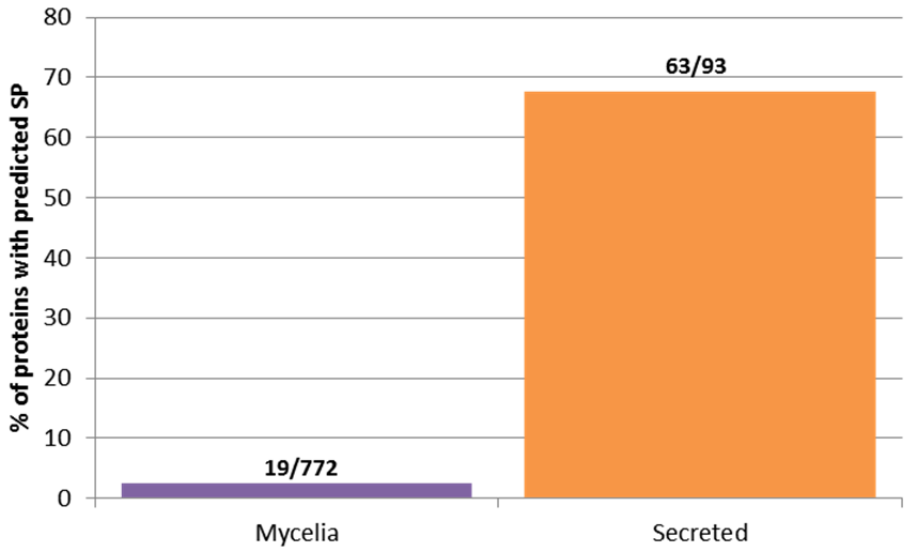


Figure 6 Secreted set is enriched for proteins with predicted signal peptides. Signal peptides for both mycelia and secreted set were predicted using SignalP 4.1. Histogram shows percentages of proteins with predicted signal peptides in each set. Numbers of the proteins with predicted signal peptides out of the total proteins in the set are also shown.

Proteomic approach allows the identification of *P. capsici* putative effectors.

With our proteomics approach we identified 93 *P. capsici* proteins that are putatively secreted during *P. capsici* growth in V8 media. To assess if these proteins could play roles in *P. capsici* virulence, we looked at their expression profiles during various stages of the *P. capsici* infection cycle. A microarray experiment assessing *P. capsici* gene expression in vitro and during infection of tomato leaves identified a set of 3,691 genes (Jupe et al., 2013), including a large effector repertoire, differentially regulated during infection. We therefore asked whether genes encoding the proteins identified in this study feature expression patterns that indicate a role during infection. From the 93 proteins identified in our secreted set, 45 were shown to be differentially expressed in the above described microarray experiment. The same analysis was performed with the proteins from the mycelial set and 254 proteins (out of 772) were identified to be differentially expressed. Thus, the secreted set is enriched for the presence of differential expressed genes (DEGs) (33% for the mycelia set and 48% for the secreted set) (Figure 8a). However it is difficult to evaluate if this enrichment could not be achieved by chance.

The analysis of the expression profiles of the 45 putative secreted proteins that are differentially expressed during *P. capsici* life stages shows that they have different expression patterns with proteins being over-expressed during all the tested *P. capsici* in vitro and infection stages (Figure 8b). Cluster analysis allowed us to identify four main gene classes that feature different expression profiles (Figure 8c): class 1 consists of three genes mainly expressed in the necrotrophic stages of infection (48 and 72 hours post-infection); class 2 contains two genes mostly expressed in mycelial stages; class 3 contains 14

genes mostly expressed in sporangia; and class 4 is composed of 26 genes expressed mainly at earlier stages of infection process.

