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Identification and characterization of effector proteins from pine needle pathogens

A thesis presented in partial fulfilment of the
requirements for the degree of

Doctor of Philosophy (PhD)

in

Genetics

at Massey University, Manawatū, New Zealand

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2022

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As a consequence of the first and second nation-wide COVID-19 lockdowns, all *Nicotiana benthamiana* and *Nicotiana tabacum* plants used in *Agrobacterium tumefaciens*-mediated transformation assays (ATTAs) involving *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* candidate effectors could not be watered and therefore died. The plants that were lost were ready for experimental work, and it took at least six weeks for new *Nicotiana* plants to be ready for research (from seed to germination).

Also as a consequence of the first nation-wide COVID-19 lockdown, *Pinus radiata* tissue culture shoots provided by Scion died. Those were intended for infiltration assays with purified candidate proteins identified by the ATTAs mentioned above. Because of the extended period of the lockdown, the shoots could not be used when return to the University was possible. A new request for new shoots was made with Scion and it took approximately 2 months for the new shoots to be ready for use.

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Abstract

Collectively, *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* cause serious foliar diseases on *Pinus radiata* in New Zealand and on many other pine species worldwide. Considering the ecological and economic importance of forest trees, understanding how these pathogens interact with their hosts on a molecular level is critical as it could lead to new and durable approaches to control the diseases they cause. Pathogens have the ability to deliver proteinaceous virulence factors, termed effectors, into the apoplast and cell cytoplasm of their host plants. Effectors typically promote host colonization through suppression of the plant immune system. However, in resistant host plants, one or more of these effectors can be recognized by corresponding immune receptors to activate the plant immune system. Often, one of the main outputs of this immune system is a localised cell death reaction, termed the hypersensitive response (HR), which renders the pathogen unable to cause disease (avirulent). The general goal of this thesis was to identify shared candidate effector (CE) proteins between the three foliar pine pathogens and to characterise their virulence (or avirulence) functions. This is important because disease resistance based on core effectors that are vital for a pathogen's ability to cause disease is more likely to be durable. Using a combination of "omics" information and bioinformatic tools, two sets of orthologous CE proteins were identified between *D. septosporum*, *C. minus* and *P. pluvialis*, while several other sets were identified between the two fungal pathogens. Some of these CEs had the ability to trigger cell death responses in non-host *Nicotiana* plants, and some were shown to activate *Nicotiana benthamiana* genes involved in pathogen-associated molecular pattern-triggered immunity and HR. CEs were also screened in the host, *Pn. radiata*, using a method developed in this thesis, where it was determined that some of these CEs also trigger cell death. Two conserved cell death elicitor families, Ecp20 and Ecp32, were identified from *D. septosporum* and its close relative *Fulvia fulvum*, and the cell death triggered by some family members in *N. benthamiana* was shown to require membrane-localized receptor-like proteins. Tertiary structure predictions of CEs provided insights into the possible roles and host targets of these proteins during pine infection. Moreover, a shared β -trefoil fold was found between sequence-unrelated CE proteins from the three pine pathogens, along

with evidence that they are also present in many other fungal species. A CRISPR/Cas9 gene editing methodology was applied to *D. septosporum* for the first time, which allowed for the functional characterization of three *D. septosporum* CE genes, two of which are also present in *C. minus* and *P. pluvialis*. Collectively, this thesis provides a significant advance in our understanding of pine-pathogen interactions at the molecular level and provides a blueprint for similar studies in other forest pathosystems.

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List of abbreviations

AMA	apical membrane antigen
ATTA	<i>Agrobacterium tumefaciens</i> -mediated transformation assay
Avr	avirulence
BAK1	brassinosteroid Insensitive 1-associated kinase 1
bp	base pairs
BSR	broad-spectrum resistance
CAP	cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins'
Cas	CRISPR-associated
CAZymes	carbohydrate active enzymes
CBEL	cellulose binding elicitor lectin
CBM	caveolin-binding motif
CC	coiled-coil
CDD	Conserved Domain Database
CDS	coding sequence
CE	candidate effector
Cm	<i>Cyclaneusma minus</i>
Cmn	<i>Cyclaneusma minus</i> morphotype novus
Cms	<i>Cyclaneusma minus</i> morphotype simile
Cmv	<i>Cyclaneusma minus</i> morphotype verum
CNC	<i>Cyclaneusma</i> needle cast
CRISPR	Clustered regularly interspaced short palindromic repeat
crRNA	CRISPR RNA
Ct	cycle threshold
DAMP	damage-associated molecular pattern
dDNA	donor DNA
DEG	differently expressed gene
dH ₂ O	distilled water
DNB	<i>Dothistroma</i> needle blight
Ds	<i>Dothistroma septosporum</i>
DSB	double-strand break
DW	dry-weight
Ecp	extracellular protein
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered immunity
EV	empty vector
Ff	<i>Fulvia fulva</i>
FLS2	flagellin-sensing 2
gDNA	genomic DNA
GH	glycoside hydrolase
GPI	glycophosphatidylinositol
H	hour
ha	hectares
hai	hours after infiltration

HDR	homology directed repair
HR	hypersensitive response
HST	host-specific toxin
IDR	intrinsically disordered regions
IMAC	immobilized metal ion affinity
IP	invasion pattern
IPR	IP receptor
IPTR	IP-triggered resistance
JA	jasmonic acid
JGI	Joint Genome Institute
LB	lysogeny broth
LRR	leucine-rich repeat
LysM	lysine motif
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
mg	milligram
min	minute
mL	milliliter
MLO	Mildew Resistance Locus O
Nb	<i>Nicotiana benthamiana</i>
NBS	nucleotide-binding site
NCBI	National Center for Biotechnology Information
NEP1	necrosis and ethylene-inducing peptide 1
ng	nanogram
NHEJ	non-homologous end joining
NLP	NEP1-like proteins
NLR	NBS, LRR-like receptor
NO	nitrogen oxide
NRQ	normalized relative quantity
Nt	<i>Nicotiana tabacum</i>
nt	<i>nucleotide</i>
PAM	protospacer adjacent motif
PAMP	pathogen-associated molecular pattern
PFT	pore forming toxin
pLDDT	predicted local-distance difference test
Pp	<i>Phytophthora pluvialis</i>
PR	pathogenesis related
PRR	pattern recognition receptor
Pry1	pathogen-related yeast 1
PTI	PAMP-triggered immunity
QTL	quantitative trait locus
R	resistance protein
RCSB PDB	Research Collaboratory for Structural Bioinformatics Protein Data Bank
RLK	receptor-like kinase
RLP	receptor-like protein
RNC	red needle cast

ROS	reactive oxygen species
RPM	rotations per minute
RPMK	Reads Per Million per Kilobase
RPW8	resistance to powdery mildew 8
RQ	relative quantity
qPCR	real time-quantitative PCR
S	susceptibility
SA	salicylic acid
SCOP	Structural Classification Of Proteins
s	seconds
sgRNA	single-guide RNA
SNP	single nucleotide polymorphism
SOBIR1	suppressor of BAK1-interacting receptor-like kinase 1
TALEN	transcriptional activator-like effectors nuclease
T-DNA	Transfer-DNA
Ti	Tumour-inducing
TIR	toll/interleukin-1 receptor-like
TLP	thaumatin-like protein
TM	template modelling
tracrRNA	trans-activating crRNA
TRV	tobacco rattle virus
V	Volts
v	volume
VIGS	virus-induced gene silencing
vir	virulence
w	weight
WAK	cell wall-associated kinases
WPBR	white pine blister rust
WT	wild-type
ZFN	zinc-finger nuclease
μL	microliter

1 Introduction

Climate change, biodiversity loss and the emergence of new diseases are the result of intense environmental deterioration (FAO, 2022). Deforestation of native ecosystems not only contributes to increased greenhouse gas levels, but also facilitates the emergence of infectious forest diseases, by altering pathogen distribution patterns and population structures (Linnakoski et al., 2019). The conservation of trees and forests can help mitigate these problems, moving the world into a more sustainable condition. Forests cover 31% of Earth's land surface area (FAO, 2022), and sequester carbon, making this ecosystem essential to combat climate change (Law et al., 2018). However, forests are subjected to biotic and abiotic stresses, many of them with anthropological causes. Increased temperatures worldwide, drought, and deficiency of nutrients in the soil due to deforestation and erosion are the major abiotic stresses (Allen et al., 2015). Pathogens and pests that have an important impact on this ecosystem are major causes of biotic stress. The Forest Resources Assessment reported that more than 140 million ha of forests across 75 countries were greatly affected by these abiotic and biotic stresses over a ten year period (van Lierop et al., 2015). With these numbers expected to increase, there is an urgent need to protect forests and minimize the impact of diseases.

The *Pinus* (pine) genus has over 100 species and is considered to be among the most ecologically and economically important genus of forest trees. These species are threatened by different pathogens which can affect roots, bark, needles, and the plant's vascular system, causing symptoms such as leafspots, defoliation and reduced uptake of water and nutrients, all of which can ultimately lead to the death of the tree. Amongst these, fungal and oomycetes are two very distinct types of microorganisms that can cause disease not only in pine, but in many other economically and ecologically important forest trees. **Table 1.1** shows the main pathogens causing different diseases in pine trees.

Table 1.1. Fungal and oomycete forest pathogens that cause disease in pine species.

Pine pathogen	Lifestyle ^a	Disease	Reference
<i>Dothistroma septosporum</i>	H	Dothistroma needle blight	Drenkhan et al. (2016)
<i>Dothistroma pini</i>	H	Dothistroma needle blight	Drenkhan et al. (2016)
<i>Lecanosticta acicola</i>	-	Brown spot needle blight	van der Nest et al. (2019)
<i>Cronartium ribicola</i>	B	White pine blister rust	Kinloch (2003)
<i>Gremmeniella abietina</i>	-	Scleroderris canker	Botella and Hantula (2018)
<i>Fusarium circinatum</i>	H	Pitch canker	Nirenberg and O'Donnell (1998)
<i>Diplodia sapinea</i>	-	Diplodia shoot blight	Adamson et al. (2015)
<i>Elytroderma deformans</i>	-	Elytroderma needle cast	Childs (1971)
<i>Cyclaneusma minus</i>	H	Cyclaneusma needle cast	Bednářová et al. (2013)
<i>Pseudocercospora pini-densiflorae</i>	-	Cercospora needle blight	EFSA Panel on Plant Health (2017)
<i>Heterobasidion annosum</i>	N	Conifer root and butt rot	Asiegbu et al. (2005)
<i>Cronartium quercuum</i> f. <i>sp. fusiforme</i>	B	Fusiform rust disease	Hamelin et al. (1994)
<i>Phytophthora pluvialis</i>	H	Red needle cast	Reeser et al. (2013)
<i>Phytophthora pinifolia</i>	H	Daño foliar del pino	Durán et al. (2008)
<i>Phytophthora kernoviae</i>	H	Phytophyhora needle blight	McDougal and Ganley (2021)

^a B: biotrophic; H: hemibiotrophic; N: necrotrophic. The dash symbol indicates that the pathogen's lifestyle is not known.

Different pathogens have different lifestyles and interact with their hosts in different ways. Pathogens can be classified as biotrophic, necrotrophic or hemibiotrophic. Biotrophs, such as *Cronartium ribicola*, which causes white pine blister rust (WPBR) and is one of the most threatening forest diseases in North America, derive nutrients from living host tissue. WPBR has decreased the *Pinus monticola* (western white pine) population by up to 90% in this area, and also caused high mortality to other important white pine species (Kinloch, 2003). Necrotrophs, on the other hand, such as the root rot pathogen *Heterobasidion annosum*, kill their host cells in order to acquire nutrients. Root rot is a problem in many parts of Europe, North America and Asia, causing decay and, in severe cases, tree death (Asiegbu et al., 2005). Pathogens like *Fusarium circinatum* that have an initial biotrophic phase and later switch to a necrotrophic one, are known as hemibiotrophs. *Fu. circinatum* is responsible for pitch canker, one of the most important diseases of pines in North America, South Africa, parts of South America and Europe (Raitelaitytè et al., 2016).

Despite the serious impact these pathogens have on pine trees, not much is known about how they interact with their hosts at the molecular level. Most of these forest pathogens have had their genomes sequenced and many have been studied to determine their genetic diversity and population structure (Brar et al., 2018; Gross et al., 2014; Menkis & Burokienė, 2012; Mullett et al., 2021). Some other studies have putatively identified genes involved in the infection process of pines; however, few studies have actually functionally characterized the molecular mechanisms involved in pine-pathogen interactions (e.g., through gene deletion or disruption methods).

Pathogens interact with their hosts to promote host colonization by delivering proteinaceous molecules, termed effectors, into plant cells and the surrounding apoplastic environment, where they typically suppress host immune defence responses (Uhse & Djamei, 2018; Wang et al., 2020). In resistant host plants, however, one or more of these effectors can be recognized as invasion patterns (IPs) by corresponding immune receptors (termed resistance (R) proteins) to activate the plant immune system (Cook et al., 2015; Jones & Dangl, 2006). The identification and characterization of such effectors through genomic, transcriptomic, and proteomic approaches is known as effectoromics, and can be used as a way to screen for proteins that elicit or suppress plant defence responses. Knowledge of the molecular basis of plant-pathogen interactions can help with breeding strategies and new ways to manage diseases, by helping to identify host *R* genes. Based on this, the focus of this thesis was to identify and characterize effector proteins from three important *Pinus radiata* (radiata pine) pathogens with the ultimate goal of addressing knowledge gaps of molecular plant-pathogen interactions in a gymnosperm tree pathosystem.

1.1 *Pinus radiata*

Pn. radiata is one of the most common tree species in plantations in the Southern Hemisphere. There are over four million hectares (ha) of planted radiata pine worldwide, with the largest plantations in Chile, New Zealand (about 1.5 million ha each) and Australia (0.77 million ha). These three Southern Hemisphere countries are responsible for over 90% of the world's *Pn. radiata* plantations (Mead, 2013). There are five locations where *Pn. radiata* is native: Ano Nuevo, Monterey and Cambria (Coastal California, USA),

and both the Guadalupe and Cedros Islands (Mexican Islands). Radiata pine is grown commercially in New Zealand as an exotic species, originating from the Monterey peninsula in California, USA (Rogers, 2002).

Forestry is a significant industry in New Zealand. Total exports of forestry products from New Zealand are worth about \$6 billion a year, making it the country's third largest export earner, only behind dairy and meat. Approximately 90% of trees in all New Zealand-planted commercial forests are *Pn. radiata* trees, with most of the other species grown in the South Island (NZ Forest Owners Association, Facts and Figures 2020/2021).

Radiata pine plantations have been established in different types of climates; however, they have only really been successful in temperate areas, where the summers are relatively dry (Mead, 2013). The New Zealand climate varies from subtropical in the north to more temperate further south. Rainfall in the country is variable, but normally is evenly distributed throughout the year, with summer drought not being uncommon (Mead, 2013).

The first introduction of *Pn. radiata* to New Zealand occurred in 1859. The species is very adaptable to domestication and has high genetic variability (Mead, 2013). For those reasons, plus commitment to a large planting programme and intensive research efforts, rapid genetic improvement has been achieved in pines. The New Zealand breeding programme for *Pn. radiata* began in the 1950s. Current radiata pine breeding programmes focus on fast growth, stem straightness, good wood properties and resistance to common diseases (Jayawickrama & Carson, 2000; Li et al., 2017; Mead, 2013).

1.2 Foliar *Pinus radiata* diseases

There are three main *Pn. radiata* foliar diseases in New Zealand. Dothistroma needle blight (DNB) and cyclaneusma needle cast (CNC) are diseases caused by the fungal pathogens *Dothistroma septosporum* and *Cyclaneusma minus*, respectively, and red needle cast (RNC) is caused by the oomycete *Phytophthora pluvialis* (**Table 1.2**). *Phytophthora kernoviae* also has significant impact on New Zealand forests and is associated with a disease known as physiological needle blight (McDougal & Ganley, 2021), but was not included in this thesis. It is important to note that DNB has been

identified as a problem for longer than the other two diseases and is a model system for foliar pine pathogens; for these reasons, it has been extensively studied over the years.

These three foliar diseases can result in reduced tree growth and losses in productivity in affected areas, and sometimes tree death. It was estimated that a 10% increase in the proportion of trees affected by DNB or CNC can lead to a wood volume loss of 7-13 m³ ha⁻¹ (Bulman & Gadgil, 2001; Ismael et al., 2020). Translated to costs, according to the most recent available data, economic losses because of DNB of *Pn. radiata* were around \$20 million annually during the 2000s (Watt et al., 2011) and, in 2009, the losses caused by CNC to New Zealand forests were around \$38 million per annum (Bulman, 2009), all of which has a substantial effect on New Zealand's economy. In the United Kingdom, in 2010, losses due to DNB were estimated as £8 million a year in commercial timber forests and £50 million a year in landscape and recreational forest (Brown, 2011). There are not many studies quantifying losses due to RNC; however, one reported a 35% growth loss in the year following a severe incidence of RNC, and an average of 16% growth loss within three years of the RNC event (unpublished data from Ismael et al. (2020)).

Table 1.2. Characteristics associated with the three main *Pinus radiata* diseases in New Zealand.*

Symptom	Dothistroma Needle Blight (DNB)	Cyclaneusma Needle Cast (CNC)	Red Needle Cast (RNC)
Causal agent	Fungus – <i>Dothistroma septosporum</i>	Fungus – <i>Cyclaneusma minus</i>	Oomycete – <i>Phytophthora pluvialis</i>
Time of year (Southern Hemisphere)	All year, first appears on current foliage about December	September to November	April to October
Tree ages susceptible	Up to 15 years	6 to 20 years	All ages, but usually over 3 years
Incidence and severity	General distribution, almost every tree in affected areas, but tree to tree variation is apparent	Scattered individuals, up to 90% severity on very susceptible trees	Localized/general distribution, almost all trees in affected areas
Needle symptoms	Brick red bands on green needles with black spots within bands	Yellow, then gold, then brown needles	Oily green band, then yellow, then red needles
Needle retention	Needles are retained but die	Needles detach easily	Needles detach easily

* Adapted from the Ministry for Primary Industries website (<https://www.mpi.govt.nz/forestry/new-zealand-forests-forest-industry/forestry-pest-disease-management/pine-needle-diseases/>).

1.2.1 *Dothistroma* needle blight

DNB is one of the most important foliar diseases of pine trees. Over 80 pine species have been recorded with DNB, with different levels of susceptibility (Drenkhan et al., 2016). DNB is caused by two filamentous Dothideomycete fungal species, *D. septosporum* and *Dothistroma pini*, distinguishable only by molecular tools (Barnes et al., 2004). The origin of these species is not known, with hypotheses including Central America, the Himalayas and parts of North America and Europe (Drenkhan et al., 2016). A study that examined a worldwide collection of almost 4000 isolates suggested the origin of *D. septosporum* to be Eurasian, based on microsatellite data of these isolates. *D. septosporum* isolates could be grouped into three main population clusters (North America, Western and Eastern Europe), with subsequent spread to the Southern Hemisphere (Mullett et al., 2021).

D. septosporum is responsible for the majority of DNB, especially in North America and the Southern Hemisphere, where it causes devastation of *Pn. radiata* plantations (Bulman et al., 2016). *D. pini* is not present in New Zealand but causes DNB in Europe and USA. At first a large problem mainly in the Southern Hemisphere, in the last three decades DNB has increased in incidence and severity throughout the Northern Hemisphere, in both natural and managed forests (Barnes et al., 2022). One of the reasons this disease has become a global problem is the use of monocultures of susceptible hosts in areas that provided ideal conditions for pathogen and disease development. Increased incidence and severity is also attributed to changes in climate, with increased temperatures and wetter summers (Barnes et al., 2022; Woods et al., 2016). In New Zealand, DNB was first observed in 1962 (Bulman, 1993), and by the late 1960s had spread throughout most of the North Island. It is now present in New Zealand wherever suitable hosts are grown.

Early DNB symptoms usually appear on lower needles of pines as yellowish or pale brown spots that later become brown and widen to a necrotic band around the needle (**Figure 1.1A**). Within weeks of infection, those bands typically develop a characteristic brick red colour which can remain after the needles have died (Bradshaw, 2004; Gilmour, 1967). The characteristic red bands develop around the necrotic lesions

due to accumulation of a host non-selective broad-spectrum toxin, dothistromin (Bassett & Buchanan, 1970). Dothistromin is a virulence factor of *D. septosporum*; although not essential for pathogenicity, it is required for development of disease lesions (Kabir et al., 2015a). Small black spots (fruiting bodies; pycnidia) erupt from the needle in the red disease lesions (Bulman et al., 2016). Symptoms normally appear at the base of the crown first and then proceed towards the top part of the tree (Bulman et al., 2016) (**Figure 1.1B**). In contrast to other needle diseases, such as CNC and RNC, the dead needles are held on the tree for some time before dropping (Bulman et al., 2013).

Most DNB infections occur from late spring to late summer, taking place when the temperature is between 16 and 20°C and with continuous leaf wetness periods (Bradshaw, 2004; Bulman, 1993). Defoliation is most apparent between September and October but begins in summer on current foliage. The most serious impact of defoliation of needles is retardation of growth, which in severe cases can lead to tree mortality (Bradshaw, 2004; Drenkhan et al., 2016).

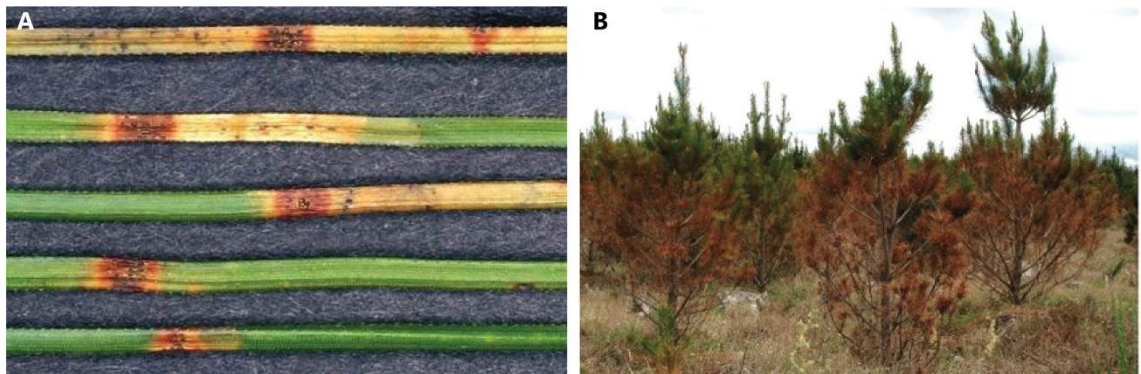


Figure 1.1. Dothistroma needle blight (DNB) symptoms caused by *Dothistroma septosporum*. **(A)** Red band and necrotic lesions, with fruiting bodies (black spots) on *Pinus radiata* needles infected with *Dothistroma septosporum*. **(B)** Needle death and premature defoliation caused by DNB in the lower parts of a *Pn. radiata* tree. Figure adapted from Bulman et al. (2008).

Dothistroma species are heterothallic, meaning that individuals carry one of two mating type genes with both mating types being necessary for sexual reproduction (Groenewald et al., 2007). Sexual reproduction is known to occur predominantly in the Northern Hemisphere, while in the Southern Hemisphere only three African countries have reported the sexual state (Drenkhan et al., 2016). With only one mating type and very low diversity, the New Zealand population of *D. septosporum* has been essentially

clonal for more than six decades (Bradshaw et al., 2019a). This is consistent with the hypothesis that *D. septosporum* was introduced into the Southern Hemisphere (Mullett et al., 2021). The sexual state of *D. pini* has never been reported (Drenkhan et al., 2016).

D. septosporum produces asexual and sexual spores, termed conidiospores (conidia) and ascospores, respectively. Conidia are dispersed by rain splash, which makes dispersal in the field very localized (Gadgil, 1967; Ivory, 1972; Mullett et al., 2016). Conidia are released during rain then splash on to fresh needles, where they germinate and cause new infections, with the main source of inoculum being needles attached to, or suspended in, the tree (Bulman et al., 2013; Gadgil, 1970; Kabir et al., 2015b). Long-distance conidiospore dispersal is believed to occur when spores are suspended in clouds, fog or mist, and by anthropogenic means such as the transportation of infected seedlings (Ivory, 1972). On the other hand, ascospores are dispersed by wind, which makes it possible for them to reach distant areas with susceptible trees, where the presence of both mating types leads to development of ascostromata, releasing more ascospores (Mullett et al., 2016).

D. septosporum has a hemibiotrophic lifestyle (Kabir et al., 2015b). The pathogen spends the first part of its lifecycle without causing any visible damage to cells of *Pn. radiata*, living first as an epiphyte, then penetrating the needle via stomata and colonizing the epistomatal chamber. This is followed by colonization of the intercellular apoplastic environment, located in the mesophyll layer, and gradual mesophyll cell disintegration, then endodermal collapse and eruption of fruiting bodies. Kabir et al. (2015b) found that the epiphytic phase lasts two weeks under glasshouse conditions, with colonization of the apoplast only starting three to five weeks after inoculation. Six-to-twelve weeks after inoculation, mature sporulating lesions appear near the red bands on the needles, indicating the late stage of DNB. Significantly higher levels of dothistromin are found in sporulating lesions compared to early lesions (Kabir et al., 2015b).

Disease management strategies that have been used for DNB include silvicultural methods, such as pruning and thinning, which can lower the levels of inoculum available to initiate new infection (Bulman et al., 2016). Aerial applications of fungicides, especially copper-based ones, provide relatively good control of the disease, where 3–4

applications are typically needed before stands reach the age of 15 years, at which time natural resistance against DNB occurs (Bulman & Gardner, 2014). However, their use can be heavily restricted in some countries, in which other more environmentally friendly methods need to be applied. Breeding for DNB resistance in *Pn. radiata* is possible, since *Dothistroma* resistance is moderately to highly heritable (Bulman et al., 2016), however no regions associated with resistance have been mapped through quantitative trait locus (QTL) analysis. The clonal population of *D. septosporum* in New Zealand makes breeding for disease resistance more durable than in places where both mating types, and subsequently sexual recombination, occur.

1.2.2 *Cyclaneusma* needle cast

CNC is a needle disease of many *Pinus* species worldwide (Bednářová et al., 2013), with 37 species being affected, including *Pn. radiata* in New Zealand and Australia. Scots pine (*Pinus sylvestris*) is also prone to the disease in forests of Europe and North America (Merrill & Wenner, 1996; Müller & Kurkela, 2012). The disease is caused by the Leotiomycete fungus *C. minus*.

Differences in ascospore length and characteristics of the fungus grown on agar cultures indicated that there are two morphological types of *C. minus* in New Zealand, termed *C. minus* "simile" and *C. minus* "verum" (Bulman & Gadgil, 2001). However, there seems to be considerable variation within each morphotype and, therefore, modern molecular techniques were required to correctly classify these isolates. DNA analysis was able to differentiate these morphotypes, serving as a tool to classify them, and showed that *C. minus* "simile" is more common across New Zealand than *C. minus* "verum" (Hunter et al., 2016). The DNA-based study also identified one isolate that could not be classified as either "verum" or "simile", suggesting that additional morphotypes might exist, highlighting the need for further characterization of this pathogen.

C. minus appears to occur first as an endophyte of pine needles, colonizing the tree for two months to two years before the occurrence of symptoms (Helander et al., 1994). Needles more than one-year old turn a mottled yellow-green and then a mottled yellow-brown a few weeks later. Transverse reddish-brown bands are also a common symptom of the disease (Bulman & Gadgil, 2001). However, the most characteristic

symptom of CNC is premature needle-cast in spring and autumn. In highly susceptible trees, almost the whole crown can be affected (Van der Pas et al., 1984). Symptoms of CNC can be seen in **Figure 1.2**.

Needles showing CNC symptoms are easily detached from the infected tree and most are shed prematurely, generally in September and October (for the spring peak) and April to June (for the second, less severe peak during autumn). By December, all affected needles are usually shed and the tree crown looks thin, holding only the newly flushed foliage, which is unaffected by the disease up to about 8–9 months of age (Bulman & Gadgil, 2001).