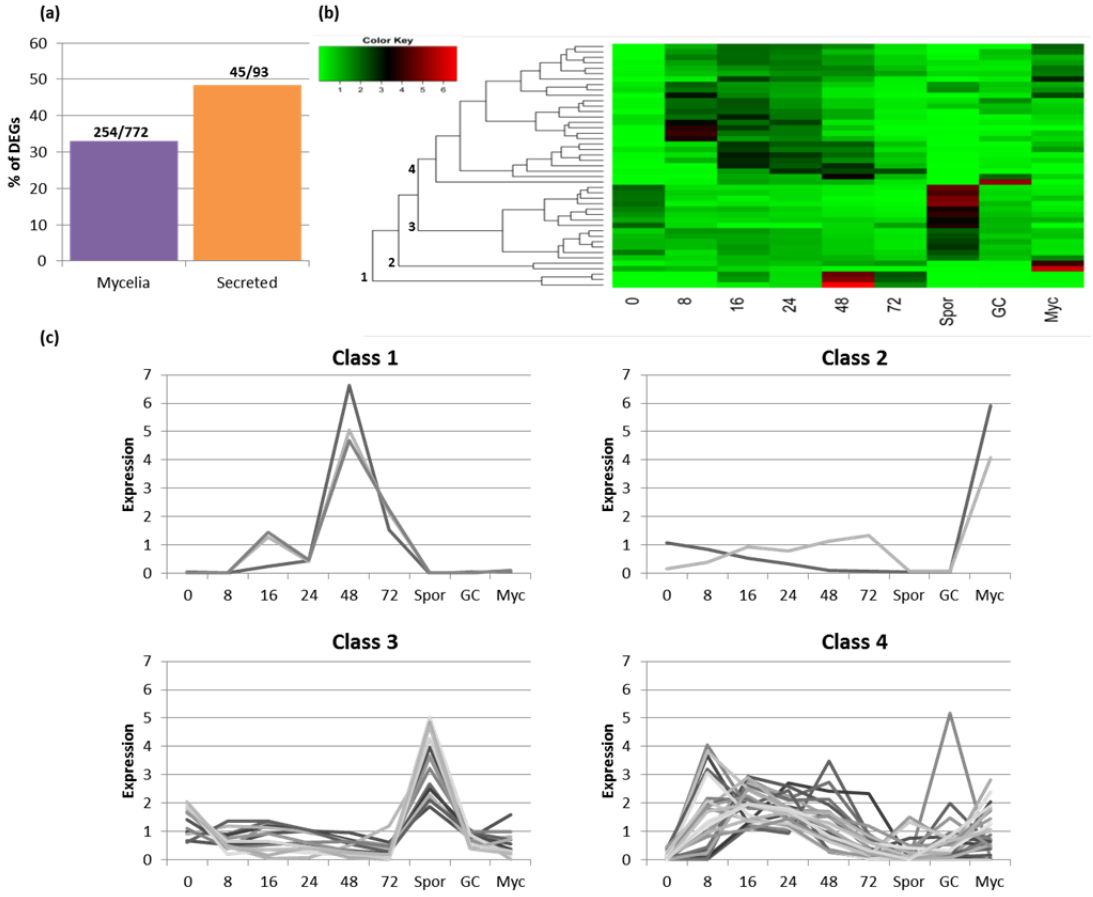


Figure 7 Expression profiles of *P. capsici* putative secreted proteins. (a) Proteins from both mycelia and secreted set were searched against a dataset of DEGs (Jupe et al., 2013). Histogram shows percentages of proteins that are encoded by DEGs in each set. Numbers of the proteins encoded by DEGs out of the total proteins in the set are also shown. (b) Cluster analysis with proteins encoded by DEGs present in the secreted set. Color key indicates fold changes over mean expression values across all treatments. Numbers (one to four) indicate classes of genes that were selected for further analysis. (c) Expression (fold changes over mean expression values across all treatments) for the four cluster classes was analysed separately. Numbers (0, 8, 16, 24, 47 and 72) stand for hours post-infection. Spor, GC and Myc stand for sporangia/zoospore, germinating cysts and mycelia stages respectively.

An analysis of the protein domains of these 45 putative secreted proteins that are differentially expressed during *P. capsici* life stages showed that the majority of these proteins encode predicted hydrolases, peptidases and pathogenesis related proteins suggesting a virulence role of these proteins (Table 2). Interestingly, some proteins do not contain any annotated domains

leading to the possibility that they are effector proteins, lacking homologues in publicly available databases.

Table 2 Description of *P. capsici* proteins from the secreted set encoded by DEGs

Class 1	iprDesc	SP
Phyca11_9247		N
Phyca11_7941	Peptidase C1A, papain C-terminal	Y
Phyca11_8418	Peptidase C1A, papain C-terminal	N
Class 2	iprDesc	SP
Phyca11_509045	SCP-like extracellular	Y
Phyca11_534296		N
Class 3	iprDesc	SP
Phyca11_507518	SMP-3 /Gluconolaconase/LRE-like region	Y
Phyca11_503383	Necrosis inducing protein-1	Y
Phyca11_507175		N
Phyca11_552671	Thaumatin, pathogenesis-related	Y
Phyca11_507030	EGF	N
Phyca11_528056		Y
Phyca11_504431		N
Phyca11_131909	Glycoside hydrolase, catalytic core	N
Phyca11_507766	Metallophosphoesterase	N
Phyca11_572227	Glycolipid anchored surface protein GAS1	Y
Phyca11_507979	Rare lipoprotein A	Y
Phyca11_505468	TonB box, conserved site	Y
Phyca11_508012		N
Phyca11_506007		Y
Class 4	iprDesc	SP
Phyca11_506445	Alpha/beta hydrolase fold-1	N
Phyca11_577335	Carboxylesterase, type B	Y
Phyca11_552045	Glycosyl hydrolase 53	Y
Phyca11_102803	Pectate lyase, catalytic	N
Phyca11_11949	Elicitin	Y
Phyca11_15125	Glycoside hydrolase, family 11	N
Phyca11_98516	Glycoside hydrolase, family 43	Y
Phyca11_126683	Protein of unknown function DUF676, hydrolase-like	Y
Phyca11_573718		N
Phyca11_508581	Glycoside hydrolase, catalytic core	Y
Phyca11_565318		Y
Phyca11_6934		Y
Phyca11_125481		Y
Phyca11_508441	Glycoside hydrolase, family 3	Y
Phyca11_555398	Ribonuclease T2	Y
Phyca11_109236	Thaumatin, pathogenesis-related	Y
Phyca11_509157	Glycoside hydrolase, family 17	Y
Phyca11_572121		Y
Phyca11_57493	EGF, extracellular	Y
Phyca11_508589	Glycoside hydrolase, family 17	Y
Phyca11_551419		Y
Phyca11_504748	Proteinase inhibitor I25, cystatin	Y
Phyca11_511894	Peptidyl-prolyl cis-trans isomerase, cyclophilin-type	Y
Phyca11_506995	Glycoside hydrolase, family 17	Y
Phyca11_505466		Y

Phyca11_540242	Glycoside hydrolase, family 6	N

Note: SP stands for Signal peptide. Y or N indicate the presence or absence of predicted signal peptides respectively.

Discussion

This study aimed to identify secreted proteins from the plant pathogenic oomycete *P. capsici*. For that *P. capsici* was grown in two different media (PB and V8), and samples of the mycelial mat or of the media with and without *P. capsici* presence were analysed by mass-spectrometry. In this study we generated two datasets containing proteins that appear consistently in *P. capsici* mycelia samples (791 proteins) or consistently in V8 media in which *P. capsici* was grown (99 proteins).

As the *P. capsici* strains used in this study expressed constitutively free GFP, we tried to use the presence of GFP to assess the quality of the separation between mycelia and secreted proteins in our dataset (as GFP should be detected only in the mycelia set). However, GFP was not detected by our proteomic approach, suggesting that GFP presence was under the detection threshold. Nonetheless our mycelia and secreted sets were distinct, with only 21 proteins common in both sets (Figure 5a), pointing to a low contamination of secreted samples with mycelia derived proteins. An analysis of LFQ intensities of these 21 shared proteins showed that 15 of them were at least two times more abundant in secreted samples while 2 of them were more abundant in the mycelia set (Figure 5b). Thus these 15 and 2 proteins were added to our final secreted and mycelia set respectively leading to a final set size of 93 proteins for the secreted set and 772 for the mycelia set.

To gain better insights into the possible virulence role of the 93 proteins predicted to be secreted in our study we looked at their expression profiles obtained in a composite microarray looking at both *P. capsici* and tomato transcriptomes during *P. capsici*-tomato interactions (Jupe et al., 2013). 45 out of the 93 identified secreted proteins were shown to be differentially expressed during *P. capsici* growth *in vitro* and in tomato leaves (Figure 7). These proteins showed different expression profiles and contained distinct predicted domains that help on predicting their possible virulence functions (Figure 7, Table 2). For instance, in our analysis we identified a set of glycoside hydrolases that are up-regulated during early stages of infection, allowing us to hypothesise that they could be working at these infection stages to degrade plant cell walls and therefore mediate successful pathogen ingress in the host plant. Also proteins with no predicted functional domains and no predicted signal peptide were identified, leading to the hypothesis that these proteins could encode undescribed families of *P. capsici* effectors.