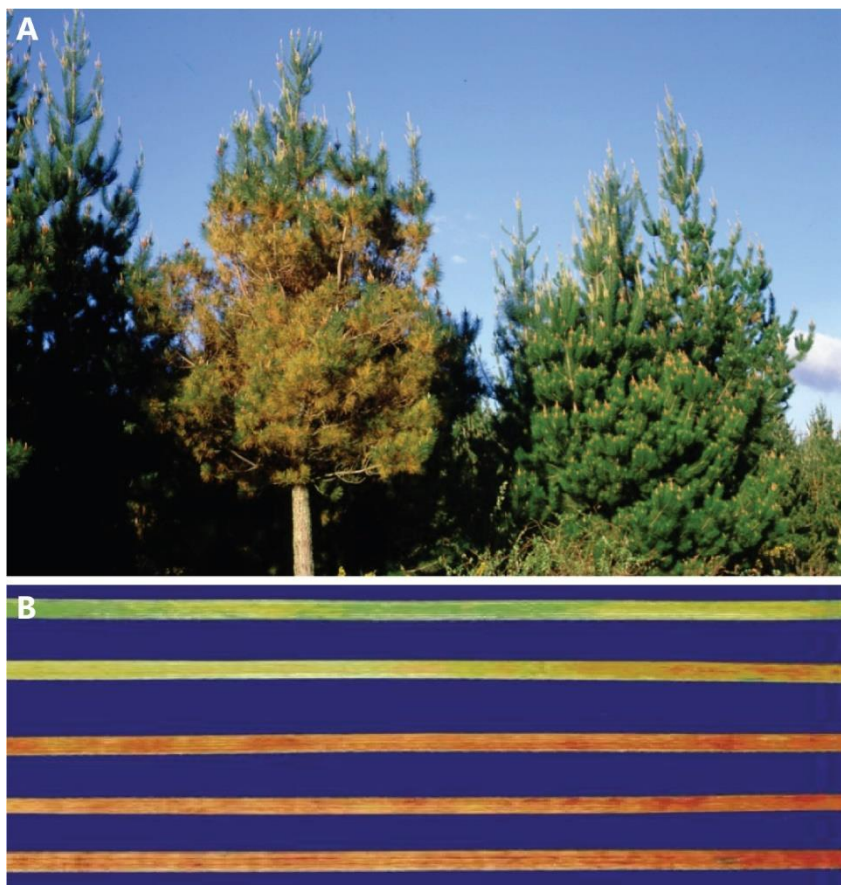


Figure 1.2. Cyclaneusma needle cast (CNC) symptoms caused by *Cyclaneusma minus*. **(A)** The pruned tree shows yellowing typical of CNC. Figure reproduced from Bulman & Gardner (2014). **(B)** Stages of CNC development (starting from the needle at the top) from green to yellow to brown with transverse stripes developing on the bottom needle. Figure reproduced from: <https://www.nzffa.org.nz/farm-forestry-model/the-essentials/forest-health-pests-and-diseases/forestry-diseases/Cyclaneusma-needle-cast/cyclaneusma-needle-cast-of-pines/>.

The infective propagules of *C. minus* are airborne ascospores that are discharged from apothecia on dead needles lying on the forest floor, but rarely seen on needles attached to trees (Gadgil, 1984). Fruiting body formation occurs during autumn and winter (Gadgil, 1984), in which they split open and release spores into the air, which can land on needles, leading to new infections. During autumn and winter, when needles are more susceptible to infection and inoculum levels are high, wet periods of about 5 hours of duration and temperatures between 10–25°C provide good conditions for infection (Gadgil, 1984).

From 1983 to 1985, the distribution of CNC in New Zealand was surveyed in 15 forests (totalling more than 70,000 ha of *Pn. radiata*) (Bulman, 1988). It was discovered that regions of the North Island had the highest severity of the disease, while the lowest disease incidence was in regions of the South Island (Bulman & Gadgil, 2001). Stands aged 11 to 20 years old had the highest disease severity, while stands under 5 years old and over 25 years had the lowest disease severity (Bulman, 1988).

The use of *Pn. radiata* cultivars with increased CNC resistance gained through breeding, along with the practice of removing diseased trees during thinning (Bulman & Gardner, 2014), may be the most effective way to control the disease since copper-based fungicides (as used for DNB) do not work for CNC. Resistance to *C. minus* is heritable and trees that show resistance have been selected for breeding programmes (Bulman, 1993). A recent study showed a positive correlation between resistance to DNB and CNC, which suggests that it is possible to select families of *Pn. radiata* that are resistant to both diseases (Ismael et al., 2020).

1.2.3 Red needle cast

Fungi and oomycetes are two very distinct groups of microorganisms that are responsible for the destruction of numerous important plants worldwide. Although oomycetes share some morphological features with fungi, they possess unique characteristics that separate them in a different kingdom: Chromista. One of the main differences between them is the composition of their cell walls. Cellulose is the main component of oomycete cell walls, while chitin is a major cell wall component of fungi (Fawke et al., 2015). Other distinguishing features include the fact that oomycetes

normally have diploid cells, nonseptate hyphae and mitochondria with tubular cristae. Belonging to the Oomycota phylum, many *Phytophthora* species are aggressive pathogens of agricultural crops and forest trees. In forests, they are mostly recognized as soil-borne pathogens, causing root rots in trees. However, in the past decade, *Phytophthora* diseases associated with conifer foliage have emerged as a growing concern for economically important crops (Dick et al., 2014; Durán et al., 2008).

In 2005, needle cast events unlinked to any recognized pathogen were observed in New Zealand pine plantations. A few years later, DNA analysis showed a match with *P. pluvialis*, a newly described species from Oregon, USA (Reeser et al., 2013). *P. pluvialis* was first isolated as a part of a study to determine the diversity of *Phytophthora* species in Oregon and Alaska, in 2002. At the time, it was named “new species 3”, since it was classified as a clade 3 *Phytophthora* based on its ITS sequence, then was officially described as *P. pluvialis* in 2013 (Reeser et al., 2013; Reeser et al., 2011). *P. pluvialis* was confirmed to be the causal agent of RNC disease in radiata pine in New Zealand (Dick et al., 2014). The first report of *P. pluvialis* in Europe happened in 2022, in the UK, with the pathogen causing resinous cankers in *Tsuga heterophylla*, a coniferous tree belonging to the Pinaceae family (Pérez-Sierra et al., 2022).

RNC affects trees of all ages but is more common on trees over three years old and most severe on trees over ten years old. Younger trees and seedlings usually only show symptoms when growing near heavily infected older stands. Early disease symptoms occur during late autumn or winter in areas exposed to prolonged periods of wet conditions and continue to develop until mid to late spring (Bulman & Gardner, 2014).

Sporangia from *P. pluvialis* are produced on the surface of infected needles. When there is high humidity, the sporangia release zoospores that are subsequently disseminated by water splash. *P. pluvialis* infects through stomata on the needles and colonizes the apoplastic environment. Although appressoria are common infection structures in *Phytophthora* species (Fawke et al., 2015), they have not been observed on needles inoculated with *P. pluvialis* to date (Dick et al., 2014; Gómez-Gallego et al., 2019; Reeser et al., 2013).

Infected needles generally have a pale olive-coloured lesion with a distinctive dark resinous mark, which often expresses as a narrow lateral band within the lesion (**Figure 1.3A and B**). Lesions are more abundant on the base of the needle than on distal parts (Dick et al., 2014). Lesions expand along the needles, turning yellow and then brown, giving infected trees a reddish appearance (**Figure 1.3C**). Following infection, needles senesce and are cast, with new needles emerging in late spring often unaffected by the disease (Dick et al., 2014). Initial studies showed that severe defoliation caused by RNC can reduce tree growth by up to 38% in a year (Ganley et al., 2014).

Management of RNC can be achieved using chemicals such as phosphorus acid and copper (Ganley et al., 2014; Rolando et al., 2019). Since resistance to needle loss due to RNC is a heritable trait, breeding for resistance to *P. pluvialis* in *Pn. radiata* seems to be a way to diminish the effects of RNC in New Zealand forests, and therefore to mitigate the losses caused by the disease (Dungey et al., 2014). This is likely to be facilitated by the New Zealand *P. pluvialis* population having very low diversity and, therefore, being essentially clonal (Brar et al., 2018).



Figure 1.3. Red needle cast (RNC) symptoms caused by *Phytophthora pluvialis*. **(A)** White arrows show *Pinus radiata* needles with black resinous bands. **(B)** *Pn. radiata* needles showing colour changes following infection with *P. pluvialis* (black resinous bands are shown with white arrows). **(C)** *Pn. radiata* trees affected by RNC, showing the reddish appearance of the affected trees prior to needle drop. Figure adapted from Dick et al. (2014).

1.3 Pine defences against pathogens

Plants have an extremely efficient defence system against pathogens. The plant defence system is comprised of structural and biochemical mechanisms, that can be pre- or post-formed in relation to the penetration by the pathogen. Structural mechanisms constitute physical barriers to penetration and/or colonization of the pathogen. Biochemical mechanisms include substances capable of inhibiting the development of the pathogen or generating adverse conditions for survival in the host (Spoel & Dong, 2012). Most of what is known about plant defence mechanisms is due to studies in

angiosperms. There are, however, studies that show pre- and post-penetration mechanisms involved in *Pinus* defence responses against some needle pathogens (Fraser et al., 2016).

Pre-formed mechanisms, known as constitutive defences, are barriers that already exist before contact with the pathogen and are aimed at containing colonization. They are represented by structural mechanisms, such as waxes, cuticles, increased cell wall thickness, trichomes, adaptations in stomata and vascular fibres, as well as pre-formed biochemical substances such as phenols, phytotoxins, protein inhibitors and hydrolytic enzymes (Dixon, 2001). Features such as a rougher needle surface, needle wettability for spore adhesion, stomata morphology, composition of waxes and other epicuticular exudates can all contribute to defence mechanisms used by pines to inhibit pathogen penetration. For example, stomata from older *Pn. radiata* are smaller in size compared with those from young trees, which is consistent with young trees being more susceptible to DNB than older ones (Franich et al., 1977). Similarly, *Pn. monticola* trees susceptible to WPBR had bigger stomata than resistant trees. Some waxes are able to inhibit *in vitro* growth of *D. septosporum*, and there are differences in composition of waxes from pines that are resistant and susceptible to WPBR (Woo et al., 2001).

On the other hand, post-penetration resistance mechanisms can increase the concentration of pre-formed compounds after infection by the pathogen and seem to play a bigger role in controlling the spread of pathogens. These mechanisms involve the production of papilla, lignification, cork layers, formation of filaments and gum deposition, all considered structural defence mechanisms. The compounds of post-penetration biochemical mechanisms include phytoalexins, reactive oxygen species (ROS), and proteins related to pathogenesis (PR proteins). The process by which plant resistance is activated and the events subsequent to induction lead to accumulation of compounds contributing to resistance (Kuć, 2001). Some of these are constitutively present in pine, such as benzoic acid, a phenolic compound with antifungal activities against *D. septosporum* that accumulates upon infection. Other phenolic compounds involved in pine-pathogen interactions include flavonoids which accumulate in pines infected with *Elytroderma* and *Lophodermella* species, compared to uninfected trees, suggesting a potential role in defence (Wallis et al., 2010). Cell wall-bound compounds,

such as lignin, function as physical barriers limiting pathogen growth, and also have an important role in defence. There are several examples of lignin accumulation *in vitro* and *in planta* after pine exposure to *D. septosporum*, or its toxin dothistromin (Franich et al., 1986; Wallis et al., 2010). PR proteins are a diverse group of proteins with antimicrobial activities that are also induced after pathogen infection. For example, the PR-2 and PR-3 families are comprised of hydrolytic enzymes active against the fungal cell wall (Veluthakkal & Ghosh Dasgupta, 2010). Several PR proteins from different families were found to be up-regulated in different pine species inoculated with pathogens (reviewed in Fraser et al. (2016)).

1.4 The plant immune system and host specificity

Unlike animals, plants do not have an adaptative immune system or mobile defence cells. However, on top of the abovementioned pre- and post-formed mechanisms, plants have developed a relatively simple, yet efficient, immune system that is activated after perception of microorganisms (Spoel & Dong, 2012). Over the years, different models have attempted to describe the process of specific plant immunity, as detailed below.

Gene-for-gene, the first of these models, was described by Flor (1942). In this model, two components, an avirulence (Avr) protein in the pathogen (encoded by an *Avr* gene), which is recognized by a matching R protein in the host (encoded by an *R* gene), are required for resistance (termed an incompatible interaction). In the absence of the matching R protein, Avr products can function as virulence factors (*avr*), promoting disease (compatible interaction) (**Figure 1.4**). The fungal pathogen *Fulvia fulva* (syn. *Cladosporium fulvum*) is the causal agent of tomato leaf mould disease and a close relative of *D. septosporum* (de Wit et al., 2012); the *F. fulva*–tomato interaction is a model pathosystem which helped to establish the molecular basis of the gene-for-gene concept of plant resistance to fungal pathogens (de Wit, 2016). Many *Avr* genes from *F. fulva*, and their respective *R* genes in tomato, have been identified. One of the limitations of this model is that an Avr protein can be recognized by more than one R protein and different Avr proteins can be recognized by the same R protein (Anh et al., 2018; Ghanbarnia et al., 2015). For example, the tomato R protein Cf-2 is required for resistance against two

different pathogens: the fungus *F. fulva* (Luderer et al., 2002) and the nematode *Globodera rostochiensis* (Lozano-Torres et al., 2012). In another example, two Avr proteins from the fungus *Leptosphaeria maculans* (AvrLm10A and AvrLm10B) are required for resistance involving the Rlm10 R protein of brassica (Petit-Houdenot et al., 2019).

		Pathogen genotype	
		<i>Avr</i>	<i>avr</i>
Plant genotype	<i>R</i>	Incompatible	Compatible
	<i>r</i>	Compatible	Compatible

Figure 1.4. Interaction types in the gene-for-gene model proposed by Flor (1942).

Even though many *Avr*–*R* gene pairs have been characterized, recognition of a pathogen protein can also occur in an indirect manner. In the guard model, secreted proteins from the pathogen have one or more plant targets, and modifications in these targets are monitored by the corresponding R protein, leading to its activation, and triggering plant disease resistance. In plants without the corresponding R protein, the interaction between the pathogen protein and its target (or guardee) increases pathogen fitness. In some cases, plant targets have evolved to become decoys, in which they can mimic the target and trick the pathogen protein into a recognition state. Here, the alteration in the target does not increase pathogen fitness in plants without the corresponding R protein (van der Hoorn & Kamoun, 2008). These host proteins are called decoys and are described by the decoy model.

Another well-known model that describes the plant immune system is the zig-zag model (**Figure 1.5**) (Jones & Dangl, 2006). In this model, recognition by the plant immune system happens in two different ways. The first is defined as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), also known as non-host defence. The other is called effector-triggered immunity (ETI), or host-specific defence. PTI is activated by highly conserved PAMPs, or microbe-associated molecular patterns (MAMPs) upon their recognition by extracellular plant pattern recognition receptors (PRRs). This defence mechanism is considered the first line of innate immunity in plants, allowing for a quick and efficient response to a wide range of pathogens. In response to this, pathogens can secrete small molecules, termed effectors, that can suppress PTI, resulting in effector-triggered susceptibility (ETS). On the other hand, plants encode R

proteins which recognize effectors (similar to the gene-for-gene model) and trigger strong defence responses, in a process called ETI, in attempts to halt the growth of the pathogen. The zig-zag denomination comes from the fact that, under selection pressure, pathogens alter/lose or develop new effectors to evade recognition by R proteins, resulting in a new ETS phase, shifting the selection pressure back onto the plant. New *R* genes can emerge and trigger a new ETI phase, restoring immunity. These processes can be repeated indefinitely.

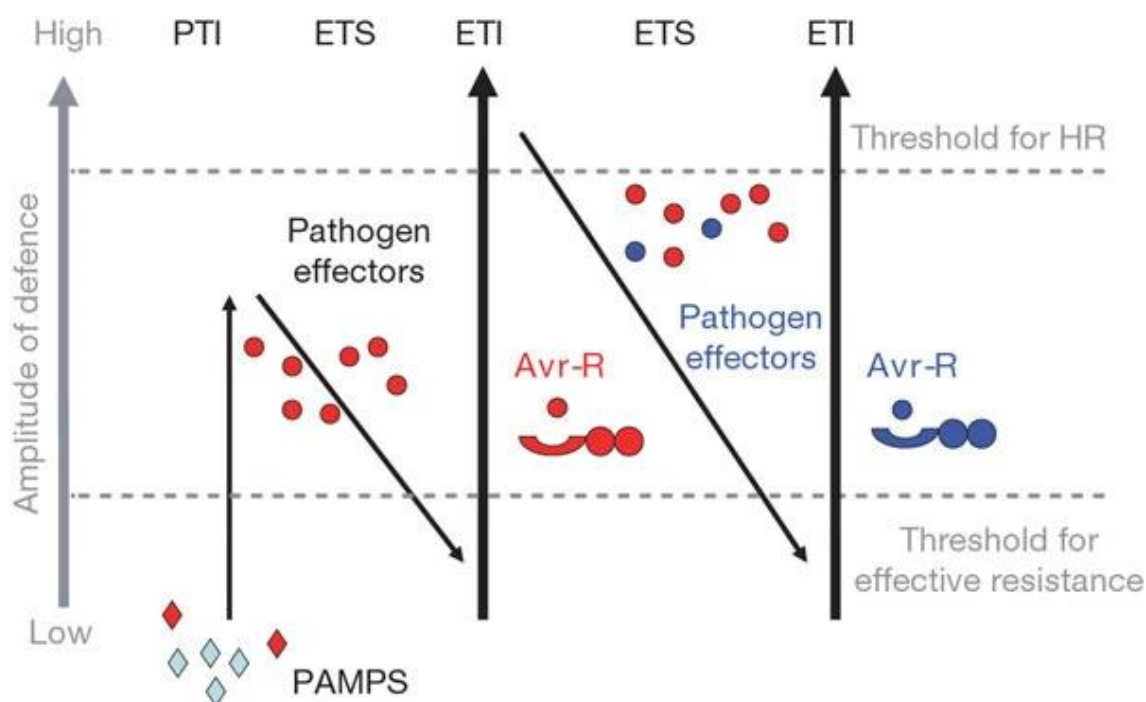


Figure 1.5. Schematic of the zig-zag model. Under pathogen attack, pathogen-associated molecular patterns (PAMPs) activate host immune receptors leading to PAMP-triggered immunity (PTI). Pathogens have the ability to secrete effectors, suppressing PTI and triggering effector-triggered susceptibility (ETS). A recognized effector (avirulence protein – Avr) by a host resistance (R) protein leads to a hypersensitive response (HR) and effector-triggered immunity (ETI). Pathogens then mutate or secrete new effectors, in a new ETI phase. The selection pressure shifts to the plant and new *R* genes can emerge, leading to a new ETI phase. Figure reproduced from Jones and Dangl (2006).

This model, however, fails to address some important points. As well as PAMPs and MAMPs, damage-associated molecular patterns (DAMPs) are also sensed by plant receptors. DAMPs are host molecules released, for example, as a consequence of the hydrolytic activity by fungal cell-wall degrading enzymes, and can trigger PTI.

Furthermore, a strong ETI response triggering localized cell death works to stop the spread of pathogens with a biotrophic lifestyle but may be beneficial to hemibiotrophic or necrotrophic pathogens. Also, in this model, there is a clear distinction between PTI and ETI and separation of the responses involved in each type of immunity, which is not always the case. Under this model, PAMPs are considered highly conserved across genera, and effectors are normally considered species- or race-specific. Several examples, however, demonstrate that this might not always be the case. Necrosis and ethylene-inducing peptide 1 (NEP1)-like proteins (NLPs) are defined as effectors found in bacteria, fungi, and oomycetes, contributing to virulence, but have a conserved region that induces plant defence responses, therefore, acting as a PAMP (Pemberton & Salmond, 2004). Normally, a PTI response is considered weaker than an ETI response, which is also not always the case. For example, a CBEL (cellulose binding elicitor lectin) is a *Phytophthora* PAMP that triggers cell death-like responses in *Nicotiana* and *Arabidopsis* plants (Khatib et al., 2004).

Considering these limitations, a new model was proposed. In the invasion model, PAMPs, MAMPs, DAMPs and effectors are essentially perceived in the same manner by the plant, which unifies them under the term invasion patterns (IPs) (Cook et al., 2015). This model suggests that extracellular and intracellular plant IP receptors (IPRs) detect IPs, resulting in an IP-triggered response (IPTR). This detection leads to one of two outcomes: continued symbiosis, with the establishment of a compatible interaction leading to pathogen proliferation, or the end of symbiosis, where an incompatible interaction stops pathogen growth (**Figure 1.6**) (Cook et al., 2015).

More recent models include the danger model, in which PAMPs, MAMPs, effectors and DAMPs are grouped under the term 'danger signals'. In this case PAMPs, MAMPs and effectors are grouped as "exogenous non-self" signals, while DAMPs are grouped under primary or secondary "endogenous self" signals. Secondary endogenous signals are generally derived from large proteins that have been processed and cleaved into small peptides and secreted after infection. For example, Pep is a small peptide family from *Arabidopsis* and maize, derived from a protein called PROPEP, that is recognized by kinase receptors (Gust et al., 2017). A similar model takes into account the danger model but differentiates between danger signals that trigger extracellular or

intracellular immunity, leading to plant resistance (van der Burgh & Joosten, 2019). The most recent model, the iceberg model, suggests that pathogens secrete effectors that function as a second ETS effector, suppressing the ETI previously caused by the recognition of a first effector by a host receptor, explaining why plants and pathogens have such a large number of these receptors and effectors (Thordal-Christensen, 2020).

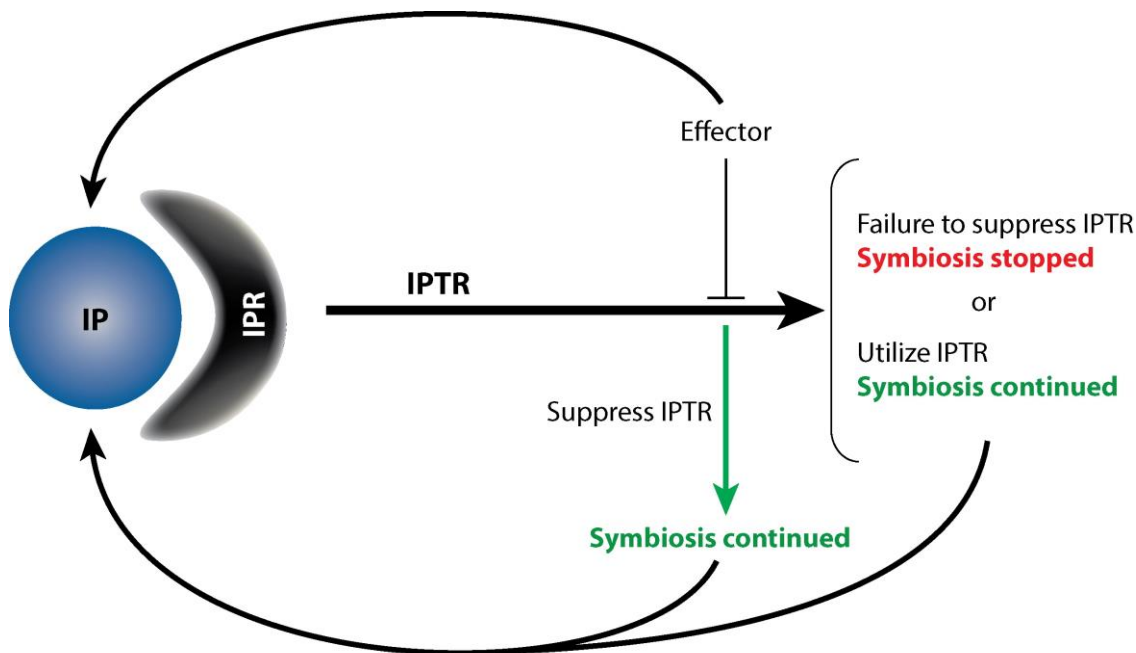


Figure 1.6. Schematic diagram of the invasion model. Pathogens secrete or present invasion patterns (IP) during host colonization. These IPs can be recognized by host IP receptors (IPRs), leading to IP-triggered responses (IPTRs). If a biotrophic pathogen is unable to suppress these IPTRs or if one or more of its effectors are recognized as IPs, then symbiosis is stopped. Necrotrophic pathogens are able to use IPTRs to their own advantage, allowing the symbiosis to be continued. Figure reproduced from Cook et al. (2015).

1.4.1 Plant immune receptors

IPs are recognized by extracellular or intracellular plant immune receptors in order to activate plant defence responses. The recognition of an IP by a plant receptor can lead to a hypersensitive response (HR), which is generally characterised by localised plant cell death that can limit pathogen growth (Win et al., 2012). Associated with the HR, chlorosis can be observed, and other defence responses are activated via different signalling pathways. These pathways can be activated based on calcium levels in the cytoplasm, rapid production of ROS and nitrogen oxide (NO), which have antimicrobial

effects and can also activate the mitogen-activated protein kinase (MAPK) signalling cascade. The amplification of these signals occurs through the actions of regulatory hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), resulting in the activation of transcription factors, including those of the WRKY superfamily, defence genes, PR proteins, phytoalexins, lignification of tissues and deposition of callose and other cell wall reinforcements.

The recognition of IPs by plant immune receptors can be direct, supported by the gene-for-gene model, or indirect, described in the Guard Model or Decoy Model (van der Hoorn & Kamoun, 2008).

1.4.1.1 Extracellular receptors

Extracellular receptors are transmembrane proteins and can be either receptor-like proteins (RLPs) or receptor-like kinases (RLKs). These receptors are generally involved in the recognition of MAMPs, PAMPs and DAMPs, providing the plant its first line of defence against pathogens, but also have the ability to recognize effector proteins.

RLPs and RLKs have an extracellular domain, which binds to different ligands, a transmembrane domain, and a cytoplasmic domain. The main difference between these two types of receptors is in the cytoplasmic domain. RLKs have an intracellular kinase domain that enables signalling upon ligand recognition. In contrast, RLPs do not have a kinase domain and therefore depend on other proteins with kinase domains to transduce signals to the cytoplasm after IP recognition (Rathore & Ghosh, 2018). For this reason, RLPs must associate with kinase adaptors, such as brassinosteroid insensitive 1-associated kinase 1 (BAK1) and suppressor of BAK1-interacting receptor-like kinase 1 (SOBIR1). RLKs are also known to interact with BAK1, as a coreceptor (Gust & Felix, 2014). For example, one of the most studied RLKs, flagellin-sensing 2 (FLS2), first identified in *Arabidopsis*, recognizes a 22-amino acid sequence (flg22) from the N-terminus of the flagellin protein of *Pseudomonas syringae*. This flg22 sequence binds directly to the extracellular domain of FLS2 and interacts with BAK1, which leads to the activation of plant immune responses (Gómez-Gómez & Boller, 2000) (**Figure 1.7A**). CEBiP and CERK1 are extracellular receptors from rice that have the capacity to bind to fungal chitin (Kaku et al., 2006). CEBiPs, as all RLPs, do not have a cytoplasmic kinase domain, and therefore

need to form a protein complex with RLK CERK1 to initiate signalling to activate plant defence responses (Shimizu et al., 2010). Unlike the direct association between flg22 and FLS2, IPs can be indirectly recognized by extracellular receptors. The effector Avr2 from *F. fulva* interacts with the tomato protease Rcr3, and this interaction is required for Cf-2 recognition (Rooney et al., 2005) (**Figure 1.7B**).

As mentioned above, the extracellular domain has the ability to bind and recognize different ligands. Most extracellular receptors have a leucine-rich repeat (LRR) domain, binding to numerous different ligands, such as hormones, proteins, and peptides. Other domains include lysin motifs (LysMs), that bind to chitin and peptidoglycan, and carbohydrate-binding domains, found in lectins. Cell wall-associated kinases (WAKs) also have an extracellular galacturonan-binding domain, and are involved in pathogen recognition. For example, the cell wall-associated kinase receptor 1 (WAK1) elicits plant defence responses through the recognition of oligogalacturonides, which are residues released from the plant cell wall (Brutus et al., 2010).

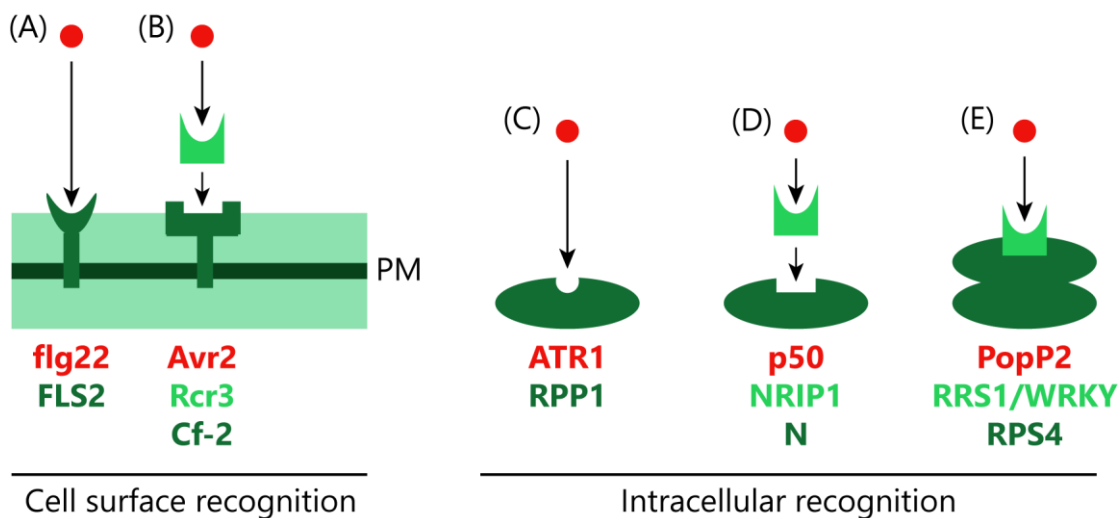


Figure 1.7. Recognition mechanisms of secreted pathogen proteins. **(A)** Direct and **(B)** indirect recognition at the cell surface. **(C)** Direct, **(D)** indirect and **(E)** integrated intracellular recognition. PAMPs/effectors are shown in red, main receptor for direct recognitions are shown in dark green and interacting receptor for indirect recognitions are shown in light green. PM: plasma membrane. See **Section 1.4.1** for further explanations of each recognition mechanism. Figure adapted from Kourelis and van der Hoorn (2018).

1.4.1.2 Intracellular receptors

Intracellular receptors are R proteins that are localized in the plant cytoplasm. Most intracellular R proteins are known as NLRs because they have a central nucleotide-binding site (NBS), an LRR domain, and a third variable domain at the protein N-terminus. This variable domain can be categorised into eight different groups, with the most common being the toll/interleukin-1 receptor-like (TIR) domain, a coiled-coil (CC) domain and a small N-terminal 'resistance to powdery mildew 8' (RPW8) domain. However, there are classes of NLRs with more than one N-terminal domain, for example NLRs with both TIR and CC domains; for this reason, NLR classification is not always precise (reviewed in Dubey and Singh (2018)).

NLRs recognize effectors either directly or indirectly, triggering defence responses. A direct interaction occurs when the NLR binds directly to the pathogen effector, as described in the gene-for-gene model (Flor, 1971). ATR1, an effector secreted by the oomycete *Hyaloperonospora arabidopsidis* directly interacts with the C-terminal LRR from RPP1, an NLR from *Arabidopsis thaliana* (Steinbrenner et al., 2015) (**Figure 1.7C**). NLRs can also associate with a protein partner and recognize modifications in said protein when targeted by an effector. In this case, the protein partner can be called a guardee, as described in the Guard Model, or a decoy, as described in the Decoy Model. For example, a tobacco NLR requires the additional protein NRIP1 to form a complex with the p50 effector from *Tobacco mosaic virus* in order to be activated (Caplan et al., 2008) (**Figure 1.7D**). Another example of indirect recognition is by Prf, a tomato NLR that interacts with Pto kinase, subsequently perceiving the *Ps. syringae* effector AvrPto. AvrPto acts as a kinase inhibitor, binding to Pto (Kim et al., 2002; Kourelis & van der Hoorn, 2018). In other cases, the protein partners are integrated into the NLR receptor, which is the case for a WRKY transcription factor that is integrated in the NLR RRS1 from *Ar. thaliana* that, in conjunction with NLR RPS4, is responsible for recognition of the PopP2 effector from *Ralstonia solanacearum* (Sarris et al., 2015) (**Figure 1.7E**).

Irrespective of whether recognition of an effector is direct or indirect, it triggers the activation of signalling pathways, resulting in plant immune responses. As mentioned

before, the most common of these responses is an HR, characterized by a localized cell death to contain pathogen spread.

1.5 Effectors

In the invasion model, effectors are perceived as IPs, together with DAMPs, MAMPs and PAMPs. Effectors are generally small proteins, but can also be secondary metabolites, such as toxins, small RNA molecules or short peptides. They are secreted by a microorganism and have the ability to modify the physiology or metabolism of another organism in favour of the invader's multiplication, thereby affecting, positively or negatively, the outcome of the interaction between the two organisms (Lo Presti et al., 2015). For example, the effector MiSSP7 from the mutualistic fungus *Laccaria bicolor* promotes symbiosis with the host plant by blocking JA, a hormone known to be involved in plant defence signalling pathways. This repression aids fungal colonization, which is beneficial to the plant as the symbiotic relationship provides the plant with important growth nutrients (Plett et al., 2014).

However, since effectors often have negative effects on the plant, the ability to recognize effectors among IPs that result in plant immune responses will be selected for. This means the invader must modify its effector arsenal in order to avoid recognition, shifting the pressure to adapt onto the plant host, leading to a constant "arms race" in plant-pathogen interactions. Therefore, from a pathogen's perspective, the emergence of new effectors is important to substitute an effector that was lost, or to interact with new plant targets (Lo Presti et al., 2015). For this reason, effectors require faster evolution rates than the rest of the genome. Many effectors can be found in gene-poor, repeat-rich regions, with these effector-encoding genes often found on accessory chromosomes (Ma et al., 2010; Raffaele & Kamoun, 2012). In some *Citrus* species, *Alternaria alternata* produces a host-specific toxin (HST) effector that is essential for pathogenicity. The genes responsible for the biosynthesis of the HST are grouped on a small dispensable chromosome of approximately 2 Mb (Wang et al., 2016). They are thought to have been acquired through horizontal gene transfer, which is a common way for pathogenic fungi to gain new genetic material, especially in populations where the pathogen has no or limited sexual reproduction (Wang et al., 2019b).

Effector proteins frequently possess common characteristics that help with their identification through bioinformatics tools (Dalio et al., 2018). One of those features is the presence of an N-terminal signal peptide for secretion into the host and the absence of transmembrane domains and glycosylphosphatidylinositol (GPI) anchor modification sites, both related to fungal membrane attachments. Another feature is that effectors are frequently small (≤ 300 amino acids) and cysteine-rich (≥ 4 cysteines). An even number of cysteine residues facilitates the formation of disulphide bonds that help effector proteins survive the harsh environment of the plant apoplast. High gene expression levels during host colonization can also be used to identify effectors and to determine during which stage of the pathogen's life cycle they are likely to be deployed. Some effectors do not possess any conserved motifs or domains and are species-specific. In contrast, some effectors are highly conserved between fungal species, known as "core effectors". These effectors provide a core function that is likely important to the virulence, pathogenicity and/or survival of multiple microorganisms that produce them. The protein family Extracellular protein (Ecp) 2 that was firstly characterized from *F. fulva* and triggers an HR in tomato lines that carry a *Cf-Ecp2* gene (Laugé et al., 1997), provides an example of a core effector. Ecp2 homologues are present in many fungal species, including plant- and animal-pathogens, as well as non-pathogenic species (Thomma et al., 2011). More broadly, in a few cases, effectors are shared across kingdoms, with fungi, oomycetes and bacteria sharing conserved domains. For example, the NLPs and the glycoside hydrolase (GH) 12 protein XEG1 are broadly distributed in oomycetes and fungi, and both trigger necrosis in multiple plants (Fellbrich et al., 2002; Ma et al., 2015).

Effector genes can also form part of multigene families. For example, the biotrophic apple pathogen *Venturia inaequalis* secretes numerous effector proteins and more than half of them are in protein families, based on sequence similarity alone (Deng et al., 2017; Rocafort et al., 2022). Similarly, *Ciborinia camelliae*, a necrotrophic pathogen responsible for flower blight disease of camellias, also has a large proportion of its predicted secretome belonging to a single expanded candidate effector (CE) family (Denton-Giles et al., 2020). Also, some Avr effectors have been found to form part of protein families from pathogens like *Magnaporthe oryzae* (rice blast disease), *Blumeria graminis* (cereal powdery mildew disease), *Melampsora lini* (flax rust disease) and

Le. maculans (stem canker disease of brassica) (Anderson et al., 2016; Catanzariti et al., 2006; Dodds et al., 2004; Kang et al., 1995; Khang et al., 2008; Praz et al., 2017; Spanu, 2017; Talbi et al., 2022). These effector families likely evolved from a common ancestor by duplication events, followed by sequence diversification that could be translated into diverse functions. In some cases, the sequence diversification is such that there is no longer similarity between proteins from the same family at the sequence level, but only at the tertiary structure level. This is the case for PevD1 from *Verticillium dahliae* and MoHrip1 from *M. oryzae*, which both have structural similarity to the characterized β -barrel fold of Alt a 1 from *Al. alternata* (Research Collaboratory for Structural Bioinformatics protein data bank [RCSB PDB] ID: 3V0R), despite having low amino acid sequence identity (Chruszcz et al., 2012; Han et al., 2012; Zhang et al., 2017a).

Effectors are divided into apoplastic and cytoplasmic types, depending on where they act in the host. Effectors secreted by pathogens during infection may accumulate on the exposed surfaces of cells, or in the intercellular environment, being classified as apoplastic effectors. In contrast, intracellular or cytoplasmic effectors are those that can translocate through the cell membrane and have direct contact with subcellular compartments and organelles (Block et al., 2008).

1.5.1 Apoplastic effectors

Recent work suggests that the intercellular plant apoplast, where interactions between microbes and plants first occur, is an important battleground where common resistance processes can be found. Pathogens secrete effector molecules to protect themselves from the harsh conditions of the apoplast as well as to degrade host cell walls and other components, and some of these effectors can trigger defence responses if they are recognised by extracellular receptors (Wang & Wang, 2018). They have different functions, such as inactivating toxic metabolites, shielding the fungus from plant defences, suppressing host immune responses, or degrading the host cell wall to facilitate infection (Lo Presti et al., 2015; Wang et al., 2020; Wu et al., 2022). One of the best characterized apoplastic effectors is Avr4 from the tomato pathogen *F. fulva*. Avr4 binds to chitin on the fungal cell wall, shielding it against plant chitinases (van den Burg et al., 2006). *F. fulva* Ecp6 and the closely related Slp1 from the rice pathogen *M. oryzae*

bind and sequester chitin oligosaccharides released from the fungal cell wall, thereby suppressing elicitation of host immune responses (de Jonge et al., 2010; Mentlak et al., 2012). Detoxifying ROS can also promote successful pathogen colonization; Pep1 from the corn pathogen *Ustilago maydis* accumulates in the apoplast and suppresses plant defences by inhibiting a secreted maize peroxidase (POX12) that reduces ROS levels (Hemetsberger et al., 2012). Plant proteases can also be the target of effectors; for example, Pit2 from *U. maydis*, EPI1 from *Phytophthora infestans* and Avr2 from *F. fulva* inhibit proteases that are produced as plant defences (Mueller et al., 2013; Song et al., 2009; Tian et al., 2007). Apoplastic effectors can also be involved in the necrotrophic switch that hemibiotrophic pathogens undergo. This is the case for apoplastic effector ChCAP from *Colletotrichum higginsianum*, CtNUDIX from *Colletotrichum truncatum* and BAS4 from *M. oryzae*, triggering cell death responses at later infection stages to help make the transition from biotrophy to necrotrophy (Bhadauria et al., 2013; Wang et al., 2019a; Zhu et al., 2019).

1.5.2 Cytoplasmic effectors

Cytoplasmic effectors are those translocated into host cells. Some effectors have the ability to interfere in hormone signalling pathways. For example, Cmu1 is a protein secreted by *U. maydis* that is translocated into the cytoplasm of plant cells during colonization, where it neutralizes SA, which has a role in plant defence (Djamei et al., 2011). Some effectors from necrotrophic pathogens induce host cell death by secreting HSTs. This cell death can occur when the HST is recognized by the product of a plant susceptibility (*S*) gene. For example, the *acrs* gene codes for a HST receptor in the chloroplast of *Citrus jambhiri*, making the plant sensitive to the HST (ACR) produced by *Al. alternata* (Ohtani et al., 2002). Other effectors interfere with secondary metabolite synthesis. This is the case for the *Avr* gene *ACE1* from *M. oryzae*, which encodes a polyketide synthase localized in the appressorium cytoplasm (Böhnert et al., 2004). Apart from fungi, oomycetes also produce cytoplasmic effectors. Most oomycete effectors contain a conserved N-terminal RxLR (Arginine, x, Leucine, Arginine) motif (Fawke et al., 2015). One of the most studied RxLR effectors is AVR3a from *P. infestans*, which suppresses host cell death induced by the apoplastic elicitor INF1, also secreted by *P. infestans*. INF1 acts as a cell death elicitor. In suppressing the action of this elicitor,

Avr3a contributes to pathogen virulence during its biotrophic phase (Bos et al., 2010). The crinkling and necrosis (CRNs) proteins are another common class of cytoplasmic effectors from oomycetes known to trigger cell death responses (Fawke et al., 2015).

1.6 Effectors as tools to achieve durable and broad-spectrum resistance

Effector proteins can serve as guides to search for potential host targets (*R* and/or *S* genes). With advances in generating fast and reliable genomic, transcriptomic, and proteomic information from plant pathogens, the search for these proteins has led to the identification and functional characterization of many effector proteins (Wu et al., 2022). Common features shared between effectors made it possible to create a diverse number of bioinformatic pipelines and online servers that predict such proteins. Tools are available to predict signal peptides, transmembrane domains, localization in host tissues and to differentiate between apoplastic and cytoplasmic effectors (reviewed in Prasad et al. (2019) and Wilson and McDowell (2022)). Sequence size (normally ≤ 300 amino acids) and number of cysteine residues (≥ 4) are other criteria often used when identifying such proteins from pathogens (Lo Presti et al., 2015). Protein tertiary structure prediction tools, such as AlphaFold2, make it possible to identify structurally related (but sequence-unrelated) proteins, predicting structures with a high degree of confidence.

There are, however, examples of effectors that do not fit these criteria. Some of them do not follow the classic endoplasmic reticulum-Golgi secretory pathway and do not have a signal peptide (Liu et al., 2014a), while others are larger in size (Nirmala et al., 2011) or have a low number of cysteines (Catanzariti et al., 2006). For this reason, effector prediction must not rely solely on computational methods, but also combine other information, such as high expression *in planta*, through transcriptomic and/or proteomic studies, and functional characterization, such as transient expression assays in non-host plants and/or gene disruption/deletion/overexpression.

Transient expression of effector genes of interest *in planta* is an established method to study effector function in molecular plant pathology. In these assays, the transformation of tissues of non-host model plants is done using *Agrobacterium tumefaciens* as a vector to carry the gene of interest into plant cells. The advantages of using model plants such as the angiosperms *Nicotiana benthamiana* and *Nicotiana*

tabacum are their short life cycle, large leaves and the fact that the two species carry many characterised plant receptors, making them ideal for broad screenings of pathogen CE proteins (Ma et al., 2012). Once these CEs are expressed *in planta*, it is possible to determine whether they trigger defence responses (e.g., a localized cell death might suggest the effector is being recognized by a plant immune receptor), or suppressing defence responses (in this case if the effector is co-expressed with a known cell death elicitor there is a loss of the cell death response).

Although these model plants are a good and fast alternative to improve the understanding of forest pathogen effector proteins, there are limitations to these assays. These proteins are secreted by pathogens when infecting their host, which are not *Nicotiana* species, and even though some defence responses between angiosperms and gymnosperms might be conserved (Carella et al., 2019; de Vries et al., 2018), there is the possibility of misrepresentation of what is occurring during the pathogen's interaction with their actual host. However, applying transient transformation assays to gymnosperms, such as forest trees, is difficult due to their size and long life cycle, which makes transformation difficult and time consuming. Hence, the need to develop rapid and reliable ways to screen these fungal and oomycete proteins in *Pn. radiata*. Further characterization of these effector proteins can be done through gene deletion or disruption using genome editing techniques, such as CRISPR/Cas9. Gene loss-of-function is a possible way to determine if a candidate gene is involved in virulence. For example, the gene encoding Avr4/6, an RxLR effector from *P. sojae*, was disrupted, preventing its recognition by matching R proteins in soybean (Fang & Tyler, 2016).

As mentioned before, plants have evolved the ability to detect a pathogen's proteins through its own R proteins, triggering strong defence responses. However, a single R protein can confer resistance to only a subset of pathogen effectors, i.e., those that carry the corresponding Avr protein to enable recognition. This resistance can be fast and easily overcome by the pathogens ability to silence, mutate, or acquire new effector proteins, hence the continuous need to identify and integrate new *R* genes into commercial cultivars. For example, resistance of poplar trees to the biotrophic pathogen *Melampsora larici-populina* was mediated by the recognition of an Avr effector by the protein encoded by the *RMlp7* *R* gene, which has been overcome by emergence of new

virulent pathotypes of the fungus (Louet et al., 2021). Resistance in trees carrying the *R* gene *Cr2* can be overcome by virulent pathotypes of *Cr. ribicola*, such as *vcr2* (Liu et al., 2021).

A way to overcome this is to breed crop varieties with durable and broad-spectrum resistance (BSR). Host BSR refers to resistance against multiple pathogen species or races of a pathogen species (Wisser et al., 2005), while durable resistance is defined as resistance in a cultivar that remains effective after that cultivar has been extensively cultivated (Johnson, 1983). Durable resistance is particularly beneficial for trees due to their long life cycles. One strategy to achieve durable and BSR is known as pyramiding, in which several *R* genes are incorporated into the same plant (Delmotte et al., 2016). Identifying core effectors is important, since their structures can be conserved across some microbial classes and also, due to their possible essential role in virulence, are less likely to be mutated or lost during microbial evolution (Nürnberg & Brunner, 2002). The inclusion of plant receptors that can recognize such core effectors can be beneficial to breeding programmes. The recognition by *R* proteins of this conserved group of effector molecules can confer durable resistance and BSR against microbes sharing the same effectors (Dalio et al., 2017).

As mentioned above, resistance can be achieved by incorporating *R* genes into host genomes. Conventional breeding programmes have already widely deployed *R* proteins for durable and BSR in crops. The rice receptors β -lectin *RK Pi-d2*, *LRR-RK OsBRR1* and *WAK-RK OsWAK1* confer resistance to *M. oryzae*, while the rice receptors *OsLecRK1*, *OsLecRK2* and *OsLecRK3* confer stronger resistance to the brown planthopper (Chen et al., 2006; Li et al., 2009; Liu et al., 2014b; Peng et al., 2009). Introduction of a cassette of five *R* genes in wheat greatly increased the plant's resistance against the fungal pathogen *Puccinia graminis* f. sp. *tritici* (Luo et al., 2021). In *P. sojae*, BSR was achieved by the discovery of two *R* genes, that when selected together in soybean lines, increased resistance against different races of the pathogen (Sahoo et al., 2021).

An alternative to this is the identification of the host *S* genes. *S* genes are exploited by pathogens to cause disease, and their elimination will undermine the ability of the pathogen to do so (Gust et al., 2010). One of best examples of an *S* gene, sometimes referred to as a recessive *R* gene, is the barley *Mildew Resistance Locus O (MLO)* gene.

Recessive *mlo*-alleles confer resistance to powdery mildew not only in barley, but also in tomato and *Arabidopsis* (van Schie & Takken, 2014). Even though this type of modification is more durable, modifying host genes so the effector cannot bind anymore is not trivial, with the risk of pleiotropic effects that are detrimental to the plant.

1.7 Pine-pathogen molecular interactions

Most planted forests contain trees with high genetic diversity and species heterogeneity (Hessenauer et al., 2020). The goal of forest breeding is to gradually improve the population, by selecting the phenotypically superior/best trees, then developing new varieties (Cortés et al., 2020). Genetically engineered genotypes produced through DNA recombination methods can serve as a faster approach than traditional breeding of trees (Badenes et al., 2016). With the increasing demand of forest products, breeding for durable and broad-spectrum disease resistance is an important goal. This is of immense importance for forest trees, since during their long life-cycle, they are exposed to a diverse number of diseases caused by different pathogens.

Identifying and incorporating *R* genes from forest species may be harder than in most agricultural angiosperms. Forest trees have large and complex genomes, which makes assembly and annotation of these genomes a difficult task. Because of this, the generation of genomic information is limited, which translates to a difficulty in finding the appropriate *R* genes in these genomes. Also, due to their long life-cycle, transformation of gymnosperms is a difficult and time consuming task, not only with regard to introducing the desired piece of DNA, but also ensuring regeneration of the whole plant. In the case of *Pn. radiata*, it takes seven years for the plant to reach sexual maturity (Bradshaw, 2004). Therefore, phenotypic characterization of candidate trees before selection takes time and large land space, with the risk of different responses between young and mature trees. For these reasons, there is a lack of information on how forest trees interact and respond to pathogens at the molecular level.

As mentioned above, conifer genomes are extremely large, with long intergenic and repeat-rich regions, mostly made up of transposable elements (Plomion et al., 2016). With advances in next generation sequencing and assembly methods there are, however, a few conifer genomic and transcriptomic studies available, and they provide valuable

insights on Pinaceae-pathogen molecular interactions. White pines are economically important trees that are affected by the biotrophic fungal pathogen *Cr. ribicola*, responsible for WPBR, one of the most important forest diseases in North America. The first transcriptome of a white pine was performed on WPBR-resistant and -susceptible seedlings of *Pn. monticola* infected with *Cr. ribicola* (Liu et al., 2013). There were differently expressed genes (DEGs) that were unique to resistant seedlings, and others unique to susceptible seedlings. Exclusively found in resistant seedlings were DEGs encoding two NBS-LRRs and two RLKs, suggesting their possible involvement in resistance mediated by R proteins. Downstream signalling genes also showed up-regulation in resistant seedlings when compared with susceptible, such as the those involved in calcium- and MAPK pathways, and PR proteins, such as chitinases, thaumatin-like proteins (TLPs) and other anti-microbial proteins. Plant hormones known to have a role in plant-pathogen interaction, as abscisic acid and ET, were up-regulated and also may be involved in WPBR resistance (Liu et al., 2013).

To add to the previous study, a comprehensive comparative study of four white pine species, all susceptible to WPBR, was performed, with *Pn. monticola* included. The results showed that assembled genes were highly conserved between the four white pine species, with a total of 2025 gene families shared between them, many of which were under positive selection. Genes under positive selection evolve more rapidly and have higher sequence diversity, an important feature during the arms race between plants and pathogens. Most of these genes encoded proteins that had domains related to stress responses and fungal resistance. The gene family with the highest number of members was related to antifungal activity, which could potentially be exploited in studies against WPBR. Included in those, families of genes related to drought tolerance and resistance to WPBR were also identified. These families all had similarity with characterized genes from Mediterranean pines associated with drought resistance, and with a characterized RLK gene and an NBS-LRR gene, the latter under positive selection, from *Picea glauca* and *Pn. monticola*, associated with resistance to WPBR. Another gene, coding for an F-box protein, and similar to a gene associated with resistance against necrotrophic fungi in *Arabidopsis*, was also found to be under positive selection. Identifying genes related to drought tolerance and resistance to WPBR, especially those under positive selection

can, ultimately, help breeding programmes and the understanding of other pine-pathogen pathosystems by adding information to forest tree genetic resources (Baker et al., 2018).

In a recent study (Lu et al., 2020), gene expression of tolerant and susceptible *Pinus contorta* infected with *D. septosporum* highlighted differences in defence responses between the two genotypes. The tolerant genotype had a stronger and more rapid expression of defence genes, when compared with the susceptible genotype. It was also observed that different genes were activated at different timepoints after the inoculation with *D. septosporum*, consistent with the fungal hemibiotrophic lifestyle, going from the biotrophic to the necrotrophic stage. Genes involved in PTI pathways were also identified, such as calcium-dependent protein kinases, BAK1, PR proteins and WRKY transcription factors. It was also found that some *R* genes were up-regulated when compared with the susceptible pine and that these *R* genes showed evidence of positive selection. These genes encoded proteins with conserved domains matching those of intracellular receptors, namely LRRs and CCs (Lu et al., 2020).

A similar approach was used to identify different defence responses in *Pn. radiata* and *Pinus pinea* during early infection stages with the fungus *Fu. circinatum*. *Pn. radiata* is considered to be highly susceptible to the pathogen, while *Pn. pinea* is highly resistant. Stronger defence responses were observed in *Pn. pinea* when compared with *Pn. radiata*, with increased production of cell wall-bound compounds, such as lignin, PR proteins and defence hormones. In the resistant species, many genes belonging to the PRR and WAK-like families were found to be up-regulated, in contrast to the small number found in *Pn. radiata*. RLK proteins with a lectin domain, which binds to chitin, were exclusively found in *Pn. pinea*. R proteins were also up-regulated in the resistant species, while only one was up-regulated in *Pn. radiata* and another strongly down-regulated, suggesting the pathogen may suppress host defence responses (Amaral et al., 2021; Zamora-Ballesteros et al., 2021).

The lack of known *R* genes (or even *S* genes) from forest species limits breeding for durable and BSR against pathogens, hence the importance of identifying such genes, which will help breeding strategies to enhance disease resistance. But for that, first, it is important to have a better understanding about the molecular mechanisms involved in

host-pathogen interactions and how the coevolution of these mechanisms on both sides forces the pathogen to secrete new or modified effectors in order to avoid host recognition/resistance and the host to develop new ways to defend itself, in a constant “arms race”.

1.7.1 Molecular studies on forest pathogens

Few studies have identified and characterised forest pathogen effectors, and more specifically *Pinus* species pathogen effectors.

Due to the growing negative impact of DNB around the world, there has been an effort to increase research on the disease, its pathogens, and the interactions these pathogens have with pine. Just over ten years ago, the first genome sequence of *D. septosporum* (NZE10) was published (de Wit et al., 2012), with the first transcriptomic data being published four years later (Bradshaw et al., 2016). The genome sequence was compared with that of *F. fulva*, a close relative of *D. septosporum*. Despite their phylogenetic similarity, these two pathogens have different hosts; *D. septosporum* infects pine, a gymnosperm, and *F. fulva* infects tomato, an angiosperm. The pathogens have 70% homologous genes, even though they have very different genomes sizes, due to a high repeat content in *F. fulva*. Interestingly, genes coding for the expression of dothistromin, a virulence factor of *D. septosporum*, are also present in genome of *F. fulva*, although they are not expressed *in planta* and some of the pathogen genes are pseudogenised (Chettri et al., 2013; de Wit et al., 2012).

As mentioned before, the *F. fulva*–tomato interaction is a model pathosystem, and therefore, the close phylogenetic relationship between *D. septosporum* and *F. fulva* provides a unique opportunity for comparative analysis of core effector functions across angiosperm and gymnosperm pathosystems, with some *F. fulva* core effectors having functional orthologues in *D. septosporum*. The *F. fulva* effector protein Avr4 (CfAvr4) triggers an HR in the presence of the Cf-4 immune receptor of tomato. DsAvr4, CaAvr4 and CbAvr4 are homologues of CfAvr4 from *D. septosporum*, *Cercospora apii* and *Cercospora beticola*, respectively. DsAvr4 also triggers a Cf-4-dependent HR, but CaAvr4 and CbAvr4 do not. Chimeric and mutant proteins, in which specific protein regions or single amino acid residues, respectively, were exchanged between them, were tested for

their ability to trigger an HR in transgenic *N. benthamiana* (expressing a copy of the tomato *Cf-4* immune receptor gene). This helped to identify a single region common to CfAvr4 and DsAvr4 that carried a conserved small aliphatic amino acid residue necessary for the elicitation of this HR (Hurlburt et al., 2018; Mesarich et al., 2016), highlighting the value of these types of comparative studies.

Another core effector, previously shown to cause cell death in *N. tabacum*, DsEcp2-1, is an orthologue of *F. fulva* Ecp2-1 (CfEcp2-1). The Ecp2 protein family from *F. fulva* has been characterized and causes an HR in tomato lines that carry a *Cf-Ecp2* gene (Laugé et al., 2000). *CfEcp2-1* is a virulence gene because once silenced or disrupted, *F. fulva* virulence is compromised (Laugé et al., 1997). HR-associated recognition of Ecp2-1 also occurs in *F. fulva* non-host *Nicotiana* species, such as *N. tabacum* (De Kock et al., 2004). As mentioned above, Ecp2 homologues are found in many other fungal pathogenic and non-pathogenic species (Thomma et al., 2011). *D. septosporum* DsEcp2-1 is highly expressed *in planta*, which suggests it may be required for virulence during infection of pine. However, deletion of *DsEcp2-1* led to bigger necrotic lesions and increased fungal biomass per lesion compared to the wild-type. This results suggests that DsEcp2-1 may act as an Avr factor that could trigger defence responses or may inhibit a host target which is involved in triggering necrosis during the biotrophic infection stage (Guo et al., 2020b).