The number of candidate secreted proteins identified in this study is far from representing the predicted total secretome of *Phytophthora* species. In *P. infestans* 1415 proteins were predicted to encompass the *P. infestans* secretome as they had predicted extracellular localisation, signal peptide and no transmembrane domains (Raffaele et al., 2010). Nevertheless, a proteomic study aiming to identify *P. infestans* secreted proteins identified only 283 proteins from which 201 had predicted signal peptides (Meijer et al., 2014). These values are in line with the ones obtained in our approach, as we only took into account secreted proteins during *P. capsici* growth in V8 media, while for *P. infestans* secretome seven different media were used, greatly enhancing secreted protein identification (Meijer et al., 2014). Nonetheless, this

discrepancy obtained between the predicted secretome of plant pathogens and the secreted proteins identified using proteomic approaches is expected as not all predicted genes encoding secreted proteins will be expressed. It is well established that during infection, effector gene expression appears timed and finely regulated, reflecting their unique roles towards pathogen virulence. Proteomics on samples generated in vitro thus is likely to limit our ability to identify effector proteins.

Although our analyses is limited in its scope, this work allowed the identification of *P. capsici* putative secreted proteins that would not be picked up using genome annotation approaches as they do not contain predicted signal peptides. This a common feature of proteomic studies aiming for secretome characterisation as, for example in plants, these studies were shown to identify 40 to 70 % of putative secreted proteins without predicted signal peptides (Tanveer et al., 2014). This signal peptide absence is not solely attributed to possible incorrect signal peptide predictions but also to the possible existence of incorrectly annotated gene models. For instance, manual curation of gene models of *P. infestans* proteins identified as secreted in a proteomics approach lead to the re-annotation of nearly 150 gene models (Meijer et al., 2014). In addition, absence of signal peptides in putative secreted proteins could also be explained by the existence of unconventional secretion mechanisms that do not require the presence of signal peptides and that are yet unknown in *Phytophthora* species (Malhotra, 2013). In this context, a detailed effort towards the identification of alternative secretion signals is desirable and may be feasible by employing datasets generated in this and other studies.

This study did not identify any effector in the secreted set from the highly characterised RXLR and CRN families. The lack of these proteins in our supernatants could be due to low abundance, differential expression/secretion mediated by plant signals and requirement of haustoria structures for effector secretion. It is also not clear to what extent cytoplasmic effectors can withstand the extracellular environment.

Notwithstanding, in our analysis we observed that around 2% of the identified proteins were responsible for more than 50% of the intensities in our dataset, suggesting that the presence of highly abundant proteins could be hampering an increase on the number of proteins identified (Figure 3a). Moreover, tomato and contaminant proteins (as keratin and trypsin) were shown to be part of these highly abundant proteins (Figure 3b). As the tomato highly abundant proteins are thought to originate from the V8 media, the use of a media with a lower protein content that still allowed *P. capsici* growth would seem a solution to avoid highly abundant detection of media proteins. However, the use of a less rich media could possibly inhibit the expression of *P. capsici* secreted proteins. In relation to the contaminant presence, it is common and unavoidable and we only can aim to minimize it with extremely careful sample handling (Hodge et al., 2013).

In summary, a proteomic approach was used to characterise the *P. capsici* secretome, allowing the identification of 93 putative secreted proteins. Validation of the secretion of these proteins and characterisation of their virulence importance could lead to the discovery of new *P. capsici* effectors that would not be identified using conventional genome annotation approaches. Moreover, as some of the identified candidate secreted proteins do not contain

signal peptides, they could be valuable tools in aiding the characterisation of unconventional secretion mechanisms in oomycetes.

Chapter 6. General discussion and concluding remarks

T. M. M. M. Amaro, E. Huitema.

Huge research efforts have led to the view that effectors are important factors that help determine disease outcomes during plant-microbe interactions (Asai and Shirasu, 2015; Bozkurt et al., 2012; Selin et al., 2016). Thus, and with plant pathogens continuously harming crop production worldwide (Oerke, 2006), it is important to characterise the virulence functions of pathogen effectors, when aiming for new and informed breeding strategies for improving resistance in crop plants (Gawehns et al., 2013).

Phytophthora capsici is a highly destructive plant pathogen that infests a variety of hosts worldwide (Kamoun et al., 2015; Lamour et al., 2012b). Nevertheless, the virulence functions of *P. capsici* encoded effectors are still mostly uncharacterised. One of the effector families encoded by *P. capsici* for which virulence mechanisms only now start to be unveiled is the Crinkling and Necrosis (CRN) effector family.