More recently, the genome sequences of 18 other *D. septosporum* isolates sampled from different geographic locations worldwide were sequenced (Bradshaw et al., 2019b). The results showed a great level of polymorphism between isolates, when compared with the New Zealand strain NZE10, especially in the Guatemalan and Colombian isolates. Comparisons also showed that some isolates have chromosome duplications and vary in levels of dothistromin production, with a German isolate producing particularly high levels of dothistromin.

To provide more insight into the molecular interactions between *D. septosporum* and *Pn. radiata*, a time-series transcriptomic study was performed (Bradshaw et al., 2016) by RNA sequencing of different fungal infection stages in the host. Stages were divided into Early, Mid and Late. In the Early, or biotrophic stage, *D. septosporum* hyphae have penetrated the needle through the stomata, but no symptoms are observed. The Mid

stage marks the beginning of the necrotrophic stage, or the necrotrophic switch the fungus undergoes, with apoplastic colonization and appearance of first lesions. In the Late stage, spores are released from a mature lesion in the needle. At the Early stage, many carbohydrate-active enzyme (CAZyme) genes that were highly up-regulated were related to cell wall-degradation, while many CEs were more highly expressed at the necrotrophic than the biotrophic stage. Using this time-series *D. septosporum*–*Pn. radiata* infection transcriptomic study, combined with a bioinformatic effector prediction pipeline, a set of CE proteins from *D. septosporum* was recently identified (Hunziker, 2018). These CEs were transiently expressed in non-host model plants, *N. benthamiana* and *N. tabacum*, to identify those possibly involved in infection and/or in triggering host defence. Several of those proteins elicited a plant cell death response, suggesting they are being recognized by plant receptors in those non-host plants, triggering non-host resistance. One of the objectives of this thesis is to build on these results.

Compared to other *Phytophthora* species, the understanding of the *P. pluvialis*–pine interaction on a molecular level is very limited. Generating *Phytophthora* genomic sequence data, along with transcriptomic data, is important to identify effector proteins secreted by pathogens and their receptor in the host. The association of host receptors with resistance to oomycete pathogens, once evaluated in field trials, can lead to the development of molecular markers which can be used for breeding BSR. Genome sequencing has been performed for two *P. pluvialis* strains, collected from New Zealand and the USA (Studholme et al., 2016), with the New Zealand one serving as the reference genome in this thesis. A population study comparing the genetic diversity between *P. pluvialis* from New Zealand and the USA showed that the USA population had twice the genetic diversity than the New Zealand population; however clonal reproduction is ongoing in both countries (Brar et al., 2018). In this case, the hypothesis is that *P. pluvialis* was introduced into New Zealand from a single population from the western USA, followed by clonal expansion. This was further supported by a follow-up study that included more isolates from both countries, in which it is believed that the pathogen originated in the USA Pacific Northwest (PNW) forests and was introduced into New Zealand, where *P. pluvialis* infected a larger range of hosts than in the PNW (Tabima et al., 2020). Transcriptomic data from *Pn. radiata* naturally infected with *P. pluvialis* was

also generated and showed highly up-regulated genes with orthologues to known pathogenicity-related genes in other *Phytophthora* species (Brar, 2018).

From the three foliar pine pathogens in this study, *C. minus* is the one for which there is the least molecular information. A recent study sequenced the genomes of eight *C. minus* isolates and compared isolates using both culture-based and genome-based analyses (McDougal et al., unpublished). Results suggested the presence of a new morphotype, not identified previously (Hunter et al., 2016), which highlights the importance of molecular studies in *C. minus* to better understand the interactions the pathogen has with *Pn. radiata*.

Other molecular studies of forest pathogens include analysis of the infection process of *Cr. ribicola*, responsible for WPBR, in *Pn. monticola*, in which putative effectors secreted during different infection stages were described (Liu et al., 2015). One of these CEs was functionally characterized as a possible Avr, triggering plant defence responses in tobacco (Ma et al., 2019). As mentioned above, *Cr. ribicola* virulent pathotypes, such as *vcr2*, can overcome resistance in trees carrying the *R* gene *Cr2*. Analysis was performed to better understand the mechanisms that enable the *vcr2* pathotype to overcome *Cr2* resistance, by comparing virulent and avirulent pathotypes, and a high level of genomic differences was found between these pathotypes. Single nucleotide polymorphisms (SNPs) in *vcr2* pathotypes were found in functional fungal virulence-related genes and may become important molecular markers in breeding programmes. Through the expression profile, it was also possible to detect different pathotype-specific pathways, with *vcr2* expressing many genes related to the detoxification of plant antimicrobial compounds, suggesting a possible explanation for the breakdown of *Cr2* resistance (Liu et al., 2021).

The oomycete *Phytophthora agathidicida*, the causal agent of kauri dieback disease, secretes RxLR effectors to interact with its hosts. Several of those trigger cell death responses in *N. benthamiana* and *N. tabacum*, and one was shown to suppresses cell death triggered by Avr3/R3a from *P. infestans* and by a cell death elicitor from *P. agathidicida* (Guo et al., 2020a). The pathogen also secretes several GH proteins that are able to trigger cell death responses in *Nicotiana* species (Bradley, 2022). GHs are part of the CAZyme family, many of which have been shown to function as IPs from fungi and

oomycetes, being recognized by the plant immune system and triggering defence responses (Bradley et al., 2022).

In another study, eight effector proteins secreted from the necrotrophic forest pathogen *H. annosum* were transiently expressed in *N. benthamiana* and were found to trigger plant cell death. Activation of *N. benthamiana* defence genes that are consistent with the response against necrotrophic pathogens were observed upon infiltration of one of these elicitors (Raffaello & Asiegbu, 2017). Another study that used similar methodology, identified several secreted effector proteins from *Me. larici-populina* that can suppress plant immunity (Germain et al., 2018).

All of these studies and the genomic/transcriptomic information generated helps to better understand the molecular basis of forest pathogens interactions with trees and can be used to identify and characterize CEs secreted by pathogens and, later, screen the host to find potential plant targets.

The identification of shared effectors from the three foliar pine pathogens can ultimately lead to the identification of cognate *Pn. radiata* immune receptors. This would be particularly beneficial since the *D. septosporum* and *P. pluvialis* population in New Zealand are clonal. For this reason, DNB and RNC resistance mediated by the recognition of an Avr product by a host R protein would be more durable, when compared to other places where both mating types of the pathogen are found. It is not known whether the *C. minus* population in New Zealand is clonal or if it has low diversity. Nevertheless, as mentioned above, selecting DNB-resistant trees is likely to also increase the resistance to CNC (Ismael et al., 2020), which might indicate that both pathogens use similar mechanisms when infecting the host, and might also be recognized in the same way by pine. Identifying these common mechanisms, or common secreted effectors shared between the three pine pathogens, will be beneficial in finding potential host targets and informing breeding programmes in order to achieve both durable and broad-spectrum resistance.

1.8 Aims and objectives

Oomycete and fungal pathogens are two very different types of organisms. Using the foliar pine pathogens, *D. septosporum*, *C. minus* and *P. pluvialis*, together with the development of a pine screening assay, the aim of this project was to find common IPs, such as effectors, that will be recognized by R proteins in the host and could be exploited to achieve durable and broad spectrum resistance (BSR). This could ultimately lead to the development of molecular markers for rapid screening in breeding programs and provide detailed insight into the molecular basis of BSR in pines.

Aim 1. Characterization of two conserved cell death elicitor families from the Dothideomycete fungal pathogens *D. septosporum* and *F. fulva* (syn. *Cladosporium fulvum*).

- Use bioinformatic prediction tools to further characterize and gain insight into the functions of two conserved protein families, Ecp20 and Ecp32, from *D. septosporum* and *F. fulva*, previously identified by Hunziker (2018).
- Use pre-existing transcriptomic information from *D. septosporum*–pine and *F. fulva*–tomato interaction to determine the expression levels of all Ecp20 and Ecp32 family members.
- Transiently express all members of *D. septosporum* and *F. fulva* Ecp20 and Ecp32 protein families in *N. benthamiana* and *N. tabacum* using an *Agrobacterium tumefaciens*-mediated transient transformation assay (ATTA) to determine if they trigger plant cell death and/or chlorosis.
- Compare the nucleotide and protein sequences of *D. septosporum* strain NZE10 Ecp20 and Ecp32 family members with those of 18 other sequences from *D. septosporum* isolates sampled globally and *D. pini*, the other causal agent of DNB, and determine if these proteins also trigger plant cell death in non-host plants.
- Further develop a method for CE protein screening in pine, employing vacuum infiltration-mediated delivery into tissue culture-generated pine shoots to determine if they trigger cell death.

- Heterologously express CE proteins in the yeast *Pichia pastoris*, purify them by immobilized metal ion affinity (IMAC) and infiltrate the purified proteins into tissue culture-generated pine shoots.
- Identify the potential plant immune receptors responsible for the recognition of cell death elicitors from the Ecp20 and Ecp32 family members.

Aim 2. Identification and characterization of candidate effector proteins from the three foliar pine pathogens *D. septosporum*, *C. minus* and *P. pluvialis* in non-host and host species.

- Using the pre-existing *D. septosporum* NZE10 genomic and transcriptomic information and an effector prediction pipeline, identify CE proteins from *D. septosporum* NZE10 and their orthologues from *C. minus* NZFS809 and *P. pluvialis* NZFS3000.
- Compare the nucleotide and protein sequences of *D. septosporum* strain NZE10 CE proteins with those of 18 other sequences from *D. septosporum* isolates sampled globally and with *D. pini*.
- Use bioinformatic prediction tools to further characterize and gain insight on the function of CE proteins from the three pine pathogens.
- Transiently express CE proteins in *N. benthamiana* and *N. tabacum* using an ATTA to determine if they trigger plant cell death and/or chlorosis.
- Heterologously express CE proteins in the yeast *Pi. pastoris*, purify and apply the newly developed pine screening method to delivery proteins into tissue culture-generated pine shoots to determine if they trigger cell death.
- Determine if the cell death triggered by CEs is associated with plant immunity responses by examining the expression of some HR-specific/defence related marker genes.

Aim 3. Functional characterization of candidate effector genes in *D. septosporum* using CRIPR/Cas9-genome editing.

- Design sgRNAs and generate sgRNA-CRISPR/Cas9 plasmids for deletion/disruption of CE genes from *D. septosporum*.

- Generate the donor DNA plasmid for homologous recombination in *D. septosporum*.
- Perform protoplast-mediated co-transformation of *D. septosporum* with sgRNA-CRISPR/Cas9 and donor DNA plasmids, and complementation of the selected genes.
- Study the function of disrupted effector genes through phenotypic characterization (radial growth and sporulation assays) and *Pn. radiata* infection virulence/pathogenicity assays.

Aim 4. Identification and characterization of candidate effector proteins from three morphotypes of *C. minus*.

- Predict effector proteins from seven additional *C. minus* isolate genomes and compare the nucleotide and protein sequences of cell death elicitors from NZFS809 with other *C. minus* isolates to determine conservation of cell death activity.
- Transiently express candidate Ecp32 proteins from different *C. minus* isolates in *N. benthamiana* and *N. tabacum* using an ATTA to determine if they trigger plant cell death and/or chlorosis.

2 Characterization of two conserved cell death elicitor families from the Dothideomycete fungal pathogens *Dothistroma septosporum* and *Fulvia fulva*

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2.1 Introduction

Understanding how plants and fungal pathogens interact at the molecular level can lead to improvements in plant health. During infection, fungal pathogens deliver a collection of small, proteinaceous virulence factors, termed effectors, into the apoplast and/or cells of their host plants to promote the infection process (Rocafort et al., 2020; Wang et al., 2020). However, as fungal infection has a negative impact on plant fitness, there is a strong selection pressure on the host to recognize these effectors as invasion patterns (IPs) and, in doing so, activate defence responses that provide protection against the pathogen (Cook et al., 2015). In line with this, in resistant hosts, particular effectors may be recognized by corresponding specific immune receptors, called resistance (R) proteins, to activate plant defence responses. Often, one of these defence responses is the hypersensitive response (HR), a localized cell death reaction that limits or halts pathogen growth (Cook et al., 2015; Win et al., 2012). As these defence responses often lead to the pathogen being unable to cause disease, such recognized effectors are typically referred to as avirulence (Avr) determinants. To circumvent recognition, and therefore the ensuing plant defence responses, the fungal invader must then modify its effector arsenal by secreting mutated or new effectors, shifting the pressure to adapt back onto the plant host, resulting in a constant "arms race" in plant-pathogen interactions (Coll et al., 2011; Jones & Dangl, 2006).

Progress in the generation of genomic, transcriptomic, and proteomic resources has enabled the rapid identification of pathogen effectors, making it possible to screen them for elicitation or suppression of plant defence responses, a process known as effectoromics. This, in turn, makes it possible to discover host defence or susceptibility genes in different plant species. A search for conserved effectors, sometimes called 'core'

effectors, is essential as it can lead to broad spectrum resistance. The tertiary structures of some effector proteins are conserved across microbial classes because they have fundamental and important roles in infection, and for this reason are less likely to be mutated or lost during microbial evolution (Nürnberg & Brunner, 2002). Therefore, plant resistance based on the recognition of conserved effectors is more likely to be durable, as it is less likely to be overcome.

Dothistroma septosporum (Ds) is a hemibiotrophic pathogen that causes Dothistroma needle blight (DNB), one of the most important foliar diseases of pine trees (Drenkhan et al., 2016). Based on genomic information, *D. septosporum* is closely related to *Fulvia fulva* (Ff; syn. *Cladosporium fulvum*) (de Wit et al., 2012). *F. fulva* is currently considered a hemibiotrophic pathogen responsible for leaf mould of tomato (*Solanum lycopersicum*), a disease that is mainly a problem in greenhouse or high tunnel environments (Hane et al., 2020; Thomma et al., 2005). In both the *D. septosporum*–pine and *F. fulva*–tomato pathosystems, infection begins with the germination of conidia on the leaf surface and penetration of hyphae into the host through the stomata. Once inside, both pathogens colonize the apoplastic space between mesophyll cells, where they secrete effector proteins (Kabir et al., 2015b; Mesarich et al., 2018).

F. fulva is one of the best studied filamentous fungal pathogens. The *F. fulva*–tomato interaction is a model pathosystem in which early pioneering studies helped to establish the molecular basis of the gene-for-gene concept of plant resistance to fungal pathogens (de Wit, 2016). In *F. fulva*, small, secreted cysteine-rich proteins delivered to the host apoplast, termed extracellular proteins (Ecps) based on their identification in apoplastic wash fluid samples, have been well-studied (Mesarich et al., 2018). These include core effectors such as Ecp2-1, Ecp6 and Avr4 (de Jonge et al., 2010; Sánchez-Vallet et al., 2013; Stergiopoulos et al., 2010; van den Burg et al., 2006), homologues of which have been found in other plant-pathogenic fungi, including *D. septosporum* (Bolton et al., 2008; de Wit et al., 2012; Stergiopoulos et al., 2012). In contrast, few studies have identified and characterized effectors from *D. septosporum*. In one of these studies, DsEcp2-1, the orthologue of Ecp2-1 from *F. fulva*, was suggested to be an Avr protein responsible for eliciting defence responses in pine (Guo et al., 2020b). In another of these studies, DsAvr4, the orthologue of Avr4 from *F. fulva*, was found to bind chitin, a function

that is required to protect the fungal cell wall against hydrolysis by host chitinases (Mesarich et al., 2018; van den Burg et al., 2006).

In general, studies highlighting the role of effectors in molecular interactions between pathogens and gymnosperm hosts are few and far between. The close phylogenetic relationship between *D. septosporum* and *F. fulva*, however, provides a unique opportunity for comparative analysis of core effector functions across angiosperm and gymnosperm pathosystems. Recently, a set of candidate effector proteins from *D. septosporum* was transiently expressed in the non-host plants, *Nicotiana benthamiana* and *Nicotiana tabacum*, to identify those that are involved in triggering plant defence responses (Hunziker, 2018). Several of these proteins, all with orthologues in *F. fulva*, elicited a plant cell death response, suggesting that they are recognized by extracellular immune receptors in these non-host plants, triggering non-host resistance. Amongst these we found two proteins, Ds70057 and Ds70694, that were of particular interest, as they are orthologues of Ecps from *F. fulva* (Hunziker et al., 2021; Mesarich et al., 2018). Both proteins appear to be part of protein families – the Ecp20 family and the Ecp32 family (Hunziker et al., 2021). The Ecp20 family is made up of four members in both species, while the Ecp32 family is made up of four members in *D. septosporum* and five members in *F. fulva*.

In this chapter, members from the Ecp20 and Ecp32 protein family from *D. septosporum* and *F. fulva* were characterized and compared through prediction of their protein tertiary structures and assessed by their cell death-eliciting capacity in non-host angiosperm plants. Also, using a novel pine screening assay developed in this study, two proteins from the Ecp20 family from *D. septosporum* were tested for their ability to trigger cell death in the gymnosperm host, *Pinus radiata*. This work provides insights into conservation of core effector function and recognition across a broad range of plant species.

2.2 Methods

2.2.1 Bioinformatics

Members of the Ecp20 and Ecp32 protein families were identified from the predicted proteomes and/or genomes of *D. septosporum* NZE10 and *F. fulva* 0WU (de

Wit et al., 2012). Chromosome locations of the *FfEcp20* and *FfEcp32* genes were identified using the *F. fulva* Race 5 genome assembly (Zaccaron et al., 2022). Gene family members were also identified from genome sequences of 18 other *D. septosporum* isolates (Bradshaw et al., 2019b) and *D. pini* CBS 116487 (GenBank assembly accession GCA_002116355.1), using reciprocal BLASTp and tBLASTn searches in conjunction with an E-value cut-off of $<10^{-5}$. BLASTp was also employed to determine the level of conservation for each family member across fungal species in the Joint Genome Institute (JGI) MycoCosm database, using an E-value threshold of $<10^{-5}$. EffectorP v3.0 (Sperschneider & Dodds, 2021) was used to predict whether members of the Ecp20 and Ecp32 families are effectors.

Protein tertiary structure predictions were performed using AlphaFold2 (Jumper et al., 2021; Mirdita et al., 2022), and the Dali server (Holm, 2020) was used to identify proteins with structural similarity in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB). Here, a Dali Z-score of 2 or greater was used to infer structural similarity. Protein tertiary structures were visualized and rendered in PyMOL v2.5, with alignments carried out using the CEalign tool (DeLano, 2002).

Protein sequence alignments were performed using Clustal Ω (Sievers et al., 2011) in Jalview (www.jalview.org). Phylogenetic trees were constructed with the neighbour-joining method using Geneious Software v9.1.8 (Kearse et al., 2012).

2.2.2 Microorganism and plants

D. septosporum NZE10 (de Wit et al., 2012), GUA2 and COLN (Bradshaw et al., 2019), as well as *D. pini* CBS 116487 (BioSample ID SAMN02254964) and *F. fulva* OWU (de Wit et al., 2012), were used to identify candidate effectors for characterization in this study. *Escherichia coli* DH5 α (Taylor et al., 1993) and *Agrobacterium tumefaciens* GV3101 (Holsters et al., 1980) were used for gene cloning and *A. tumefaciens*-mediated transient transformation assays (ATTAs), respectively. *N. tabacum* Wisconsin 38 and *N. benthamiana* were used as model non-host plants for ATTAs, while *N. benthamiana* was also used for tobacco rattle virus (TRV)-mediated gene silencing experiments. *Pn. radiata* clonal shoots, derived from family seed lots that are, compared to each other, relatively susceptible (S6 and S11) or tolerant (R4) to DNB, and grown from embryogenic

tissue under sterile conditions on LPch agar (Hargreaves et al., 2004), were provided by Scion (New Zealand Forest Research Institute Ltd., Rotorua, New Zealand) and used in protein infiltration assays. All microorganisms and plants used in this Chapter are detailed in **Appendix Table A.1**.

2.2.3 Media

2.2.3.1 Medium for bacteria

Bacterial cultures were grown in Lysogeny Broth (LB), made with 2% (w/v) LB (Duchefa Biochemie, Haarlem, Netherlands) and 0.1% (w/v) glucose. LB agar was made with the addition of 1.5% (w/v) bacteriological agar (Acumedia®, Michigan, USA).

Antibiotics were added at the following final concentration where appropriate: kanamycin (Kan) 50 µg/mL, ampicillin (Amp) 100 µg/mL, rifampicin (Rif) 10 µg/mL and gentamycin (Gm) 30 µg/mL.

2.2.3.2 Media for *Pichia pastoris*

YPD medium: 5% (w/v) yeast peptone dextrose (YPD) (Clontech, California, USA).

Minimal Dextrose (MD) plates: 1.34% (w/v) yeast nitrogen base (YNB) (Invitrogen, Massachusetts, USA), 4×10^{-5} (w/v) biotin, 2% (w/v) dextrose and 1.5% (w/v) bacteriological agar (Acumedia®).

Buffered complex glycerol medium (BMGY): 1% (w/v) yeast extract, 2% (w/v) mycological peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% (w/v) YNB, 0.4 mg/L biotin, 1% (v/v) glycerol.

Buffered complex methanol medium (BMMY): As BMGY, glycerol substituted for 0.5% (v/v) methanol. Yeast extract and peptone were dissolved in distilled water (dH₂O) and autoclaved. Filter-sterilised solutions of the remaining ingredients were mixed into the broth at room temperature (RT).

2.2.3.3 Medium for *Dothistroma septosporum*

D. septosporum was grown in *D. septosporum* medium (DM), made with 5% (w/v) malt extract (Oxoid, Ontario, Canada), 2.3% (w/v) nutrient broth (Oxoid). DM agar was made with the addition of 1.5% (w/v) bacteriological agar (Acumedia®).

2.2.3.4 Medium for *Fulvia fulva*

C. fulvum was grown in liquid Potato Dextrose Broth (PDB), made with 2.4% (w/v) PDB (BD&Co, New Jersey, USA).

2.2.3.5 Medium for *Pinus radiata*

Tissue culture shoots were maintained on modified Quoirin and Lepoivre medium with charcoal (LPch): 18 g/L KNO₃, 1.2 g/L Ca(NO₃)₂·4H₂O, 0.4 g/L NH₄NO₃, 0.36 g/L MgSO₄·7H₂O, 0.27 g/L KH₂PO₄, 8.6 mg/L ZnSO₄·7H₂O, 3.1 mg/L H₃BO₃, 20 mg/L MnSO₄·4H₂O, 0.25 mg/L CuSO₄·5H₂O, 80 µg/L KI, 0.25 mg/L Na₂MoO₄·2H₂O, 25 µg/L CoCl₂·6H₂O, 0.4 mg/L Thiamine HCl; 40 mg/L Na₂EDTA, 30 mg/L FeSO₂·7H₂O; 5 g/L activated charcoal; 12 g/L agar (Hargreaves et al., 2004).

2.2.4 Electrocompetent cells

To make *E. coli* DH5α and *A. tumefaciens* GV3101 cells competent for electroporation with plasmids, they were prepared as follows: A single colony of each strain was incubated in 25 mL LB at 37°C (*E. coli*) or 28°C (*A. tumefaciens*), for approximately 16 h with shaking at 180 rpm (Ecotron, INFORS-HT, Switzerland). Each culture was diluted 50-fold. The cultures were incubated in 500 mL LB at the same conditions as above. After approx. 3 and 5 h, respectively for the two species, cultures were collected with an OD₆₀₀ of approx. 0.7. Cultures were chilled on ice for 30 min and then transferred to centrifuge tubes pre-cooled on ice. They were centrifuged at 4500 g for 20 min at 4°C. The supernatant was discarded and 500 mL of 10% glycerol pre-cooled at -20°C were added. Cells were resuspended by gentle swirling on ice and the centrifugation was repeated. The supernatant was discarded, and 250 mL of 10% glycerol were added for resuspension. The solution was centrifuged for 15 min, and this wash was repeated three times, halving the added volume each time. Several mL of supernatant

were retained in the tubes, sufficient to acquire a milky final cell suspension. This was aliquoted (40 μ L) for storage at -80°C in 0.6 mL tubes pre-cooled at -20°C , and snap-frozen using liquid nitrogen.

Pichia pastoris GS115 competent cells were prepared as follows: a single colony was inoculated in 5 mL YPD and grown overnight at 28°C and shaking at 250 rpm (Ecotron). This preculture was inoculated in 75 mL YPD and grown overnight at the same conditions as before until an OD_{600} of approx. 1.3 was reached. The culture was then centrifuged at 1500 g for 5 min at 4°C . The supernatant was discarded, the pellet was resuspended in 50 mL of ice cold dH_2O and centrifugation was repeated. The supernatant was discarded, and 25 mL of ice cold dH_2O were added for resuspension. Centrifugation was repeated and 10 mL of ice cold 1 M sorbitol was added before centrifugation. Lastly, the pellet was resuspended in 1 mL of ice cold 1 M sorbitol. Cells were aliquoted (80 μ L) and used the same day (Kombrink, 2012).

2.2.5 DNA isolation from fungal cultures

Genomic DNA (gDNA) for *D. septosporum* isolates GUA2, COLN and COLS and for *D. pini* were kindly supplied by Irene Barnes (University of Pretoria, South Africa).

2.2.5.1 Genomic DNA extraction by CTAB method

The hexadecyltrimethylammonium bromide (CTAB) DNA extraction method was used in this study to extract gDNA from *D. septosporum* NZE10 and *C. fulvum* 0WU (Doyle & Doyle, 1987). Mycelia from fungal liquid cultures growing for approx. 7 days, for *D. septosporum* and 14 days for *C. fulvum*, were ground to a fine powder using a sterile mortar and pestle with liquid nitrogen. Then 500 mg of ground sample was transferred into a sterile microcentrifuge tube containing 600 μ L of 2% CTAB extraction buffer (2% (w/v) CTAB, 1% (w/v) PVP40, 1.4 M NaCl, 20 mM EDTA and 0.1 M Tris, pH 8). Samples were incubated at 37°C for 20 min with 2 μ L of RNase A (100 mg/ml) (Sigma-Aldrich, Missouri, USA) and then at 65°C for 40 min with occasional inversion. After removal from the water bath, samples were cooled to RT and 600 μ L of chloroform was added. The sample was mixed by inversion and centrifuged at 17000 g for 2 min. The upper aqueous phase was transferred to a new tube containing 600 μ L of isopropanol, mixed and incubated on ice for 20 min. The mixture was centrifuged at 9000 g for 30 s to pellet the

DNA. The supernatant was discarded, 600 μ L of 70% ethanol was used to wash the precipitated DNA pellet and centrifuged again for 1 min. The ethanol washing step was repeated at least twice. Then, the ethanol was decanted completely, and the sample was left to air dry. Lastly, DNA was resuspended in 50 μ L TE buffer (10 mM Tris and 1 mM EDTA, pH 8). Sample's concentrations were estimated with a NanoDrop® ND-1000 UV-Vis spectrophotometer and nanodrop® software version 3.1.0 (Nanodrop Technologies Inc, Wilmington, USA) according to the manufacturer's instructions.

2.2.6 Effector gene cloning

2.2.6.1 Polymerase chain reaction (PCR)

To obtain the inserts for cloning, 20 μ L PCR mix contained 10 μ L Phusion® High-Fidelity DNA Polymerase Mastermix (New England Biolabs, Massachusetts, USA), 1 μ L of each primer (at 10 mM), 10 ng of template and dH₂O. Conditions were: Initial denaturation at 98°C for 1 min, 30 cycles of denaturation at 98°C for 1 sec, annealing at 55-60°C for 5 s and extension at 72°C for 15 sec, final extension at 72°C for 1 min.

For standard colony PCR, Taq DNA Polymerase with ThermoPol® Buffer (New England Biolabs) was used. The 25 μ L reaction contained 2.5 μ L of 10x ThermoPol® Reaction Buffer, 10 mM of dNTPs, 0.5 μ L of each primer (at 10 mM), 0.125 μ L of Taq DNA Polymerase. Template was a single colony using a sterile tip.

2.2.6.2 Agarose gel electrophoresis

Amplicons were visualized following after gel electrophoresis (1% (w/v) in TAE buffer (190 mM Tris, 342 mM acetic acid, 2.5 mM EDTA). DNA was loaded with a 1:6 dilution of SDS loading dye (0.2% (w/v) bromophenol blue, 20% (w/v) sucrose, 1% (w/v) SDS, 5 mM EDTA, pH 6.8). Gels were stained with ethidium bromide (1 μ g/mL) for 15 min. Gels were visualised and imaged with an UV Transilluminator Gel Documentation system (Bio-Rad, California, USA). A 1 kb plus DNA ladder (Invitrogen, Massachusetts, USA) was used to estimate amplicon size.

2.2.6.3 Gel purification

The amplicons with correct sizes were excised from the gel and purified using the E.Z.N.A.[®] gel extraction kit (Omega Bio-tek, Norcross, USA) according to the manufacturer's instructions. Product concentrations were estimated with a NanoDrop[®] spectrophotometer.

2.2.6.4 Golden Gate assembly

To amplify the candidates effector genes for subsequent cloning, individual primers adding BsaI recognition sites and 4 bp overhangs specific for Golden Gate modular cloning (Engler et al., 2009), and excluding the start codon and signal peptide sequence, were designed using Primer3 (Untergasser et al., 2012) implemented in Geneious 9.1.8 (Kearse et al., 2012). Primers were synthesized by Integrated DNA Technologies (IDT). A table of primers used in this study is in **Appendix Table A.2**. In the case of genes with introns, the coding sequence was synthesized directly into the expression vector by Twist Bioscience (San Francisco, CA, USA) (**Appendix Figure A.1**).

For the one-pot Golden Gate assembly (Engler et al., 2008), 10 fmol of purified PCR products were added to 10 fmol of pICH86988, 10 fmol of pUC19B::PR1 α -3xFLAG, or 10 fmol of pUC19B::3xFLAG (for constructs without signal peptide), 10% (v/v) 1 x T4 ligase buffer, 5% (v/v) T4 ligase (New England Biolabs), 10% (v/v) 10 x BSA buffer, 1% (v/v) BsaI restriction enzyme and sterile dH₂O in total reaction volume of 20 μ L. The conditions were: 30 cycles of restriction-ligation at 37°C for 5 min and 16°C for 10 min, digestion at 55°C for 5 min, heat inactivation at 80°C for 5 min. The reaction was cleaned by filtering through 150 μ L of sepharose beads, before the transformation of *E. coli* competent cells. For plasmid propagation, 40 μ L of electrocompetent DH5 α cells were transformed with the total volume of reaction recovered after the sepharose clean-up. After recovery (1 h at 37°C) they were streaked in LB agar supplemented with Kan, 20 μ g/mL 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and 100 μ g/mL isopropyl β -D-1-thiogalactopyranoside (IPTG) and incubated in the dark at 37°C overnight. White colonies were checked by PCR using the gene's specific primer pair. Positive colonies were transferred into 4 mL LB supplemented with Kan and incubated at 37°C overnight with 180 rpm shaking (Ecotron). Four mL of the culture were used for plasmid extraction

(E.Z.N.A.[®] Plasmid Mini Kit I, Omega Bio-tek). Plasmid concentration and purity were determined by NanoDrop[®]. Constructs were sequenced by the Massey Genome Service sequencing facility at Massey University, Palmerston North, using 300 ng of plasmid DNA and 4 pmol of primers flanking the insertion site (**Appendix Table A.2**).

To create stock cultures for the ATTAs, 40 µL of electrocompetent *A. tumefaciens* GV3101 cells were transformed with 20 ng of construct and incubated in 1 mL LB for 2-3 h at 28°C for recovery. Then, 50 µL of these cells were plated on LB agar supplemented with Kan and Rif. Plates were incubated in the dark at 28°C for 48 h. Colonies were incubated in 4 mL of LB supplemented with Kan, Rif and Gen in the dark at 28°C with shaking at 180 rpm (Ecotron) for at least 16 h. An aliquot of each culture was mixed with 60% glycerol (final concentration of 30%) and snap-frozen in liquid nitrogen and stored at -80°C.

For consistency in the experiments, the predicted native signal peptides of all candidate effectors were replaced with PR1α, a broadly used *N. tabacum* signal peptide, to direct secretion of proteins to the apoplast (Ohshima et al., 1987). A flag-tag, at N-terminal, was used to enable the detection of the protein via western blot. A representative scheme of the expression vector pICH86988 is shown in **Appendix Figure A.2**. All plasmids generated in this section are listed in **Appendix Table A.3**.

2.2.6.5 Construction of a heterologous protein expression cassette in *Pichia pastoris*

For the pine infiltration assays, the candidate proteins were heterologously produced in the methylotrophic yeast *Pi. pastoris* GS115. In order to do that, the PCR products of the genes encoding the candidate proteins were cloned into the expression vector pPic9-His₆ (**Appendix Figure A.3**), which contains the *HIS4* gene, allowing selection of transformants that are able to grow on medium without histidine. The *Pi. pastoris* strain GS115 used is defective in the histidine dehydrogenase gene. The yeast transformation is based on recombination into the alcohol oxidase 1 (*AOX1*) locus in the *Pi. pastoris* genome by means of the *AOX1* sequence in linearized pPic9 (*AOX1* has restriction sites that allows for that). The expression of the gene of interest can be

induced via the *AOX1* gene promoter that is activated upon changing the carbon source of *Pi. pastoris* from glycerol to methanol (Kombrink, 2012).

The construct preparation for *Pi. pastoris* transformation was done as follows: the sequence encoding the signal peptide of the gene of interest was determined, allowing the cloning of the sequence that encodes the mature protein. The forward primer contained the sequence for the restriction site (SmaI – New England Biolabs), followed by the FLAG-tag sequence (Flag: CCCGACTACAAGGACGACGATGACAAG). The reverse primer contained the second restriction site (EcoRI – New England Biolabs) (**Appendix Table A.2**). Primers were synthesized by IDT. Resulting PCR amplicons from all candidates were cloned into the expression vector pPic9-His₆ (Invitrogen) behind the α -factor signal peptide sequence using SmaI/EcoRI restriction enzymes and T4 DNA ligase (New England Biolabs). For plasmid propagation, 40 μ L of electrocompetent DH5 α cells were transformed with 5 μ L of the ligation reaction. After recovery (1 h at 37°C) they were streaked in LB agar supplemented with Amp and incubated at 37°C overnight. Positive colonies were transferred into 4 mL LB supplemented with Amp and incubated at 37°C overnight with 180 rpm shaking (Ecotron). Four mL of the culture were used for plasmid extraction. Constructs were sequenced by the Massey Genome Service sequencing facility, using 300 ng of plasmid DNA and 4 pmol of primers flanking the insertion site. Finally, the expression cassettes were linearized with SacI or Sall restriction enzymes (New England Biolabs) and transformed into *Pi. pastoris* GS115. For that, 80 μ L of electrocompetent GS115 cells were transformed with 300 ng of linearized DNA and plated on MD and incubated at 28°C for two days. Positive *Pi. pastoris* colonies were used for posterior expression and purification of candidate effector proteins (Kombrink, 2012).

2.2.7 *Nicotiana* species growth and assays

2.2.7.1 *Nicotiana benthamiana* and *Nicotiana tabacum* growth

N. benthamiana and *N. tabacum* seeds were germinated in pots with sterilized soil. Plants were grown in a temperature-controlled room at 22°C and 12:12 light:dark photoperiod with 80-85 μ mol m⁻²s⁻¹. After two weeks, seedlings were transferred to individual pots. Infiltration assays were carried out approx. four weeks after transfer; plants were watered 2-3 h before infiltration to promote stomata opening.

2.2.7.2 *Agrobacterium tumefaciens*-mediated transient transformation assay

To perform the ATTA, *A. tumefaciens* cells were grown from glycerol stocks into LB plates supplemented with Kan, Rif and Gm for 40 h at 28°C in the dark. Colonies were incubated in LB with shaking at 180 rpm (Ecotron) until an OD₆₀₀ between 0.4-2 was reached. Cells were centrifuged at 2500 *g* for 5 min and resuspended in 1 mL of infiltration buffer (10 mM MgCl₂·6H₂O, 10 mM MES-KOH, 100 µM acetosyringone (Sigma-Aldrich)). The OD₆₀₀ was measured again, and sterile dH₂O added to reach a final OD₆₀₀ of 0.5. Infiltration cultures were incubated for 3 h at RT before infiltration.

The abaxial side of *N. benthamiana* leaves 2, 3 and 4 (Ma et al., 2012) and the two fully expended *N. tabacum* leaves were infiltrated with the appropriate *A. tumefaciens* solution using 1 mL needleless syringes. Here, the extracellular elicitor INF1 from *Phytophthora infestans*, which is recognized by the LRK1 immune receptor in *Nicotiana* species (Kamoun et al., 1997; Kanzaki et al., 2008), was used as a positive cell death control, while the empty pICH86988 vector was used as a negative no cell death control. At least 12–24 infiltration zones were tested for each candidate effector gene or control (consisting of two leaves from each of two plants × 3–6 repeat experiments). Plants were monitored for up to 7 days, when photographs were taken. Infiltration zones were assessed on whether the protein tested elicited a strong or weak cell death response, or no visible cell death. A strong cell death response was scored when it was indistinguishable from the response elicited by INF1 and around the entire infiltration zone, while a weak response was considered when the protein elicited a weaker response around approximately 50% of the infiltration zone when compared with INF1. A no-cell death response was indicated when the infiltration zone had the same response as the negative empty pICH86988 vector control.

2.2.8 *Pinus radiata* assays

2.2.8.1 Protein expression

After transformation, positive colonies of *Pi. pastoris* were screened for protein expression following Weidner et al. (2010). Single colony isolates of *Pi. pastoris* strains were incubated in 5 mL of BMGY in 50 mL falcon tubes at 28°C and 250 rpm shaking

(Ecotron) for at least 16 h to reach an OD₆₀₀ of at least 2. Cultures were centrifuged at 3000 *g* for 5 min. For induction of protein expression, harvested cells were resuspended in 10 mL BMMY to a starting OD₆₀₀ of 1. These cells were incubated at 28°C and 250 rpm shaking (Ecotron) for different times, with methanol (for maintained induction) added to a concentration of 0.5% (v/v) after 24 h. Different time points were analysed to establish the optimal time period for protein expression after induction. The time points for taking samples were: 24 h, 48 h and/or 72 h. Samples were analysed by western blot.

Once the best conditions were selected, expression of candidate proteins was induced by incubation of *Pi. pastoris* in 200 mL of BMMY for 72 h, with the successive addition of methanol every 24 h to increase the final concentration by 0.5% (v/v). Following incubation, *Pi. pastoris* cells were collected by centrifugation at 4,500 *g* for 30 min. The supernatant, containing the secreted protein of interest, was sterilised through a 0.22 µm filter (ReliaPrep, Ahlstrom, Helsinki, Finland) and adjusted to pH 8 through the addition of NaOH.

2.2.8.2 Protein purification

The culture filtrate with the secreted candidate protein (fused with a N-terminal His₆) was purified by immobilized metal ion affinity (IMAC) using Ni Sepharose™ 6 Fast Flow (GE Healthcare, Chicago, EUA) according to the manufacturer's protocol. Before loading, the column (Glass Econo-Column, BioRad) was packed with 5 mL of resin and was equilibrated by washing the resin with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4). The culture filtrate was added to the column and passed through it at 1 mL/min. The protein was eluted with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole, pH 7.4). The elution fractions were mixed, and elution buffer was added to a final volume of 50 mL to obtain enough volume to vacuum-infiltrate the shoots. A western blot was performed, as above, to determine the presence of the proteins in *Pi. pastoris* culture filtrate and after purification.

Protein concentrations in culture filtrates and purified solutions were determined by using a Pierce™ Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA), as per the manufacturer's protocol.

2.2.8.3 Pine infiltration with purified candidate proteins

The clonal rootless pine shoots were kept in LPch agar in glass jars, with each jar containing between 6-8 shoots. The shoots were removed from the jars and completely submerged in approx. 50 mL of each purified protein, or in elution buffer or bovine serum albumin (BSA) (Thermo Fisher Scientific) as negative controls. A 1% (w/v) solution of neutral red (NR) dye (Abcam, Cambridge, UK) in sterile dH₂O was used both as a negative control for the vacuum infiltration treatment and as an infiltration efficiency reference. Samples were exposed to vacuum in a glass chamber for 5 min. Shoots were then briefly rinsed in sterile dH₂O and then placed back in the glass jar. The experiment was conducted in a 22°C room with natural light. Photos were taken 7 days after infiltration, using a Nikon D7000 camera.

2.2.9 Western blotting

In order to determine if proteins of predicted sizes were present in samples, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting was conducted.

2.2.9.1 Preparation of plant material

N. benthamiana plants were grown as previously described and fully extended leaves, ideally leaves 2-5 (Ma et al., 2012), were completely infiltrated using several infiltration sites and a maximum of 1 mL of culture per leaf. Leaves were collected 48 h after infiltration and cut in half, with the mid-vein removed, before being frozen in liquid nitrogen and stored at - 80°C until required.

2.2.9.2 Total protein extraction

Frozen plants were ground into a fine powder in liquid nitrogen using a mortar and pestle. An equal volume of extraction buffer was added (GTEN buffer (10% (v/v) glycerol, 0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol (DTT), 0.1% (v/v) Tween20, one anti-protease tablet (cOmplete mini protease inhibitor cocktail tablets, Roche, Basel, Switzerland), and 2% (w/v) polyvinylpolypyrrolidone (PVPP)) and the samples were incubated at 4°C for 30 min, shaking at 60 rpm (Ecotron). Samples were

spun at 4°C, 5000 *g*, for 20 min and the supernatant was transferred to a fresh 1.5 mL microcentrifuge tube through miracloth (Merck, New Jersey, USA). The protein extract was mixed with 3 x SDS loading dye (30% (v/v) glycerol, 3% (w/v) SDS, 94 mM (v/v) Tris (pH 7.5), 0.05% (w/v) bromophenol blue, 24 mM (v/v) DTT, in sterile dH₂O) in a 2:1 ratio in preparation for gel loading.

2.2.9.3 SDS-PAGE

For protein separation, 12% separating and 5% stacking gels were used (12% gel: 40% (v/v) 29% bis-/1% tris-acrylamide mix (BioRad), 25% (v/v) 1.5 M Tris (pH 8.8), 1% (v/v) 10% SDS, 1% (v/v) 10% fresh ammonium persulfate (APS) and 0.04% (v/v) tetramethylethylenediamine (TEMED); 5% gel: 17% (v/v) 29% bis-/1% tris-acrylamide mix, 13% (v/v) 1 M Tris (pH 6.8), 1% (v/v) 10% SDS, 1% (v/v) 10%, APS and 0.04% TEMED). The pre-stained PAGE ladder and protein samples were added to the wells in 15 µL aliquots. A Mini-Protean II Electrophoresis Cell system (BioRad) was assembled according to the manufacturers' instructions. Gels were immersed in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (v/w) SDS, pH 8.3), and run at 30 V for the first 20 min and 100 V for the remaining time.

2.2.9.4 Western blotting

After electrophoresis, the gel was transferred to an immune-blot[®] polyvinylidene fluoride (PVDF) membrane (BioRad), previously activated in 100% (v/v) methanol. The membrane was placed between four sheets of Whatman 3M (pre-soaked in transfer buffer - 25 mM Tris, 190 mM glycine, 20% (v/v) methanol (pH 8.3)). This assembly with foam buffer pieces was placed in the blotting chamber containing transfer buffer, and the transfer was carried out overnight (16 h) at 30 V in a 4°C room. The membrane was then placed in a blocking solution (TBST - 20 mM Tris, 0.15 M NaCl, pH 7.6, 10% (v/v) Tween20) with 5% (v/w) skim milk) for 1 h at RT with 20 rpm shaking (Heidolph rotator, John Morris Scientific, Auckland, New Zealand). The blocking buffer was exchanged with anti-FLAG[®] M2 antibody produced in mouse (Sigma-Aldrich) in a 1:5000 dilution on blocking buffer. After a 1.5 h incubation with the primary antibody, the solution was decanted off and three washes were performed with the membrane submerged in TBST buffer with gentle shaking (20 rpm - Heidolph rotator) at RT for 10 min per wash. The

secondary antibody (chicken anti-mouse, Santa Cruz Biotechnology, Texas, USA) was diluted in blocking solution in a 1:20,000 ratio and added to the membrane where it was incubated for 1 h at RT 20 rpm (Heidolph rotator) with shaking, followed by three washes with TBST for 10 min each. The membrane was incubated with 0.5 mL of SuperSignal® West Dura Extended Duration substrate (Thermo Fisher Scientific) for five min. The membrane was visualised with an Azure Biosystems c600 Bioanalytical Imaging system (Azure Biosystems, California, USA).

2.2.10 Tobacco rattle virus-mediated virus induced gene silencing

The work described in this section and in **Section 2.2.11** was carried out at the Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China by Zhiyuan Chen and Yan Wang.

Fragments used to generate tobacco rattle virus (TRV)-mediated virus induced gene silencing (VIGS) constructs were amplified by PCR from cDNA or genomic DNA of *N. benthamiana* using PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Shiga, Japan) with the primers listed in **Appendix Table A.2**. The TRV-mediated VIGS system is composed of pTRV-RNA1, which encodes replication related proteins, and pTRV-RNA2, which harbours the viral coat protein, and the candidate fragment sequences that target silencing to the corresponding genes (Liu et al., 2002; MacFarlane, 1999). The purified fragments were cloned into the pTRV-RNA2 vector (Dong et al., 2007) using the ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China). The resulting vectors were verified by sequencing and individually transformed into *A. tumefaciens* strain. DsEcp20-3 was screened against around 100 TRV VIGS constructs which targeted different *N. benthamiana* leucine-rich repeat (LRR)-receptor like protein (RLP) and LRR-receptor like kinase (RLK)-encoding genes. Information on this library of silencing constructs can be found in Supplementary Data 1 from Wang et al. (2018). Primers for *T26*, *T26-2*, TRV:*BAK1* (Heese et al., 2007) and TRV:*SOBIR1* (Liebrand et al., 2013) are shown in **Appendix Table A.2**.

A. tumefaciens strains carrying *N. benthamiana* gene silencing vectors (Wang et al., 2018) were grown overnight in LB medium with the appropriate antibiotics. *A. tumefaciens* cells were pelleted as mentioned above, resuspended, and incubated in

infiltration medium (10 mM 2-[N-morpholino] ethanesulfonic acid pH 5.6, 10 mM MgCl₂, 200 µM acetosyringone) for 3–4 h before agroinfiltration. *A. tumefaciens* cultures expressing pTRV-RNA2 constructs were mixed in a 1:1 ratio with *A. tumefaciens* culture expressing pTRV1 to a final OD₆₀₀ of 1.0 before infiltration into cotyledons of four-leaf-stage *N. benthamiana* seedlings. *P. sojae* NPP1 and XEG1 (Fellbrich et al., 2002; Ma et al., 2015), and *P. infestans* INF1 were used as cell death-eliciting controls for VIGS. XEG1- and INF1-triggered cell death was known to be compromised in *N. benthamiana* leaves treated with TRV:*BAK1* or TRV:*SOBIR1*, but not with TRV:*GFP*, which was used as a negative control (Wang et al., 2018).

2.2.11 RNA isolation and quantitative reverse transcription PCR

To determine the expression levels of *BAK1*, *SOBIR1* and *T26* in the TRV-mediated VIGS experiment, *N. benthamiana* leaves of similar size were selected and ground to a powder in liquid nitrogen. Total RNA was isolated from 100 mg of ground material using the E.Z.N.A.[®] Total RNA Kit I (Omega Bio-tek) and used as a template for cDNA synthesis with PrimeScript Reverse Transcriptase (Takara Bio Inc.). Real time-quantitative PCR (RT-qPCR) was performed on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems Inc., California, USA) using PrimeScript RT Master Mix (Takara Bio Inc.) with translation elongation factor 1 alpha (*EF-1α*) as an endogenous control and the primers listed in **Appendix Table A.2**. Data were analysed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.3 Results

2.3.1 Two *Dothistroma septosporum* elicitors are effector candidates that form part of protein families with orthologues in *Fulvia fulva*

In previous work, two small, secreted candidate effector proteins from *D. septosporum* NZE10, Ds70694 and Ds70057, triggered cell death in the non-host species *N. tabacum* and *N. benthamiana* (Hunziker et al., 2021). Both proteins are homologues of Ecps from *F. fulva* and are part of two different protein families, Ecp20 and Ecp32, respectively, that were shown to be located in the apoplast of tomato leaves infected with *F. fulva* (Mesarich et al., 2018). More specifically, Ds70694 is an orthologue

of FfEcp20-3 from *F. fulva* and was therefore named DsEcp20-3, whilst Ds70057 is an orthologue of FfEcp32-3 and was therefore named DsEcp32-3.

The Ecp20 family has four paralogues in each species (**Appendix Figure A.4**). Based on sequence similarities between them, DsEcp20-3 and FfEcp20-3, together with the other orthologous pairs Ds/FfEcp20-1, Ds/FfEcp20-2 and Ds/FfEcp20-4, grouped together in a phylogeny (**Figure 2.1A** and **Figure 2.2A**). All proteins have predicted signal peptides and no known functional domains (**Figure 2.3A**). Interspecies pairwise amino acid identities between all family members from *D. septosporum* and *F. fulva* ranged from 10.6% to 85.5%, while intraspecies pairwise identities between all family members ranged from 14.2% to 40.9% in *D. septosporum* and 11.8% to 44.2% in *F. fulva* (**Appendix Table A.4**). The *Ds/FfEcp20-1* genes contain one exon, with the intron located at the same position, while genes *Ds/FfEcp20-2*, -3 and -4 do not have any introns (**Figure 2.3A**). All genes encode a protein with four cysteine residues, although the location of these varies slightly from protein pair to protein pair (**Figure 2.2A** and **Figure 2.3A**).

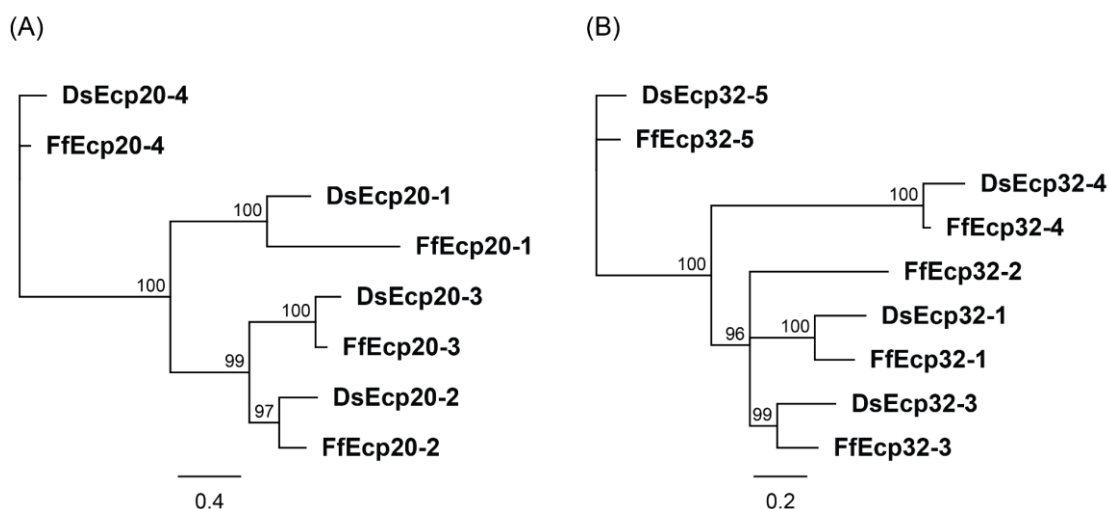


Figure 2.1. Protein phylogenies of the *Dothistroma septosporum* and *Fulvia fulva* Ecp20 **(A)** and Ecp32 **(B)** families. The trees were constructed with the neighbour-joining method using Geneious Software v9.1.8. Bootstrap values are shown at the nodes as percentages. The scale bar represents 0.4 **(A)** and 0.2 **(B)** substitutions per site. Ds: *D. septosporum*; Ff: *F. fulva*.

Based on data from a previously published transcriptome study (Bradshaw et al., 2016), all genes of the *D. septosporum* Ecp20 family are expressed during pine infection, with *DsEcp20-3* and *DsEcp20-4* being the two most highly expressed (**Table 2.1**). *DsEcp20-3* showed strong up-regulation in the Mid and Late *in planta* stages that mark

the start and end of necrotrophic infection, when compared with the Early (biotrophic) stage and *in vitro*, while *DsEcp20-4* is strongly up-regulated in the Late *in planta* stage. For the corresponding genes in *F. fulva* during tomato infection, as based on data from a previously published transcriptome study (Mesarich et al., 2018), *FfEcp20-2* and *FfEcp20-3* were highly expressed throughout the timepoints analysed, while *FfEcp20-4* was highly expressed at the beginning of infection and downregulated at later time points, when compared with *in vitro* (**Table 2.1**). It should be noted that *FfEcp20-1* had no gene model and therefore had no FPKM value assigned to it in the previous study (Mesarich et al., 2018). Thus, no expression information was available for this gene.

The Ds/FfEcp32 family was analysed in the same way as the Ds/FfEcp20 family above. The Ecp32 family has five paralogues in *F. fulva*, named FfEcp32-1 to -5, and four in *D. septosporum*, named DsEcp32-1 and DsEcp32-3 to -5 (there is no orthologue of FfEcp32-2 in *D. septosporum*) (**Figure 2.1B**, **Figure 2.2B** and **Appendix Figure A.4**). All these family members have signal peptides and no known functional domains (**Figure 2.3B**). Interspecies pairwise amino acid identity between all family members from *D. septosporum* and *F. fulva* ranged from 10.6% to 82%, while intraspecies pairwise identity between members ranged from 22.1% to 42.1% in *D. septosporum* and 21.6% to 49% in *F. fulva* (**Appendix Table A.4**). Regarding the exon-intron structure, *Ds/FfEcp32-1*, *FfEcp32-2* and *Ds/FfEcp32-3* do not have any introns. In contrast, the *Ds/FfEcp32-4* gene pair has one intron at the same position, while the *Ds/FfEcp32-5* gene pair has two introns at the same positions (**Figure 2.3B**). In terms of cysteine residues, the Ds/FfEcp32 proteins have anywhere from none (Ds/FfEcp32-5) to five (Ds/FfEcp32-4), although in the case of DsEcp32-3, an additional cysteine is present compared to the Ff orthologue (**Figure 2.2B** and **Figure 2.3B**).

All *DsEcp32* genes are also expressed during pine infection. However, only *DsEcp32-3* and *DsEcp32-5* are highly expressed *in planta*, especially at the Mid and Late necrotrophic stages when compared with *in vitro*. *DsEcp32-1* and *DsEcp32-4* are only expressed at a low level *in planta*. During *F. fulva* infection of tomato, *FfEcp32-1*, *FfEcp32-2* and *FfEcp32-3* are highly expressed at all timepoints. *FfEcp32-4* was not expressed under any condition, while *FfEcp32-5* was only expressed at 12 dpi, and was downregulated compared with *in vitro* (**Table 2.1**).

Genomes from the two species are mesosyntenic (de Wit et al., 2012; Zaccaron et al., 2022) and, in keeping with this, conservation of chromosome location between the two species for each of the *Ds/FfEcp20* and *Ds/FfEcp32* gene pairs was found (**Appendix Figure A.5**). Taken together, sequence similarity, intron position and chromosome localization suggest a common evolutionary origin for the *Ecp20* and *Ecp32* genes from both species.