CRN proteins were identified as cytoplasmic effectors encoded by oomycete genomes that in some but not all cases induced cell death responses in plants (Schornack et al., 2010; Stam et al., 2013b; Torto et al., 2003). CRNs were also shown to have virulence functions and their virulence mechanisms have recently started to be elucidated (Ramirez-Garcés et al., 2015; Song et al.,

2015; Stam et al., 2013c; Zhang et al., 2015b). However, there are a number of unanswered questions in CRN effector biology (summarised in Chapter 2). One of these questions is whether CRN mediated cell death, seen upon over-expression *in planta*, represents a virulence function.

Has CRN mediated cell death a virulence role?

CRN effectors were identified and named after their capacity to induce plant cell death (Torto et al., 2003). Despite following studies showing that cell death inducing capacities are not present in all CRN effectors (Shen et al., 2013; Stam et al., 2013b), it is still important to assess the virulence relevance of these cell death processes.

In the first described effort aimed to unveil the virulence function of CRN mediated cell death, we used PcCRN83_152 as a model as this CRN featured both cell death and virulence bosting phenotypes when over-expressed in *N. benthamiana* leaves (Stam et al., 2013b). Using a random mutagenesis screen, we were able to create and characterise PcCRN83_152 variants that lost their cell death capacities (NCD variants). Importantly, some of these NCD variants retained the capacity to enhance *P. capsici* growth when over-expressed in *N. benthamiana* leaves, indicating that cell death is not required for PcCRN83_152 virulence capacities (Chapter 3).

In pathogens with hemi-biotrophic lifestyles, as *P. capsici*, it could be hypothesised that CRN effectors could work on inducing plant cell death during necrotrophic stages of infection. While some CRNs are upregulated during late stages of infection, some of them seem upregulated during the biotrophic stages (Stam et al., 2013b). Moreover, this upregulation during biotrophy is not

connected to absence of cell death inducing capacities. For example, PcCRN83_152 was shown to induce plant cell death and also to be up-regulated in early biotrophic stages of *P. capsici* infection on *N. benthamiana* leaves (Mafurah et al., 2015; Stam et al., 2013a). Thus, there is a possibility that CRN cell death is a phenotypic outcome not desired by the pathogen which in turn implies that CRN-induced cell death is not related to virulence.

As a process undesired by the pathogen, CRN mediated cell death could be connected to plant defence responses, namely with recognition events leading to HR. Nevertheless, *in planta* over-expression of cell death inducing CRNs was shown to enhance pathogen virulence (Ramirez-Garcés et al., 2015; Stam et al., 2013b; Zhang et al., 2015b), rendering this hypothesis improbable. However, it cannot be excluded that during an infection process, plant mediated defence responses against the over-expressed CRN could be circumvented by other pathogen encoded effectors, being CRNs enhancing virulence despite plant mediated recognition and consequent cell death. In addition, CRN mediated cell death could also be an over-expression artefact that does not reflect the true function of CRN effectors during infection processes.

Our results do not provide definite answers for these questions relating the nature of CRN mediated cell death. Moreover they still leave unclear if the observations on PcCRN83_152 hold true for other CRN effectors. Nonetheless the results obtained lead to new insights into CRN functions that stress the need to further characterise the nature of CRN mediated cell death and its putative virulence roles.

PcCRN83_152 virulence function(s)

The analysis on Chapter 3 provided new insights on the virulence relevance of PcCRN83_152 mediated cell death. However, it did not provide information on the plant processes targeted by PcCRN83_152 to achieve both its virulence and cell death functions. In Chapter 4 we showed that PcCRN83_152 interacts with nuclear proteins from the host plants *N. benthamiana* (NbSIZ1 and NbSLX1) and tomato (SlSIZ1Δ867). We also showed that SIZ1 proteins are involved in plant immunity processes against *P. capsici* infections. Moreover, while no conclusive evidence was obtained for NbSLX1 involvement in plant immunity processes, both SIZ1 and SLX1 proteins are connected with DNA repair mechanisms in yeast and mammal systems (Rouse, 2009; Strunnikov et al., 2001; Westerbeck et al., 2014), connecting these targets with PcCRN83_152 mediated host chromatin re-localisation capacities. AeCRN13, a cell death inducing CRN effector from *Aphanomyces euteiches*, was recently shown to mediate increased plant susceptibility against *P. capsici* infections by inducing plant DNA damage (Ramirez-Garcés et al., 2015). Similarly, with this work, we could be unveiling a host complex involved in DNA stability that is targeted by a CRN effector from *P. capsici*. Further studies, and taking advantage of the available NCD variants of PcCRN83_152, could lead to an understanding of how PcCRN83_152 is modulating the function of its plant targets to induce both its cell death and virulence functions *in planta*.