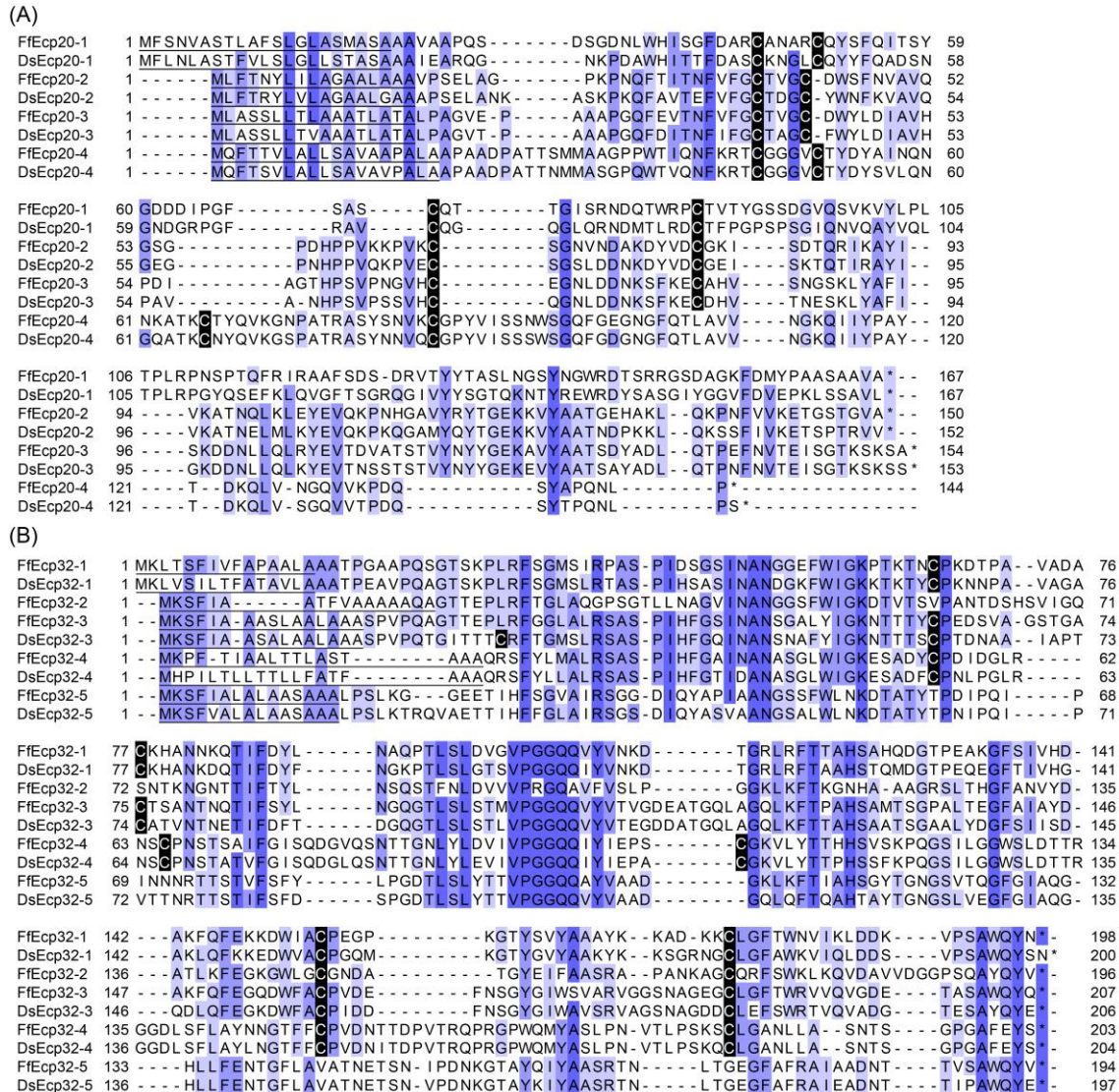
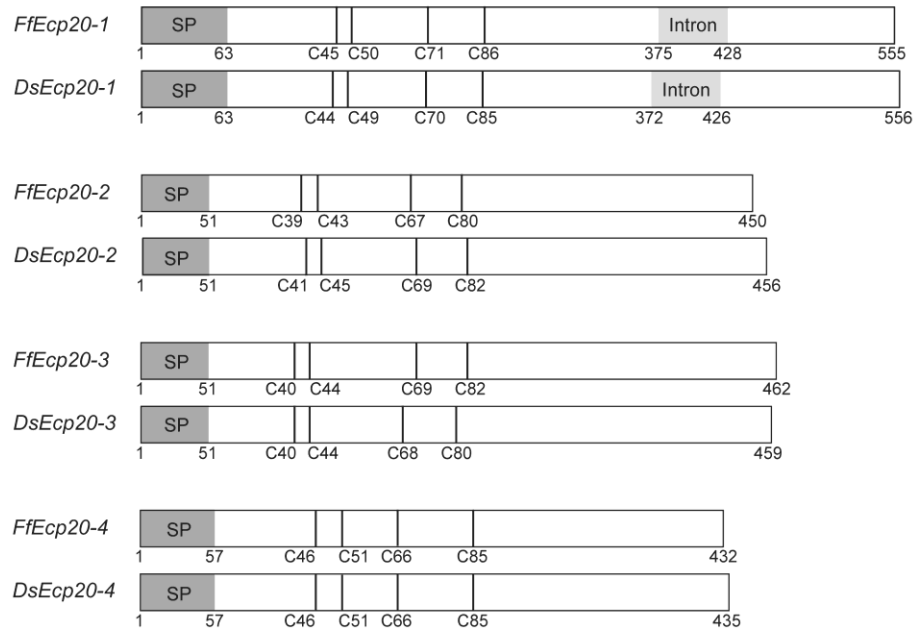


Figure 2.2. Protein alignment of *Dothistroma septosporum* and *Fulvia fulva* Ecp20 (A) and Ecp32 (B) family members. The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined. Ds: *D. septosporum*; Ff: *F. fulva*.

(A)



(B)

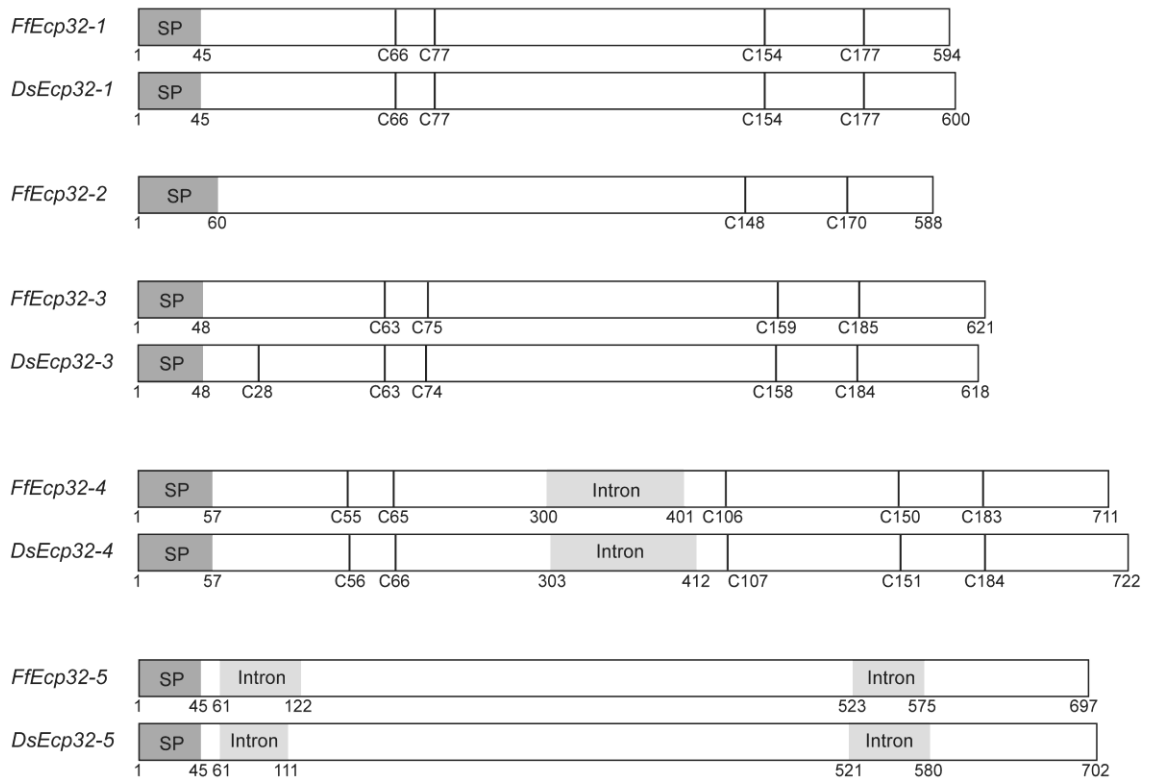


Figure 2.3. Schematic representation of *Ecp20* (A) and *Ecp32* (B) gene family members. Dark grey boxes indicate the signal peptide (SP) position and light grey boxes indicate introns. Vertical black lines indicate cysteine residues, with the respective amino acid position shown under each gene. Ds: *D. septosporum*; Ff: *F. fulva*.

Table 2.1. Features of *Dothistroma septosporum* and *Fulvia fulva* Ecp20 and Ecp32 proteins.

JGI ID ^a	Pathogen	Name	Ds expression ^b				Ff expression ^c				EffectorP prediction ^d	AlphaFold structural prediction (RCSB PDB accession ID) ^e
			FM	E	M	L	FM	4dpi	8dpi	12dpi		
189833	<i>F. fulva</i>	FfEcp20-1					*	*	*	*	Cytoplasmic	Root induced signalling protein – 7cwp (9.4)
196849	<i>F. fulva</i>	FfEcp20-2					114	1984	1827	1975	Dual	Effector protein PevD1 – 5xmz (9.1)
192674	<i>F. fulva</i>	FfEcp20-3					5	660	201	598	Apoplastic	Effector protein PevD1 – 5xmz (9.1)
191139	<i>F. fulva</i>	FfEcp20-4					485	1640	20	177	Apoplastic	Root induced effector protein TSP1 – 7cwj (20.1)
51311	<i>D. septosporum</i>	DsEcp20-1	19	62	53	57					Dual	Root induced effector protein TSP1 – 7cwj (9.3)
51158	<i>D. septosporum</i>	DsEcp20-2	10	17	57	84					Dual	Major allergen Alt a 1 subunit – 4aud (9.0)
70694	<i>D. septosporum</i>	DsEcp20-3	5	4.2	205	114					Apoplastic	Effector protein PevD1 – 5xmz (9.3)
43416	<i>D. septosporum</i>	DsEcp20-4	8	233	192	3732					Apoplastic	Root induced effector protein TSP1 – 7cwj (18.4)
190869	<i>F. fulva</i>	FfEcp32-1					97	547	1025	602	Dual	16.4 kDa salivary peptide – 7kc8 (9.8)
183790	<i>F. fulva</i>	FfEcp32-2					1	84	169	221	Apoplastic	Agglutinin – 3c9z (10.1)
189688	<i>F. fulva</i>	FfEcp32-3					2	84	8	452	Apoplastic	Trypsin inhibitor – 4j2k (9.5)
194822	<i>F. fulva</i>	FfEcp32-4					0	0	0	0	Apoplastic	Botulinum neurotoxin type A – 3v0a (9.4)
185982	<i>F. fulva</i>	FfEcp32-5					21	0	0	0.5	Apoplastic	Trypsin inhibitor – 4j2k (9.7)
75860	<i>D. septosporum</i>	DsEcp32-1	53	81	34	67					Dual	Trypsin inhibitor – 4j2k (9.7)
70057	<i>D. septosporum</i>	DsEcp32-3	310	467	2569	3312					Apoplastic	Trypsin inhibitor – 4j2k (9.1)
95543	<i>D. septosporum</i>	DsEcp32-4	9	3	4	10					Apoplastic	Botulinum neurotoxin type A – 5tpb (10.2)
174276	<i>D. septosporum</i>	DsEcp32-5	14	17	536	101					Apoplastic	Trypsin inhibitor – 4j2k (9.4)

^a Protein ID in the Joint Genome Institute (JGI) database. ^b *Dothistroma septosporum* (Ds) expression during infection stages of *Pinus radiata* (fungal mycelium (FM) (in culture), Early (E), Mid (M), Late (L) (*in planta*); Bradshaw et al. 2016) as Reads Per Million per Kilobase (RPMK). *Fulvia fulva* (Ff) expression during infections stages of *Solanum lycopersicum* (fungal mycelium FM (in culture), 4 days post infection (dpi), 8 dpi and 12 dpi; Mesarich et al. 2017) as Fragments Per Kilobase per Million (FPKM). * Gene was not detected in the RNA-seq experiment. ^d Protein sequences were queried at <https://github.com/JanaSperschneider/EffectorP-3.0>. Dual prediction: apoplastic/cytoplasmic prediction. ^e Structural predictions were made using AlphaFold (Jumper et al., 2021) and the predicted structure was queried against those in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using the protein structure comparison server, Dali (Holm, 2020). Top hit is shown, next to the PDB accession ID, and in parenthesis the Z-score (>2.0) is shown.

2.3.2 Predicted tertiary structures of Ecp20 and Ecp32 family members suggest possible roles in virulence

The tertiary structures of all Ecp20 family members from *D. septosporum* NZE10 and *F. fulva* 0WU were predicted using AlphaFold2 (**Figure 2.4** and **Appendix Figure A.6**). All proteins from the Ecp20 family are predicted to be structurally similar to the characterized β -barrel fold of Alt a 1 from *Alternaria alternata* (RCSB protein data bank [PDB] ID: 3V0R) (Chruszcz et al., 2012) (**Table 2.1** and **Appendix Table A.5**). **Figure 2.4** shows the predicted structures for DsEcp20-3 and DsEcp20-4, as well as the alignments between DsEcp20-3 and the characterized structure of Alt a 1 from *Al. alternata* and between the predicted structures of DsEcp20-3 and DsEcp20-4. The predicted structures for all the DsEcp20 and FfEcp20 proteins are shown in **Appendix Figure A.6** along with characterized crystal structures of similar proteins from other fungal species.

The tertiary structures of Ecp32 proteins were also predicted using AlphaFold2 (**Figure 2.5** and **Appendix Figure A.7**). All Ecp32 family members had significant hits with trypsin inhibitors and lectins; these are proteins that share a β -trefoil fold and are known virulence factors in plant-pathogenic fungi (Renko et al., 2012; Sabotič et al., 2019; Varrot et al., 2013) (**Table 2.1** and **Appendix Table A.5**). **Figure 2.5** shows the predicted structures for DsEcp32-3 and DsEcp32-4, as well as the alignments between DsEcp32-3 and the characterized structure of a trypsin inhibitor from the plant species *Enterolobium contortisiliquum* (RCSB PDB ID: 4J2K) (Zhou et al., 2013), which is the top hit in the RCSB PDB for most Ds/FfEcp32 proteins. The alignment between the predicted structures of DsEcp32-3 and DsEcp32-4 is also shown in **Figure 2.5**, while all the predicted Ds/FfEcp32 structures can be found in **Appendix Figure A.7** along with characterized structures of similar proteins.

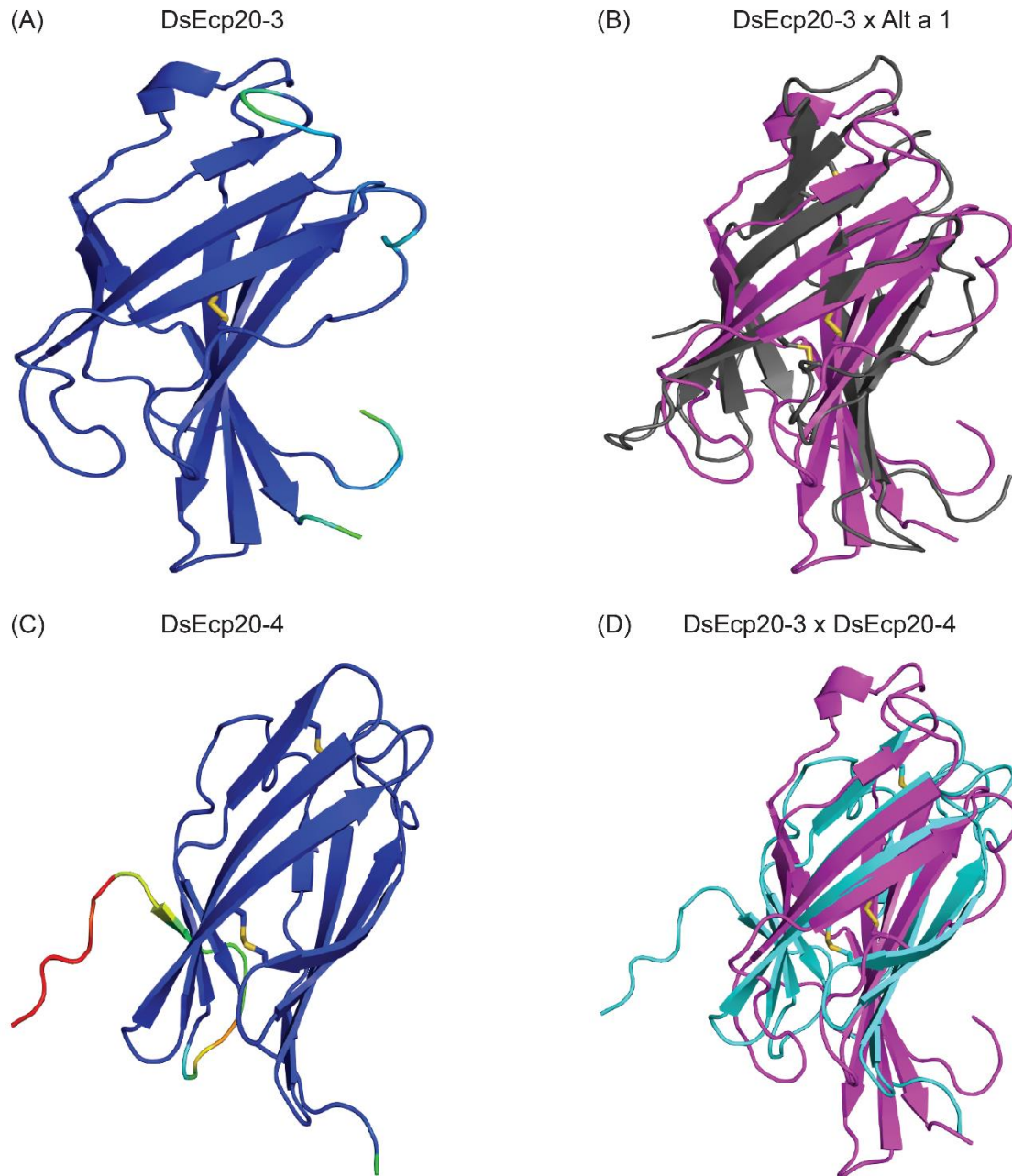


Figure 2.4. Predicted protein tertiary structures of Ecp20 family members from *Dothistroma septosporum*. **(A)** Predicted structure of DsEcp20-3. **(B)** Alignment of the predicted DsEcp20-3 structure (pink) with the characterized structure of Alt a 1 (black) from *Alternaria alternata* (RCSB protein data bank [PDB] ID: 3V0R) (Chruszcz et al., 2012). **(C)** Predicted structure of DsEcp20-4. **(D)** Alignment of predicted DsEcp20-3 (pink) and DsEcp20-4 (blue) structures. Disulphide bonds are shown as yellow sticks. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 and aligned using the CEalign tool (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022). Structures on the left were coloured in PyMOL according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Ds: *D. septosporum*.

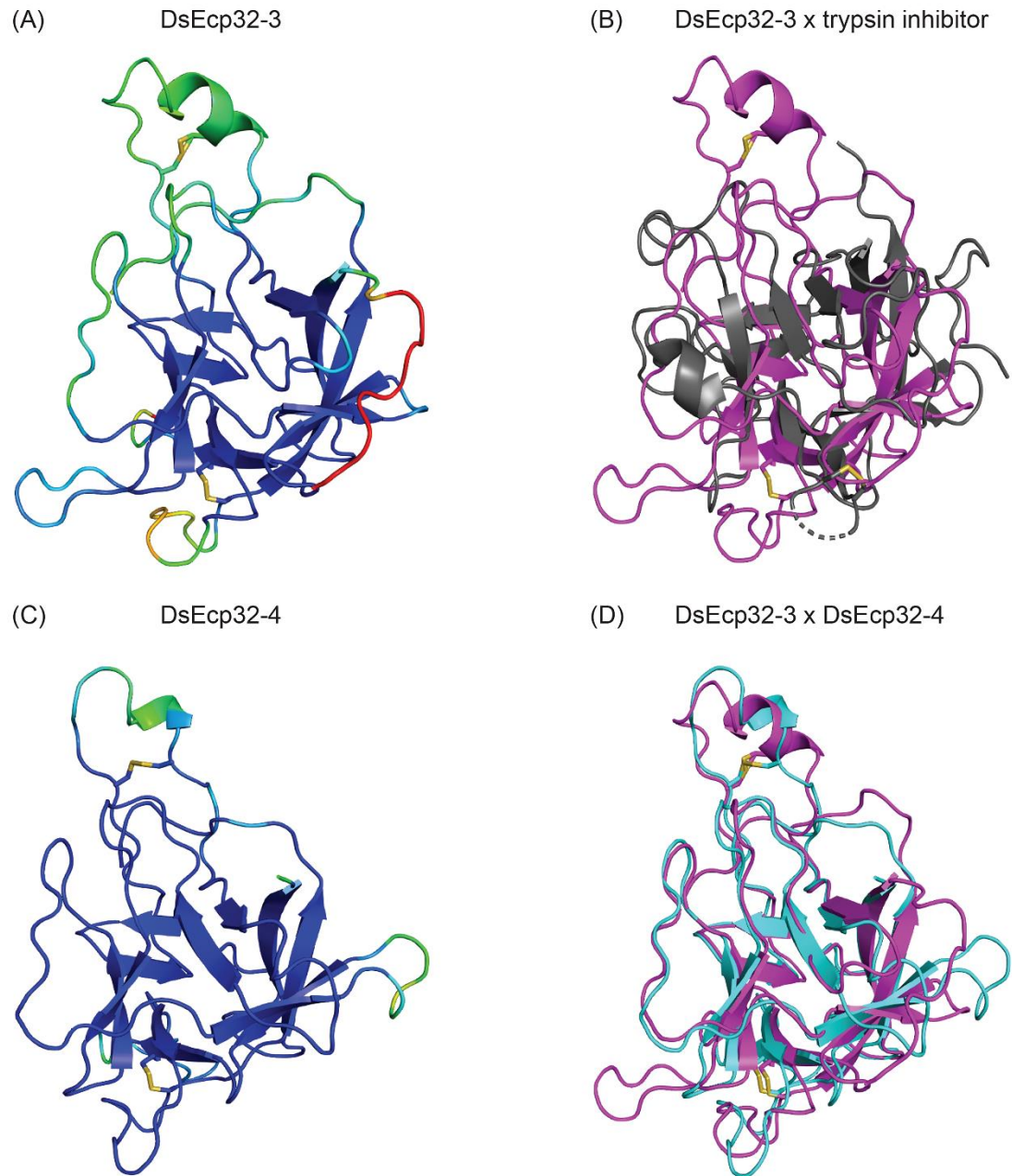


Figure 2.5. Predicted protein tertiary structures of Ecp32 family members from *Dothistroma septosporum*. **(A)** Predicted structure of DsEcp32-3. **(B)** Alignment of the predicted DsEcp32-3 (pink) structure with the characterized structure of trypsin inhibitor (black) from *Enterolobium contortisiliquum* (RCSB protein data bank [PDB] ID: 4J2K) (Zhou et al., 2013). **(C)** Predicted structure of DsEcp32-4. **(D)** Alignment of predicted DsEcp32-3 (pink) and DsEcp32-4 (blue) structures. Disulphide bonds are shown as yellow sticks. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 and aligned using the CEalign tool (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022). Structures on the left were colored in PyMOL according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Ds: *D. septosporum*.

2.3.3 Ecp20 and Ecp32 family members are conserved across other fungal species and *Dothistroma septosporum* isolates

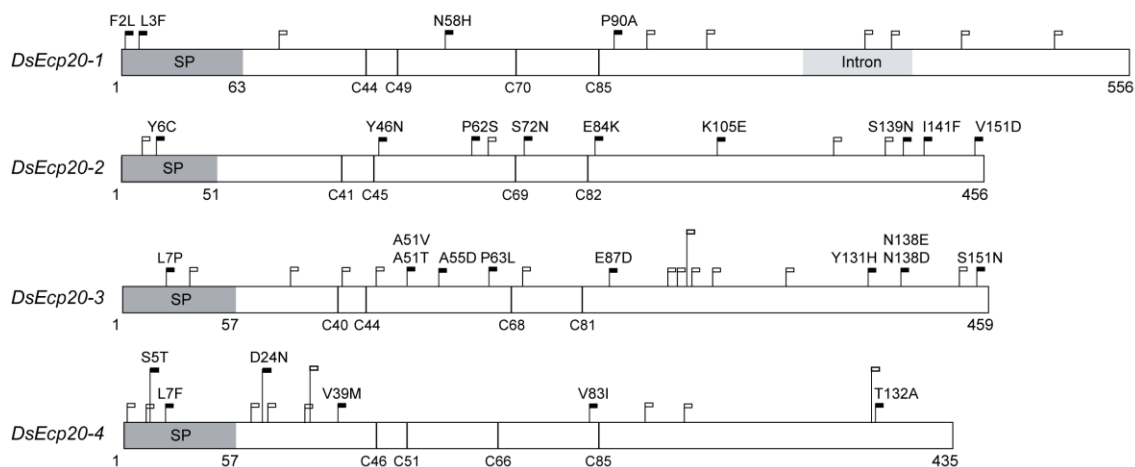
The Ecp20 family is present in fungal species from other classes. DsEcp20-1 had 27 significant BLASTp hits in the JGI MycoCosm protein database. Two of these were from other Dothideomycete species (including the orthologue in *F. fulva*), while the rest were with from species in the Eurotiomycetes class (**Appendix Table A.6**). DsEcp20-2 and DsEcp20-3, the most closely related to each other among the DsEcp20 family members (**Figure 2.1A**), both had the same 28 hits in JGI (**Appendix Table A.6**). All of these matches were with other Dothideomycete species and, of these, 24 were hits to proteins from other plant pathogens. DsEcp20-4 had 1,006 hits in the JGI MycoCosm protein database, of which 241 were proteins from plant pathogens (**Appendix Table A.6**).

In general, the Ecp32 family is more widely conserved among other fungal species compared to the Ecp20 family. Individual searches in the JGI MycoCosm protein database for each of the DsEcp32 family members yielded approximately 1,000 hits for each family member (**Appendix Table A.6**). Those hits included proteins from fungal species with different lifestyles, ranging from saprophytes, to biotrophs, hemibiotrophs and necrotrophs. For example, DsEcp32-3 had 158 hits to proteins from fungal plant pathogens in JGI. Of these, 42 were with other members from the Mycosphaerellaceae family. These included *Cercospora zea-maydis* with four paralogs, *Pseudocercospora* (*Mycosphaerella*) *eumusae* with six, *Pseudocercospora fijiensis* with five and *Zymoseptoria tritici* with three paralogs (**Appendix Table A.6**).

Dothistroma pini is also a causal agent of DNB (Barnes et al., 2004). Thus, the *D. pini* genome was searched for genes encoding orthologues of DsEcp20/32. All proteins from the *D. septosporum* Ecp20 and Ecp32 families had orthologues in *D. pini*, except Ecp32-5, for which the gene encoding this protein appears to be a pseudogene (**Appendix Figure A.4**). The protein sequences were very similar between family members from the two *Dothistroma* species, with full-length pairwise amino acid identity varying from 81.1% to 93.8% (**Appendix Table A.4**).

Next, it was determined whether the *D. septosporum* *Ecp20* and *Ecp32* family genes are conserved in other isolates of this species and whether they are identical in sequence to NZE10. Genome sequences were available from 18 *D. septosporum* isolates collected from different geographic locations (Bradshaw et al., 2019). All members of the *D. septosporum* *Ecp20* and *Ecp32* families were present in the 18 other genomes. In each of these genomes, there was at least one single nucleotide polymorphism (SNP) leading to an amino acid difference in at least one family member for both *Ecp20* and *Ecp32* (**Figure 2.6** and **Appendix Table A.7**).

(A)



(B)

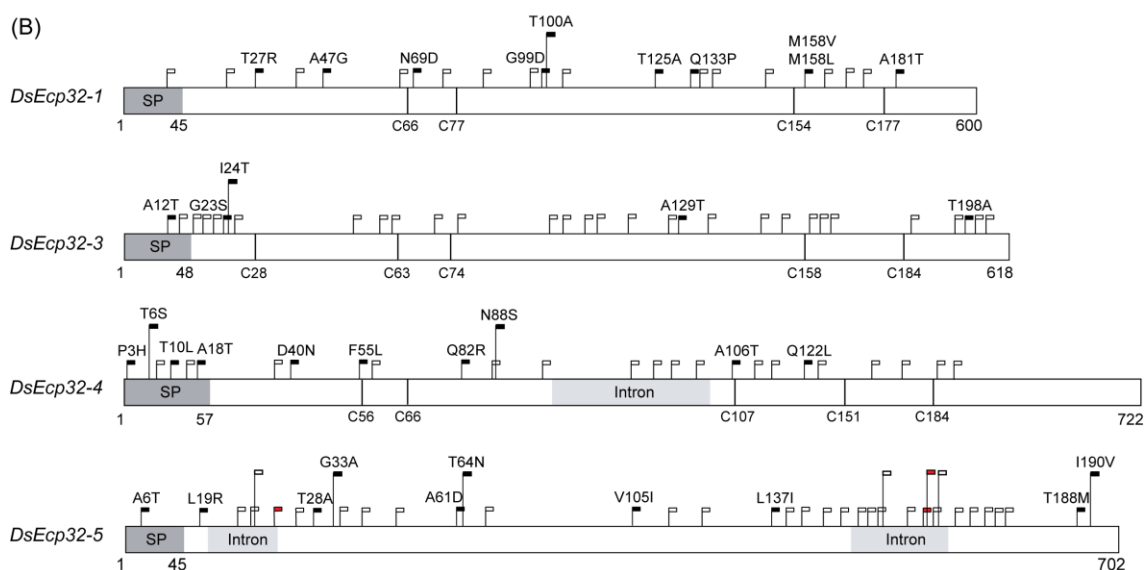


Figure 2.6. Schematic diagram showing sequence variation in *Dothistroma septosporum* NZE10 *Ecp20* (A) and *Ecp32* (B) family members across 18 other *D. septosporum* isolates. Dark grey boxes indicate signal peptide positions and light grey boxes indicate intron positions. Black flags show the position of nucleotide variations that led to non-synonymous changes, with the specific amino acid differences indicated above the flags. White flags show the position of synonymous nucleotide variations. Red flags show the position of nucleotide insertions. Vertical black lines show the positions of cysteine residues. Nucleotide positions are shown under each gene. SP: signal peptide. Ds: *D. septosporum*.