Effector targets have been proposed to be extremely good candidates for resistance breeding efforts (Gawehns et al., 2013). SIZ1 proteins could be one of these targets, as transgenic *N. benthamiana* plants over-expressing NbSIZ1

showed enhanced resistance to *P. capsici* infections. These transgenic plants showed a dwarf phenotype, due probably to elevated SA levels, which could be prejudicial for plant productivity. Nevertheless, a subversion of the dwarf phenotype, as partially achieved in *Arabidopsis* plants by over-expression of a bacterial gene encoding a salicylate hydroxylase (nahG) (Lee et al., 2006), without losing the resistance phenotype could be attempted. Nonetheless, to gain confidence of the viability of using SIZ1 proteins to achieve plant resistance against *P. capsici*, it is important to test if, coupled with an effective reversion of the described SIZ1-mediated dwarf phenotype, the observed resistance phenotypes hold true for crop plants and if they are consistent when using diverse *P. capsici* isolates.

Identification of new *P. capsici* candidate effectors

The study of pathogen effectors and their virulence host targets can lead to new insights into the mechanisms of plant immunity that can promote improvements on crop resistance breeding. In *Phytophthora* these studies are mostly focused on two classes of effectors (RXLRs and CRNs) (Anderson et al., 2015, Chapter 2). While studies aiming to characterise effectors from these two families have undoubtedly been important on characterising *Phytophthora* virulence mechanisms, better insights on these mechanisms could be obtained by identifying and studying other families of effector proteins. Effectors were shown to have redundant functions and to target similar plant immunity hubs (Mukhtar et al., 2011), thus, for instance, the blocking of the action of an RXLR effector could be complemented by the action of a effector from an uncharacterised effector family. Therefore, the identification and characterisation of other

effector families from *Phytophthora* could prove to be crucial for future resistance breeding.

In a proteomics-based approach aiming for a characterisation of the *P. capsici* secretome, we identified 93 putative *P. capsici* secreted proteins from which 45 were predicted to be differentially expressed at different stages of the disease cycle in a gene expression study published previously (Jupe et al., 2013). These proteins were therefore considered as candidate *P. capsici* effector proteins. Importantly, some of these effector candidates do not contain signal peptides and, for that reason, would not be identified by genomic- and transcriptomic-based effector identification efforts. Moreover, the proteome approach followed provides experimental evidence of high abundance of these candidate effectors in the *P. capsici* secretome, which is indicated by their consistent detection by mass spectrometry. Further studies confirming the secretion of these proteins and their virulence role could lead to the discovery of new important virulence mechanisms deployed by pathogens and consequently more possible targets for studies aiming to increase plant resistance.

Concluding remarks

In this thesis we significantly increased our knowledge on CRN effector proteins. Besides summarising the recent findings on CRN biology (Chapter 2), we addressed an important and unanswered question regarding CRN effector functions, namely the relevance of cell death mediated by CRNs for pathogen virulence (Chapter 3). In this work I provided the first direct evidence for a separation of CRN cell death and virulence roles, which could lead to important new insights on interpreting and designing experiments to assess CRN

biological relevance. For instance, if it is true that CRN mediated cell death is not a pathogen desired phenotype, there is a need for caution when interpreting the virulence relevance of cell death associated processes mediated by CRN effectors.

An identification of PcCRN83_152 plant nuclear targets and a characterisation of their putative connections with plant immunity processes against *P. capsici* infection were also achieved in this thesis (Chapter 4). Due to the characterised functions of PcCRN83_152 targets (SLX1 and SIZ1 proteins), in this work we could be unveiling a new immunity related complex putatively associated with DNA repair mechanisms with potential to be considered in future resistance breeding strategies. Therefore, with this work, I uncovered putative virulence mechanisms for a *P. capsici* effector, virulence mechanisms which for *P. capsici* effectors remain almost completely unknown. Thus I provided new clues for an increased understanding of the virulence mechanisms that render *P. capsici* so devastating in such a variety of host plants.

Lastly, in this work we identified new putative effectors from *P. capsici* that could provide with further new leads to an increased understanding of *Phytophthora* virulence mechanisms and new cues to pursue when aiming to enhance plant resistance against *P. capsici*.

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