A total of 59 different SNPs were identified among the *Ecp20* family members across the 18 *D. septosporum* isolates, compared to NZE10 (Figure 2.6). *DsEcp20-1*, *DsEcp20-2* and *DsEcp20-4* had approximately the same number of SNPs: 11, 13 and 15, respectively, while *DsEcp20-3* had a total of 20. For *DsEcp20-1*, *DsEcp20-3* and *DsEcp20-4*,

however, most SNPs (61%, 60% and 60%, respectively) were synonymous. In the case of *DsEcp20-2*, however, only 30% were synonymous and a higher number of SNPs leading to an amino acid difference (9 out of 13; 70%) were observed (**Figure 2.6A**). The *Ecp32* gene family had a higher number of DNA modifications across the 18 *D. septosporum* genomes analysed, with a total of 117 SNPs and 3 insertions. The two most polymorphic genes were *DsEcp32-3* and *DsEcp32-5*. Interestingly, while *DsEcp32-3* had 31 sequence modifications, only five of these led to an amino acid difference (16%). All other family members had 9 or 10 SNPs leading to an amino acid difference (**Figure 2.6B**).

The Guatemalan (GUA1 and GUA2) and Colombian (COLN and COLS) isolates showed the greatest amino acid sequence diversity, when compared to strain NZE10, for both the *Ecp20* and *Ecp32* family members. For *DsEcp20-1*, the most diverse isolates were COLN and COLS, with identical sequences, while COLN had the most sequence variation in *DsEcp32-1*. For *DsEcp20-2* and -4, and *Ecp32-3*, -4, and -5, both Guatemalan isolates were the most diverse and, for *DsEcp20-3*, GUA2 had greater amino acid diversity (**Appendix Table A.7**).

2.3.4 Other members of the *Ecp20* and *Ecp32* families from *Dothistroma septosporum* and *Fulvia fulva* trigger cell death responses in non-host plants

In a previous study, *DsEcp20-3* and *DsEcp32-3* were shown to elicit cell death in the non-host plants *N. benthamiana* and *N. tabacum* (Hunziker et al., 2021); these two model plant species have been used in other studies of plant-pathogen interactions and their responses to the same protein sometimes vary (Guo et al., 2020b; Hunziker et al., 2021). Thus, it was determined whether other family members of both *D. septosporum* and *F. fulva* could elicit the same response in these hosts. To achieve this, all *Ecp20* and *Ecp32* family members were transiently expressed in *N. benthamiana* and *N. tabacum* using ATTAs, and the percentages of plant responses in three different categories recorded: strong cell death, weak cell death and no cell death (**Figure 2.7** for *N. benthamiana* and **Figure 2.8** for *N. tabacum*). Here, orthologous pairs from both pathogens were tested on the same leaf, so that differences between them could be better assessed.

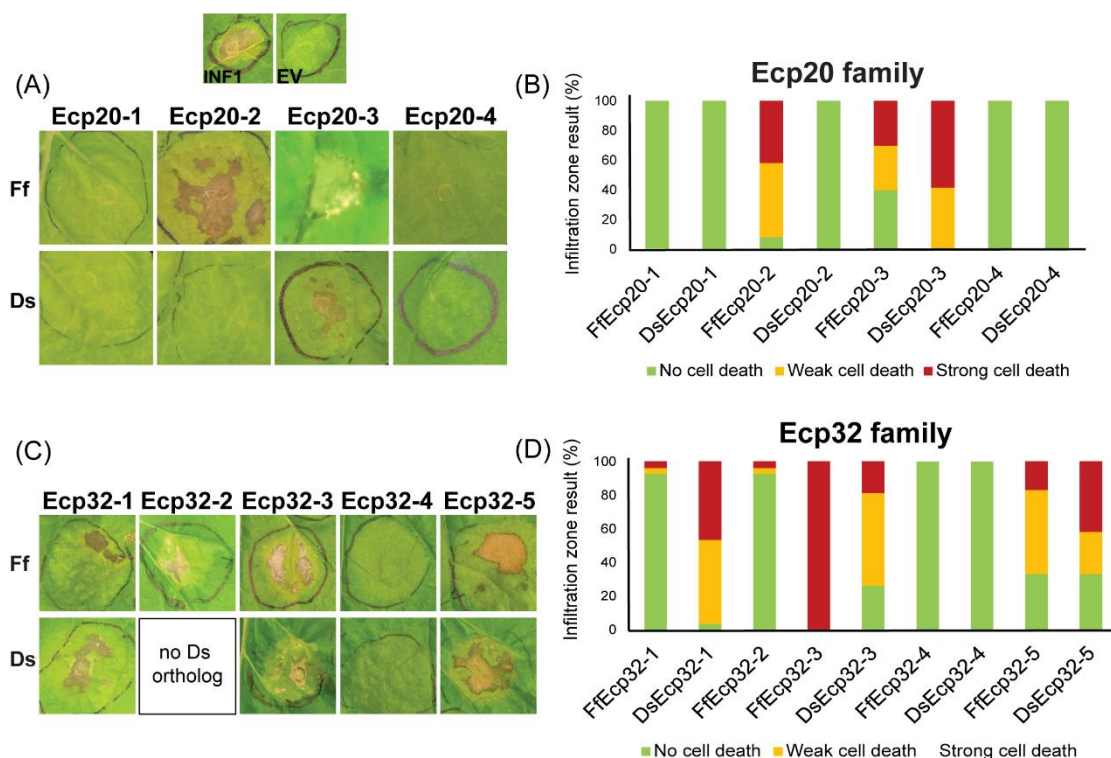


Figure 2.7. Ecp20 and Ecp32 proteins from *Dothistroma septosporum* NZE10 and *Fulvia fulva* 0WU trigger cell death responses in the non-host plant *Nicotiana benthamiana*. Ecp20 **(A)** and Ecp32 **(C)** family members from *D. septosporum* and *F. fulva* were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no cell death control; Ds: *D. septosporum*; Ff: *F. fulva*. Graphs display the percentages of infiltration zones that showed cell death in response to Ecp20 **(B)** and Ecp32 **(D)** family members from *F. fulva* and *D. septosporum*, divided into three categories: strong cell death, weak cell death and no cell death. Photos were taken 7 days after infiltration.

As expected, the positive control INF1 triggered strong cell death responses and the negative control empty vector did not elicit a response. Among Ecp20 family members, FfEcp20-2 triggered cell death responses in *N. benthamiana* in 91.7% of infiltration zones, whilst its orthologue DsEcp20-2 did not elicit any cell death responses. Both FfEcp20-3 and DsEcp20-3 caused consistent plant cell death in *N. benthamiana*, with DsEcp20-3 having 58% of infiltration zones showing strong cell death across the entire infiltration zone (**Figure 2.7A** and **2.7B**). In *N. tabacum*, DsEcp20-3 was the only family member that triggered a cell death response (**Figure 2.8A** and **2.8B**). Interestingly, DsEcp20-4, one of the most highly expressed secreted proteins during infection of pine

by *D. septosporum* (Table 2.1), and its orthologue from *F. fulva*, FfEcp20-4, did not induce cell death responses in either *N. benthamiana* or *N. tabacum* (Figure 2.7 and Figure 2.8).

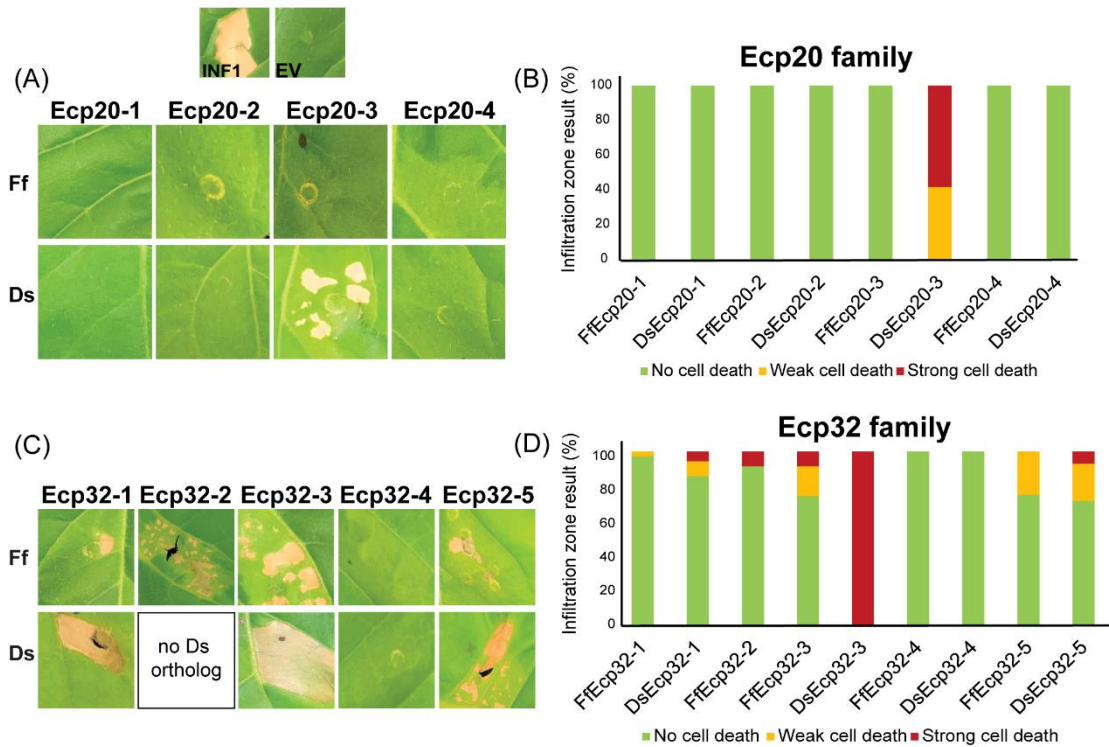


Figure 2.8. Ecp20 and Ecp32 proteins from *Dothistroma septosporum* NZE10 and *Fulvia fulva* OWU trigger cell death responses in the non-host plant *Nicotiana tabacum*. Ecp20 (A) and Ecp32 (C) family members from *D. septosporum* and *F. fulva* were expressed in *N. tabacum* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no cell death control; Ds: *D. septosporum*; Ff: *F. fulva*. Graphs display the percentage of infiltration zones that showed cell death in response to Ecp20 (B) and Ecp32 (D) family members from *F. fulva* and *D. septosporum*, divided into three categories: strong cell death, weak cell death and no cell death. Photos were taken 7 days after infiltration.

The ability of Ecp32 family members to trigger cell death was also assessed in both *Nicotiana* species. In this case, all proteins from the Ecp32 family, apart from the Ds/FfEcp32-4 protein pair, induced some degree of cell death in both plant species (Figure 2.7 and Figure 2.8). Interestingly, DsEcp32-1 consistently triggered cell death in *N. benthamiana*, while its orthologue from *F. fulva*, FfEcp32-1, only caused a strong cell death response in 3.5% of infiltration zones across all replicates (Figure 2.7C and 2.7D).

In contrast, FfEcp32-3 was capable of triggering consistent strong cell death responses, while DsEcp32-3 only triggered a strong response in approximately 20% of infiltration zones. The Ds/FfEcp32-5 pair triggered similar degrees of cell death responses in *N. benthamiana* (**Figure 2.7D**). In *N. tabacum* the pair Ds/FfEcp32-4 did not trigger cell death responses, while the other proteins triggered different levels of responses, with DsEcp32-3 triggering consistent strong cell death responses (**Figure 2.8C and D**).

To determine whether apoplastic localization is required for members of the Ecp20 and Ecp32 families to trigger cell death, proteins that previously elicited a plant response, but now lacked the PR1 α signal peptide for secretion to the apoplast, were transiently expressed in *N. benthamiana*. Without the signal peptide, none of proteins tested were able to cause cell death (**Figure 2.9** and **Appendix Figure A.8**).

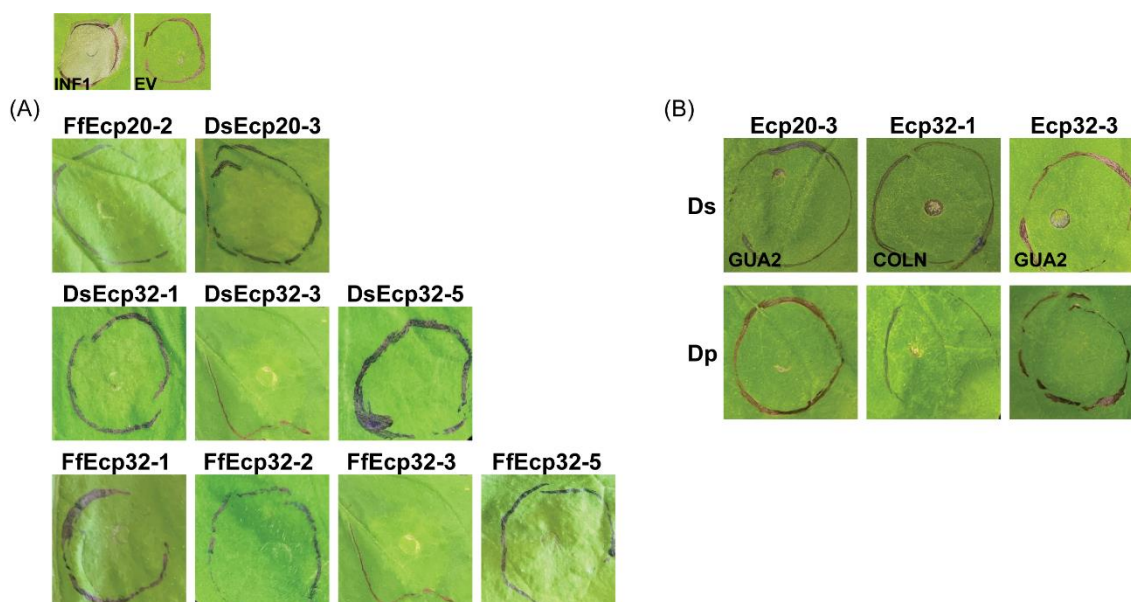


Figure 2.9. Apoplastic localization is required for cell death elicitation by members of the Ecp20 and Ecp32 protein families from *Dothistroma septosporum* and *Fulvia fulva* in *Nicotiana benthamiana*. Ecp20 and Ecp32 proteins from *D. septosporum* NZE10 and *F. fulva* 0WU (**A**), and from other *D. septosporum* strains and *Dothistroma pini* (**B**), were expressed without a signal peptide in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no cell death control; Ds: *D. septosporum*; Ff: *F. fulva*; Dp: *D. pini*; GUA2: Guatemalan isolate; COLN: Colombian isolate. Photos were taken 7 days after infiltration.

In cases where a cell death response was not observed at all, or where only weak cell death was observed, a Western blot analysis was performed to confirm the presence of the proteins in the tissue of the infiltrated plants (**Appendix Figure A.8**). All proteins that did not trigger cell death or only triggered a weak cell death response were detected via Western blot, indicating that the lack of cell death response was not due to absence of those proteins.

Taken together, these results indicate that Ecp20 and Ecp32 family members from two closely related fungal pathogens, *D. septosporum* and *F. fulva*, differ in their ability to induce a cell death response in non-host species. Furthermore, the finding that proteins without a signal peptide failed to trigger a plant cell death response, suggests that secretion into the apoplastic environment of *N. benthamiana* is essential for their cell death-eliciting activity.

2.3.5 Proteins from other *Dothistroma septosporum* isolates and *Dothistroma pini* also trigger cell death in non-host plants

Alleles of some Ecp20 and Ecp32 family genes identified from *D. pini*, and from isolates of *D. septosporum* other than NZE10, were tested for the ability of their encoded proteins to trigger cell death in *N. benthamiana* (**Appendix Figure A.4**). For the proteins from *D. septosporum* NZE10 that consistently triggered the highest percentages of cell death (DsEcp20-3, DsEcp32-1 and DsEcp32-3), orthologues of other *D. septosporum* alleles were tested and as a result, it was found they triggered the same responses as those of *D. septosporum* NZE10 (**Figure 2.10**). The same was true for the respective orthologues of DpEcp20-3, DpEcp32-1 and DpEcp32-3 from *D. pini*, which also elicited cell death responses in *N. benthamiana*. These results suggest conservation of cell death-eliciting activity between isolates of *D. septosporum* and between the two *Dothistroma* species for members of the Ecp20 and Ecp32 families.

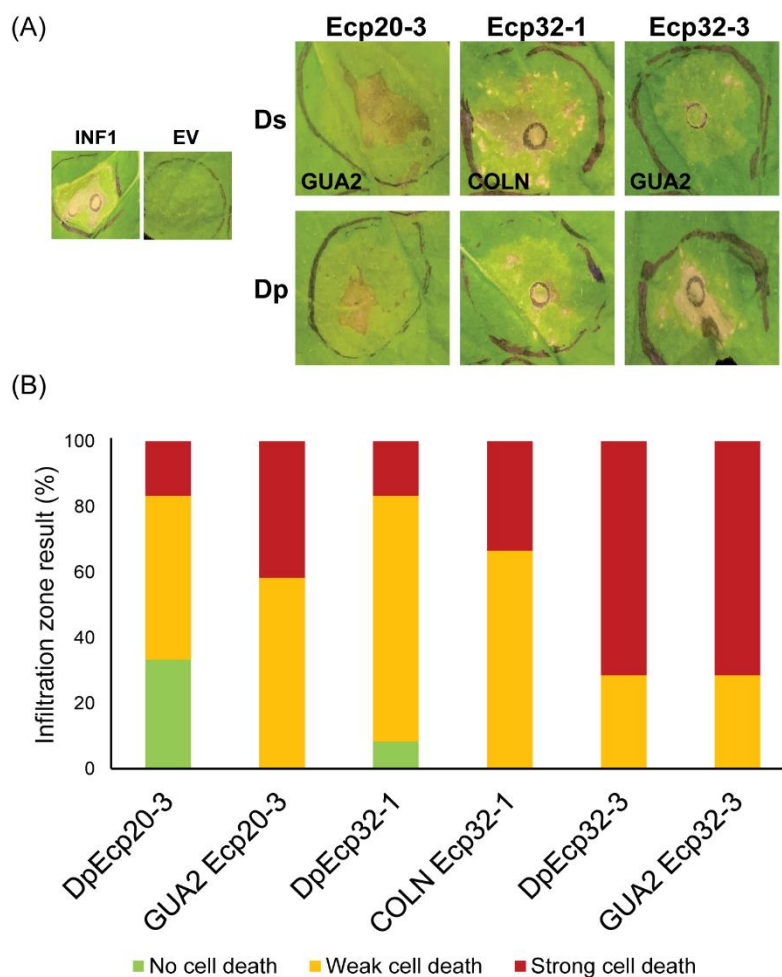


Figure 2.10. Ecp20 and Ecp32 proteins from other *Dothistroma septosporum* isolates and *Dothistroma pini* trigger cell death in non-host plant *Nicotiana benthamiana*. **(A)** DsEcp20-3 and DsEcp32-3 from the Guatemalan *D. septosporum* isolate (GUA2), DsEcp32-1 from the Colombian *D. septosporum* isolate (COLN), together with their orthologues from *D. pini*, were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Ds: *D. septosporum*; Dp: *D. pini*. **(B)** Graphical display of the percentage of infiltration zones that showed cell death in response to the respective Ecp20 and Ecp32 family members tested from *D. pini* and the most polymorphic *D. septosporum* isolates, divided into three categories: strong cell death, weak cell death and no cell death. Photos were taken 7 days after infiltration.

2.3.6 Infiltration of purified Ecp20 and Ecp32 proteins from *Dothistroma septosporum* into *Pinus radiata* triggers cell death

Transient expression of Ecp20 and Ecp32 candidate effector proteins from *D. septosporum* lead to the identification of cell death elicitors in non-host plants *N. benthamiana* and *N. tabacum*. Following that, some of these proteins were investigated to determine whether they could also induce a cell death response in pine. Preliminary results (Hunziker, 2018) combined the use of pine tissue culture plants and vacuum infiltration as a system for delivering and screening pathogen proteins for host cell death responses. In those first trials, neutral red (NR) dye was used both as a negative control for the vacuum infiltration treatment and as an infiltration efficiency reference, demonstrating efficient infiltration and showing that the liquid did not damage the shoots. Next, a *Botrytis cinerea* protein, BcSSP, that was previously identified as strong cell death inducer in *N. benthamiana* and *Camellia lutchuensis* (Denton-Giles, 2014), was heterologously expressed in *Pi. pastoris*, enabling easy generation of sufficient volumes of culture filtrate for pine infiltration. BcSSP appeared to cause a cell death-like response in pine, and for this reason, it was chosen as positive control for further experiments in this present study, along with DsEcp32-3. This *D. septosporum* protein was chosen due to its consistent strong cell death response in non-host plants (**Figure 2.7** and **Figure 2.8**).

2.3.6.1 Optimization of protein expression conditions in *Pichia pastoris* and delivery trials in *Pinus radiata*

The *Pi. pastoris* system is widely used for heterologous protein expression. It has the advantages of being a simple technique that generates high yields of proteins, and for these reasons was chosen as the method to generate the candidate effector proteins. However, *Pi. pastoris* transformants can show significant clonal variation (Zheng et al., 2014). For this reason, *Pi. pastoris* clones for each construct coding for the candidate effector protein of interest were screened to test their capacity for protein expression and to determine the best timing for protein production.

For the first experiment, the pPic9-His₆ vectors expressing BcSSP and DsEcp32-3, along with the empty pPic9-His₆ vector, were transformed into *Pi. pastoris*. After PCR

screening of transformants, the positive single colonies that grew on the selective MD plates were selected for further analysis. In order to do that, the candidate proteins were produced in *Pi. pastoris* for 24, 48 and 72 h. Western blotting analysis was performed to determine the presence of the protein in samples harvested at each of the different time points (**Appendix Figure A.9**). For BcSSP, all selected clones expressed the protein in at least one of the time points analysed, while for Ds70057 (DsEcp32-3), 72 h appeared to be the optimal time for production for both of the clones tested (**Appendix Figure A.9**).

Pi. pastoris culture filtrates with the secreted BcSSP and DsEcp32-3 proteins, along with the empty pPic9-His₆ vector, NR dye, and dH₂O, were then vacuum infiltrated into *Pn. radiata* shoots. For this experiment, one genotype (S6) from a family rated as relatively susceptible to *D. septosporum* (based on field data) was used. The NR control demonstrated good infiltration efficiency, as could be observed by the dark red colouration of most parts of the pine shoot (**Figure 2.11**). The NR treatment, together with the dH₂O control showed that the infiltration process did not damage the shoots. The empty vector gave a considerable response, as indicated by the cell death-like appearance of the shoots, and for this reason could not be used for a conclusion regarding the effects of either BcSSP or DsEcp32-3 proteins. (**Figure 2.11**). This suggested *Pi. pastoris* was secreting something else into the culture filtrate that was being recognized by the pine immune system (e.g., a MAMP). So, the next step was to purify the *Pi. pastoris* produced protein, using the histidine-tag (His₆) fused to the N-terminus of the proteins to better assess the responses of the pine shoots to BcSSP and DsEcp32-3.



Figure 2.11. Vacuum infiltration of *Pinus radiata* shoots. Whole shoots of *Pn. radiata* derived from a family seedlots that are relatively susceptible to *Dothistroma septosporum* were infiltrated with BcSSP and DsEcp32-3 proteins from *Pichia pastoris* culture filtrates. Distilled water, neutral red dye, and empty vector pPic9-His₆ were used as negative controls. Representative photos (from 6 to 8 pine shoots for each treatment) were taken 7 days after infiltration. Ds: *D. septosporum*; Bc: *Botrytis cinerea*.

2.3.6.2 Purified candidate effector proteins induce cell death in *Pinus radiata*

In the first pine infiltration experiment, culture filtrates from *Pi. pastoris* transformed with an empty vector (negative control) elicited cell death in the *Pn. radiata* genotype used. Thus, to avoid this problematic background response, candidate proteins were purified through a histidine-tag in the expression vector using IMAC prior to infiltration in pine (**Figure 2.12**). For this experiment two other *Pn. radiata* genotypes were included: S11 and R4, which are from seedlots of families relatively susceptible (S11), and relatively tolerant (R4), respectively, to *D. septosporum* infection (based on field data). Two negative controls were used: protein purification elution buffer (the same as the buffer that the candidate proteins were eluted in) and BSA protein.

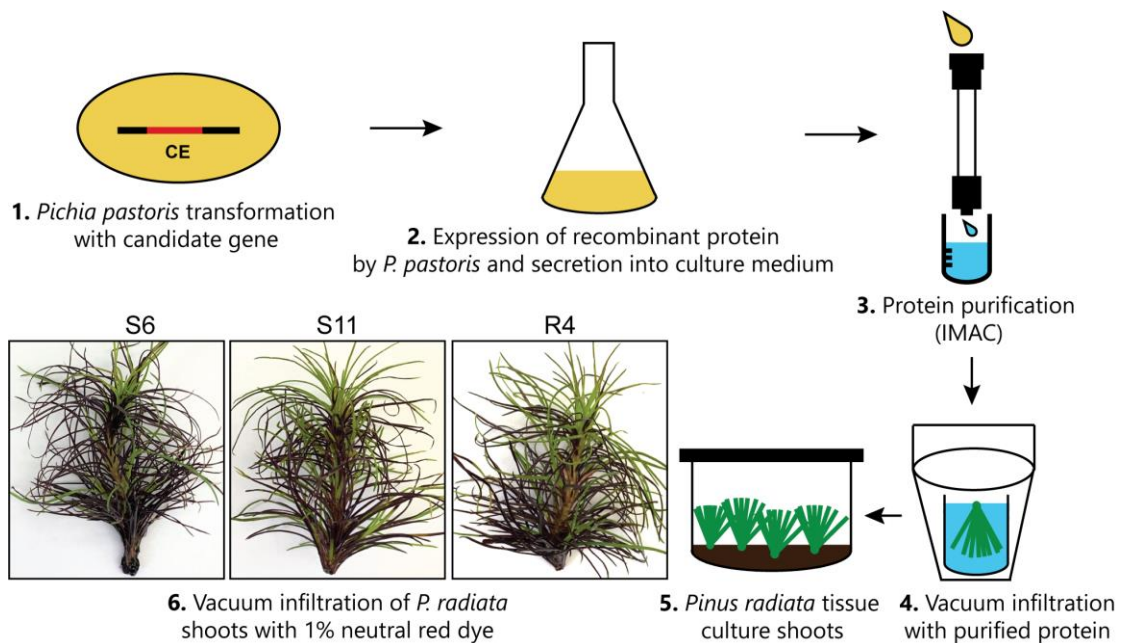


Figure 2.12. Overview of the developed pine shoot vacuum infiltration method. *Dothistroma septosporum* candidate effector DsEcp32-3 and *Botrytis cinerea* effector BcSSP2 proteins were produced through heterologous expression and secretion by *Pichia pastoris* (**1**, **2**). The proteins were purified by immobilised metal affinity chromatography (IMAC) (**3**) then vacuum-infiltrated into clonal shoots of *Pinus radiata* (**4**). Infiltrated shoots were returned to LPch medium for up to 7 days after infiltration (**5**). 1% neutral red was infiltrated to assess the efficiency of vacuum infiltration (**6**) in the different pine genotypes S6, S11 and R4. CE: candidate effector. Figure reproduced from Hunziker et al. (2021).

Infiltration of the negative controls (protein purification elution buffer or BSA protein) did not cause any damage or visible stress responses, as expected, even with

higher concentrations of BSA (**Figure 2.13**). In contrast, *Pn. radiata* shoots infiltrated with purified protein of DsEcp32-3 and BcSSP consistently showed cell death within 7 days across all three *Pn. radiata* genotypes tested (S6, S11 and R4) (**Figure 2.13** and **Appendix Figure A.10**), with these responses near-identical between both proteins. These results suggest that this novel method of effector delivery to gymnosperm tissue is a well suited medium-scale screening of multiple pine genotypes with candidate effector proteins.

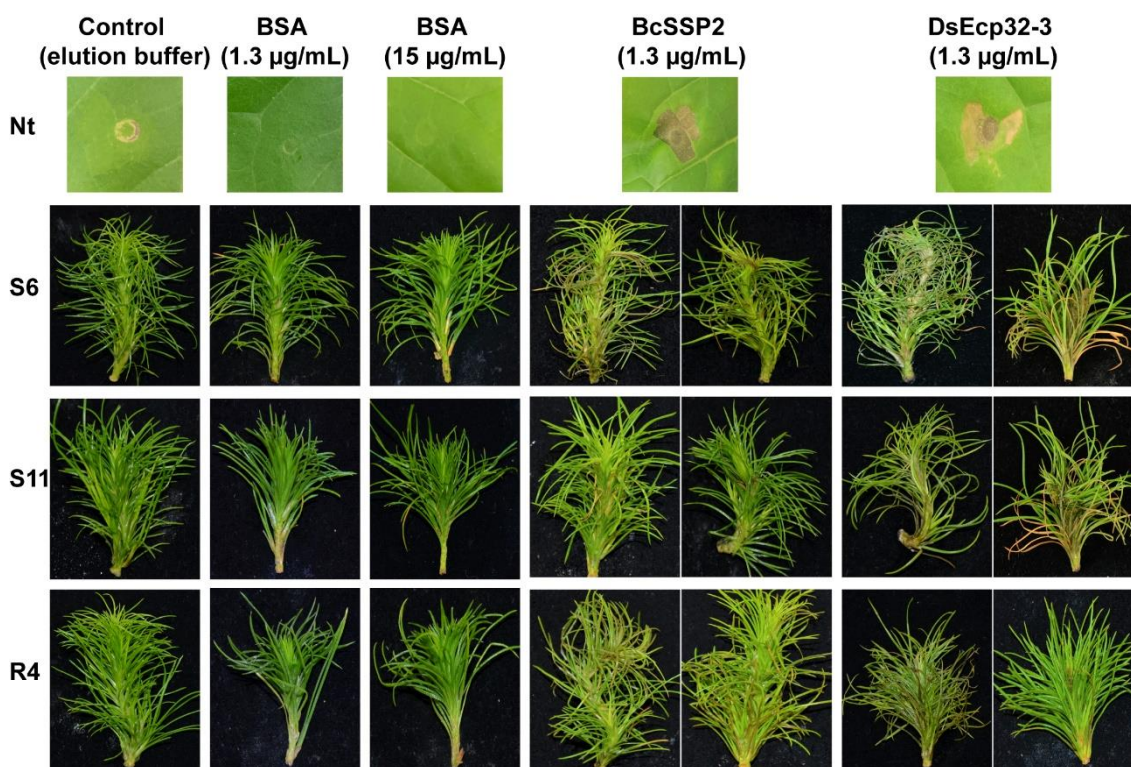


Figure 2.13. The *Dothistroma septosporum* candidate effector DsEcp32-3 induces cell death in *Pinus radiata* shoots following vacuum infiltration. Whole shoots of *Pn. radiata* genotypes that are from seedlots of families relatively susceptible (S6 and S11) or relatively tolerant (R4) to *D. septosporum* infection were infiltrated with purified *Botrytis cinerea* SSP2 (positive control) or DsEcp32-3 protein produced by heterologous expression in *Pichia pastoris*. Negative controls were elution buffer only and solutions of bovine serum albumin (BSA). Representative photographs (from 18 to 24 pine shoots for each treatment) were taken 7 days after infiltration, with further images shown in **Appendix Figure A.10**. The top panel shows infiltrations of the respective solutions into *Nicotiana tabacum* (Nt) for reference. Ds: *D. septosporum*; Bc: *B. cinerea*. Figure reproduced from Hunziker et al. (2021).

2.3.6.3 Ecp20 proteins from *Dothistroma septosporum* differ in their cell death eliciting ability in host *Pinus radiata*

The pine infiltration method described previously proved to be a suitable approach for testing the cell death-eliciting activity of candidate apoplastic effector proteins from pine pathogens in *Pn. radiata*. Using this same method, two additional proteins from the DsEcp20 family, DsEcp20-3 and DsEcp20-4 were tested, and the infiltration of DsEcp32-3 as a positive cell death control was repeated (**Figure 2.14** and **Appendix Figure A.11**). DsEcp20-3 and DsEcp20-4 were chosen due to their high expression in the Mid and Late (necrotrophic) stages of *D. septosporum* infection in *Pn. radiata* (**Table 2.1**), and the fact that DsEcp20-3 triggered cell death responses in *Nicotiana* species and DsEcp20-4 did not.

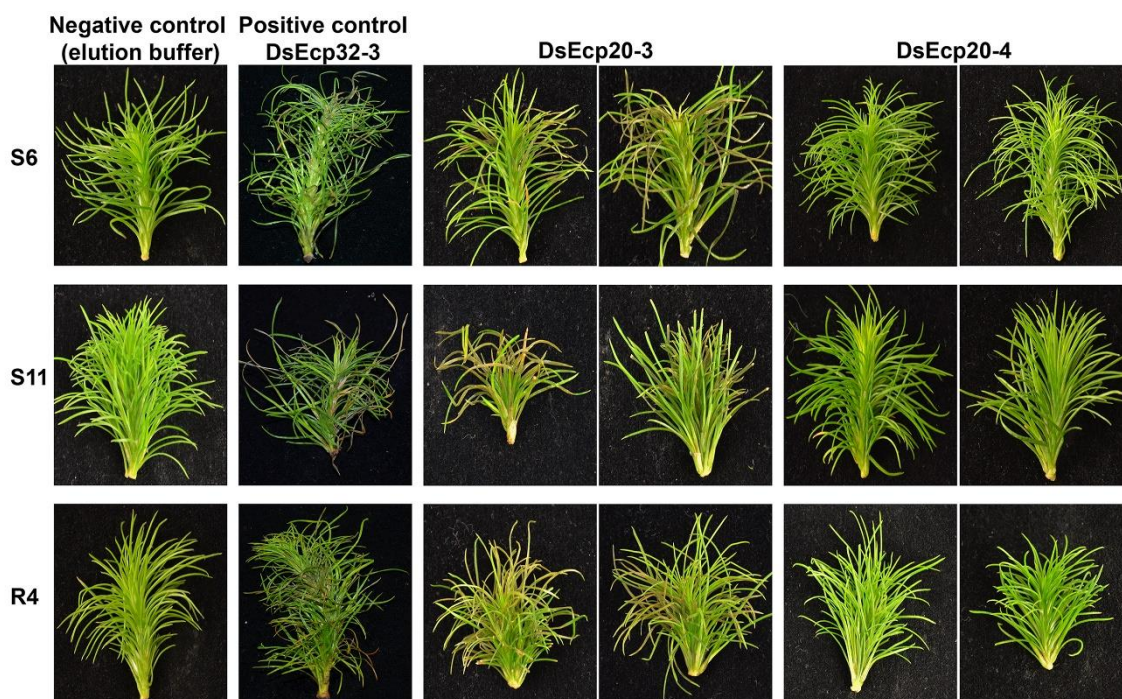


Figure 2.14. *Dothistroma septosporum* candidate effectors DsEcp20-3 and DsEcp20-4 trigger the same responses in *Pinus radiata* shoot tissues following vacuum infiltration as in non-host plants. Whole shoots of *Pn. radiata* derived from family seedlots that are relatively susceptible (S6 and S11) or tolerant (R4) to *D. septosporum* infection were infiltrated with purified DsEcp20-3 and DsEcp20-4 proteins produced by heterologous expression in *Pichia pastoris*. Elution buffer was used as negative control and purified DsEcp32-3 protein as a positive control. Representative photos (from 18 to 24 pine shoots for each treatment) were taken 7 days after infiltration, with further images shown in **Appendix Figure A.11**. Ds: *D. septosporum*.

The DsEcp32-3 positive control triggered cell death in all three pine genotypes, as expected (**Figure 2.14**). Infiltration of elution buffer (negative control) did not cause any damage or visible stress responses. Interestingly, both tested proteins gave the same response as they had shown in the non-host *Nicotiana* plants: DsEcp20-3 consistently elicited cell death in the same three pine genotypes used in the previous study, while DsEcp20-4 failed to elicit any response in the pine shoots.

2.3.7 DsEcp20-3-triggered cell death in *Nicotiana benthamiana* requires membrane-localized receptor-like proteins

The results described in this section were obtained at the Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China by Zhiyuan Chen and Yan Wang.

To determine how cell death elicitors from the Ds/FfEcp20 and 32 family were perceived by plants, the genes encoding the extracellular receptor-like kinase co-receptor genes *BAK1* and *SOBIR1*, as well as LRR receptor-like genes, in *N. benthamiana* (**Figure 2.15**) were silenced, in conjunction with agroinfiltration, as previously described by Wang et al. (2018). As expected, agroinfiltration of positive controls NPP1, INF1 and XEG triggered consistent strong cell death responses in *N. benthamiana* leaves treated with TRV:*GFP*, while INF1- and XEG1-triggered cell death was compromised in leaves treated with TRV:*BAK1* or TRV:*SOBIR1*. DsEcp20-3-triggered cell death was consistently compromised in *N. benthamiana* leaves in which the receptor-like kinase genes *BAK1* or *SOBIR1* were silenced (**Figure 2.15B**). In addition, DsEcp20-3-triggered cell death was compromised in *N. benthamiana* leaves treated with TRV VIGS constructs TRV:*T26* and TRV:*T26-2* (Wang et al., 2018) (**Figure 2.15D**). TRV:*T26* and TRV:*T26-2* both target the same two *N. benthamiana* genes but in different regions of those genes. One of these target genes encodes an LRR-type extracellular receptor-like protein (Sol Genomics Network accession number - Niben101Scf03138g01049.1) and one an extracellular LRR (Niben101Scf01834g02009.1). Other members of the Ecp20 and Ecp32 families (FfEcp20-3, DsEcp32-3 and FfEcp32-3) were screened and as a result, cell death triggered by FfEcp20-3 and FfEcp32-3 was also significantly compromised in *N. benthamiana* leaves treated with TRV:*BAK1*, TRV:*SOBIR1* or either of the T26 constructs (**Figure 2.15B**

and 2.15D). For DsEcp32-3-triggered cell death, no reduction was detected on TRV:*BAK1*- or TRV:*SOBIR1*-silenced plants (data not shown). Taken together, these results suggest that the cell death responses triggered by members of the Ecp20 and Ecp32 families from *D. septosporum* and *F. fulva* in *N. benthamiana* are the result of recognition by extracellular immune receptors, and that the transduction of defence response signals following this recognition requires the extracellular co-receptors BAK1 and SOBIR1.

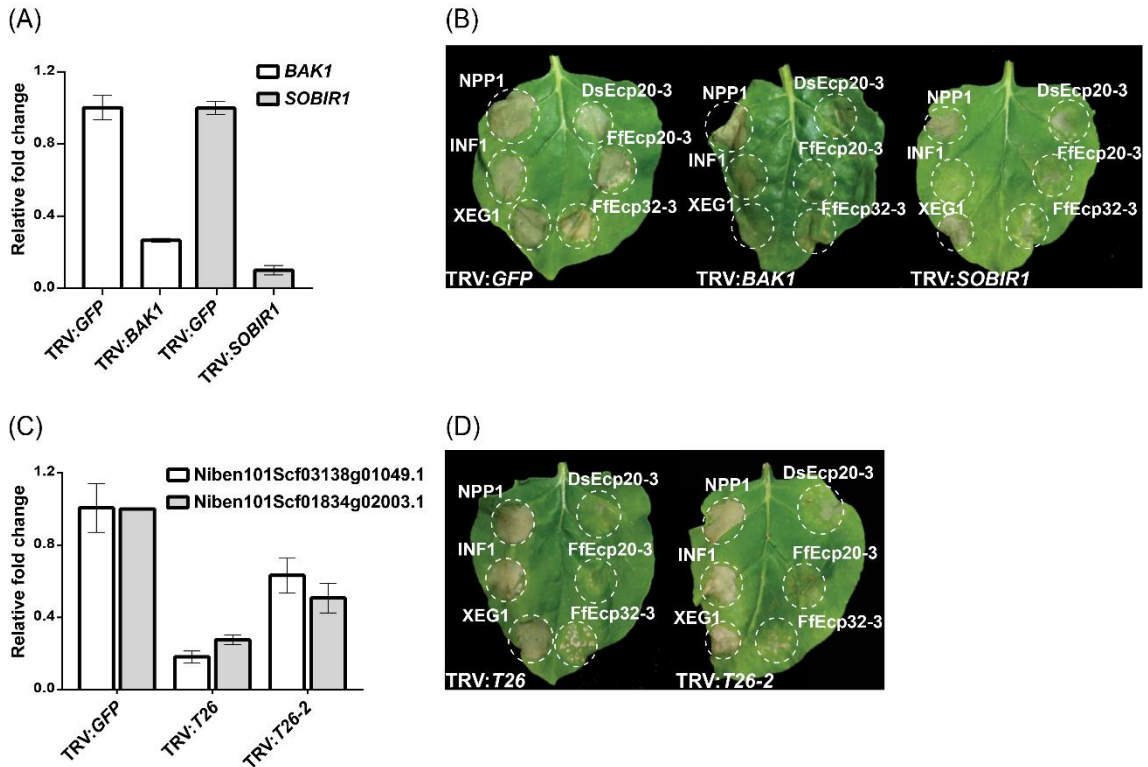


Figure 2.15. Cell death triggered by DsEcp20-3 in *Nicotiana benthamiana* is compromised in leaves silenced for different receptor-like genes. Expression levels of **(A)** *BAK1* and *SOBIR1* and **(C)** *T26* constructs in plants silenced for these genes, relative to their expression in plants silenced for the Green Fluorescent Protein (*GFP*) gene (absent in *N. benthamiana*; normalized to 1). Genes were silenced using a Virus-Induced Gene Silencing (VIGS) method based on Tobacco Rattle Virus (TRV). **(B, D)** Representative leaves showing cell death induced by expression of XEG1, INF1, NPP1, DsEcp20-3, FfEcp20-3 and FfEcp32-3 in *N. benthamiana* treated with specific TRV-mediated VIGS constructs as shown. Leaves ($n = 8$) were photographed three days after agroinfiltration (dai). Experiments were repeated twice with similar results. Ds: *D. septosporum*; Ff: *Fulvia fulva*. This work was carried out by Zhiyuan Chen and Yan Wang at the Department of Plant Pathology, Nanjing Agricultural University, Nanjing, China.

2.4 Discussion

Plant-pathogenic fungi secrete a collection of virulence factors, termed effector proteins, into their hosts to promote the infection process (Lo Presti et al., 2015; Rocafort et al., 2020). While many of these effectors appear to be singletons with no obvious sequence similarity to proteins present in the effector repertoires to which they belong, others form part of families. Indeed, in *Venturia inaequalis*, the biotrophic Dothideomycete pathogen responsible for scab disease of apple, more than half of the predicted effector repertoire is made up of protein families based on sequence similarity alone (Deng et al., 2017; Rocafort et al., 2022). Likewise, in *Ciborinia camelliae*, the necrotrophic Leotiomyce pathogen responsible for flower blight disease of camellias, approximately one tenth of the predicted secretome belongs to a single massively expanded candidate effector family (Denton-Giles et al., 2020). Notably, effector families can also be associated with avirulence. For example, in the hemibiotrophic Sordariomyce pathogen *Magnaporthe oryzae* (rice blast disease), the biotrophic Leotiomyce pathogen *Blumeria graminis* (cereal powdery mildew disease), as well as the biotrophic Pucciniomyce pathogen *Melampsora lini* (flax rust disease) and the hemibiotrophic Dothideomycete pathogen *Leptosphaeria maculans* (stem canker disease of brassica), certain avirulence effectors have been found to form part of protein families (Anderson et al., 2016; Catanzariti et al., 2006; Dodds et al., 2004; Kang et al., 1995; Khang et al., 2008; Praz et al., 2017; Spanu, 2017; Talbi et al., 2022).

The emergence of sequence-related effector families in fungi is often the result of duplication events from an ancestral gene or from other family members and is typically followed by sequence diversification through an accumulation of indels and synonymous/non-synonymous substitutions and/or recombination events between different family members (Denton-Giles et al., 2020; Franceschetti et al., 2017; Pendleton et al., 2014). Such sequence diversification is anticipated to have roles in, for example, the diversification of effector functions, as well as the evasion of host immunity. In some cases, this diversification can result in an almost complete loss of sequence similarity between family members, with similarity only retained at the protein tertiary structure level, as has recently been shown for several effector families of plant-pathogenic fungi using the ground-breaking *ab initio* protein structure prediction tool, AlphaFold2

(Rocafort et al., 2022; Seong & Krasileva, 2022). However, in many cases, sequence identity between family members is maintained, even across different fungal species. These cross-species effector families are of high interest, as their sequence conservation may indicate an important and/or conserved (i.e., core) role in fungal virulence (Stergiopoulos et al., 2010).

In this study, two candidate effector protein families, Ecp20 and Ecp32, which are highly conserved at the primary sequence and tertiary structure levels between the two closely related Dothideomycete fungal pathogens, *D. septosporum* and *F. fulva* were identified. Despite their close phylogenetic relationship, these two pathogens have very different hosts, with *D. septosporum* causing disease on pine (a gymnosperm), and *F. fulva* causing disease on tomato (an angiosperm) (Drenkhan et al., 2016; Thomma et al., 2005). An investigation of pre-existing gene expression data revealed that both protein families have members encoded by genes that are highly expressed *in planta*, while others are only moderately or lowly expressed. Such a finding is consistent with what was recently observed in *V. inaequalis*, where gene family members are expressed at different levels during apple infection (Rocafort et al., 2022).

Both the Ecp20 and Ecp32 families were found to have members that differed in their ability to trigger cell death in the non-host species *N. benthamiana* and *N. tabacum*. The differential ability of these family members to trigger a cell death response does not appear to be restricted to proteins from *D. septosporum* and *F. fulva*. Indeed, it was recently determined that an orthologue of Ds/FfEcp20-4 from the hemibiotrophic Dothideomycete pathogen *Z. tritici* (septoria leaf blotch disease of wheat), named Zt4 (JGI protein ID 104697), triggers cell death in *N. benthamiana* (Kettles et al., 2017). This is in contrast to Ds/FfEcp20-4, where, in this study, no cell death response was observed. Similarly, a homologue of Ecp32-1 from *Z. tritici* (JGI protein ID 105896; no reciprocal orthologue in *D. septosporum* or *F. fulva*) was not able to induce a cell death response in *N. benthamiana* (Kettles et al., 2017) whereas in this study, DsEcp32-1 and FfEcp32-1 consistently and infrequently triggered cell death, respectively. Of course, it remains possible that some of the family members triggered other plant responses, such as changes in host gene expression or the accumulation of reactive oxygen species (ROS), which were not measured in our assays. In any case, variation in the ability of family

members to elicit plant cell death is not restricted to the Ecp20 and Ecp32 families, with variation also observed in, for example, the cell death-inducing capacity of CcSSP effector candidate family members from *C. camelliae* in camellia petals (Denton-Giles et al., 2020).

BLASTp searches demonstrated that, like those shown above for *Z. tritici*, homologues of the Ecp20 and Ecp32 family members are present in other fungal species, with many of these species being plant pathogens. Interestingly, as observed in *D. septosporum* and *F. fulva*, these homologues were also frequently found to exist as protein families. Taken together, it is therefore likely that the Ecp20 and Ecp32 proteins are core effectors, possibly contributing to conserved virulence functions in their respective plant hosts. In addition to searching for homologues in other plant pathogens, the genomes of other *D. septosporum* isolates and of *D. pini*, the other causal species of DNB, were also screened and it was found that whilst Ecp20 and Ecp32 family members are polymorphic across other *D. septosporum* isolates, all family members are present in these isolates and also in *D. pini*, although Ecp32-5 is pseudogenised in *D. pini*. There was also conservation of cell death-eliciting function between isolates of *D. septosporum* and between the two *Dothistroma* species, since the respective orthologues showed the same responses as those from the NZE10 strain, also triggering cell death in *N. benthamiana*. Whether the identified sequence variation contributes to virulence function of these proteins outside of cell death elicitation remains to be determined.

As the primary amino acid sequence of the Ecp20 and Ecp32 family members provided no insights into their potential virulence function, the tertiary structures of these proteins were predicted using the AlphaFold2 tool and compared to proteins of characterized structure and/or function. Based on this analysis, all eight *D. septosporum* and *F. fulva* Ecp20 family members were predicted to possess structural similarity to PevD1, an effector protein with an Alt a 1 (β -barrel) fold from the hemibiotrophic Sordariomycete pathogen *Verticillium dahliae* (Chruszcz et al., 2012; Han et al., 2012). Interestingly, the Alt a 1 fold has also been found in MoHrip1, a plasma membrane-binding virulence factor from *M. oryzae* that is highly expressed during rice infection (Zhang et al., 2017b). Just like FfEcp20-2, FfEcp20-3 and DsEcp20-3, the PevD1 and MoHrip1 proteins induce cell death responses in *Nicotiana* species (Han et al., 2012; Zhang et al., 2017), suggesting that this cell death-eliciting ability could be a conserved

function of these proteins. It should be noted, however, that proteins with an Alt a 1 fold, including PevD1, have also been shown to interact with and inhibit the enzymatic activity of pathogenesis-related 5 (PR5) proteins from different host plants (Gómez-Casado et al., 2014; Kumar & Mukherjee, 2020; Zhang et al., 2019). Whether the Ecp20 proteins from *D. septosporum* and *F. fulva* perform a similar role in pathogen virulence remains to be explored.

In contrast to members of the Ecp20 family, the Ecp32 proteins were predicted to be structurally similar to proteins from diverse organisms with a β -trefoil fold. Proteins with a β -trefoil fold have the ability to recognize different ligands, such as DNA, proteins and carbohydrates, and so can be involved in different processes and interactions between organisms (Žurga et al., 2015). Among the β -trefoil fold proteins, the most abundant members are lectins, carbohydrate-binding proteins, and hydrolase inhibitors, such as protease inhibitors (Renko et al., 2012). Lectins are proteins that specifically bind carbohydrates and, in fungi, are associated with plant-pathogen interactions, fungal growth, fruiting body development and, due to their toxicity, defence against parasitic or predatory organisms (Sharon, 2007; Varrot et al., 2013). Lectins from different mushroom species, for example, are known to be toxic to organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Juillot et al., 2016; Schubert et al., 2012; Wohlschlager et al., 2011). These proteins bind to the plasma membrane and, in doing so, induce cell death. Cyt toxins from *Bacillus thuringiensis*, for instance, are insecticidal proteins with a β -trefoil fold that are known to interact with host membrane lipids (Soberón et al., 2013). Even though the mechanism of lectin toxicity is not known, it is hypothesized that this toxicity may be the direct result of host membrane damage. Taken together, this could indicate a possible role Ecp32 proteins in triggering plant cell death, at least in part, by interacting with and disrupting the host membrane.

As mentioned above, protease inhibitors can also adopt a β -trefoil fold, and in this study, it was found that most Ds/FfEcp32 proteins had a predicted tertiary structure similar to that of a plant trypsin inhibitor. Of interest, fungal pathogens frequently target host proteases involved in plant defence by secreting effectors that serve as protease inhibitors, with well characterized examples including Pit2 from the biotrophic Ustilaginomycete *Ustilago maydis* (corn smut disease) and Avr2 from *F. fulva* (Mueller et

al., 2013; van Esse et al., 2008). While it is not yet known whether Pit2 or Avr2 adopt a β -trefoil fold, another possible function for the Ecp32 proteins could therefore involve the inhibition of plant proteases during host colonization.

The fact that Ecp20 and Ecp32 family members of *D. septosporum*, *F. fulva* and *Z. tritici* are likely apoplastic (Hunziker et al., 2021; Kettles et al., 2017; Mesarich et al., 2018), and that members of both families across multiple fungal species trigger cell death in *Nicotiana* species (with this activity dependent on their secretion to the apoplast), may suggest that each family presents a conserved epitope that is recognized by an extracellular immune receptor in these non-host plants. In support of recognition by an extracellular immune receptor, silencing of the extracellular co-receptors *NbBAK1* or *NbSOBIR1* in *N. benthamiana*, which are required to transduce defence response signals following apoplastic effector recognition (Liebrand et al., 2013; Liebrand et al., 2014), compromised Ds/FfEcp20-3-dependent cell death. Notably, a compromised cell death response was also observed for FfEcp32-3, but not DsEcp32-3. One possibility here is that the expression of *NbBAK1* and/or *NbSOBIR1* was not reduced to a level sufficient for the cell death response triggered by DsEcp32-3 to be compromised. Alternatively, given its predicted structural similarity to lectins, other processes outside of immune receptor recognition, such as cell membrane perturbation, could be involved in cell death elicitation by DsEcp32-3.

One of the aims of this study was to develop a method to screen putative effectors of conifer pathogens directly in their host using a protein infiltration approach, with the long-term goal of identifying corresponding immune receptors for the effectors, and thus assisting breeding for disease resistance. By submerging pine shoots into solutions containing purified candidate effector proteins, and applying a vacuum pressure, it was possible to develop a robust and reliable infiltration process that did not harm the pine needle tissue. Two positive controls were identified that can be used as references in future studies. One was the *B. cinerea* effector BcSSP, the other a secreted protein of *D. septosporum* (DsEcp32-3) that induces cell death symptoms in pine shoots as well as *Nicotiana* leaves. DsEcp32-3 is of interest due to its very high expression and up-regulation in the mid and late (necrotrophic) stages of disease caused by *D. septosporum* on *Pn. radiata* (Bradshaw et al., 2016) and its ability to elicit cell death in

three genotypes of *Pn. radiata* as well as in *Nicotiana* species. This protein could be acting as a necrotrophic effector, triggering cell death that assists the pathogen by eliciting the destruction of needle tissue. This action would augment that of dothistromin which is required for disease lesion expansion, but not for initial elicitation of cell death (Kabir et al., 2015a).

The novel method of effector delivery to gymnosperm tissue outlined in this chapter would be well suited for medium-scale screening of multiple pine genotypes with a small number of effector proteins. If the effector-screening process were to be scaled up for high-throughput commercial use with a large number of effector proteins, the time used to grow and prepare fresh purified effector proteins in the *Pi. pastoris* system as used here could be a limiting factor. An alternative delivery method might be through *Agrobacterium tumefaciens*-based transformation of pines, in which the effector genes rather than the proteins are delivered to the host plant. *Agrobacterium tumefaciens*-based transformation of *Pn. radiata* and other *Pinus* species has been achieved (Grace et al., 2005; Maleki et al., 2018) but is a slow and technically challenging process. Despite this, future efforts to establish reliable transient transformation of pines may be worthwhile.

This study has broad implications for the understanding of fungal (and oomycete) pathogens of gymnosperm trees and how these species interact with each other at the molecular level. Importantly, the high level of conservation of effectors across fungal taxa, and conservation of plant responses across angiosperms and gymnosperms suggests that much of what the scientific community has learned about plant-pathogen interactions involving angiosperm systems may also be relevant to gymnosperms. A similar conclusion was reached by studying pine responses to *D. septosporum* infection (Lu et al., 2020). There also appear to be common 'core' interactions that could be exploited to give broad host-range resistance. Another implication relates to the pine screening method which could be adapted for use with other gymnosperm tree species and other plant hosts that are not amenable to the usual screening methods. Finally, this type of screening method has the potential, along with expression data and pathogen gene knockout studies, to identify necrotrophic effectors that could in turn identify susceptibility gene targets, leading to durable resistance.

In line with the DsEcp32-3 cell death response observed in the non-host and host species, two (purified) *D. septosporum* proteins from the Ecp20 family were investigated to determine whether they could also induce a cell death response in pine. Both proteins, DsEcp20-3 and DsEcp20-4, were of interest due to their high expression in the Mid and Late (necrotrophic) stages of *D. septosporum* infection in *Pn. radiata* (Bradshaw et al., 2016). The finding that DsEcp20-3 and DsEcp20-4 triggered different cell death responses in non-host plants, *N. benthamiana* and *N. tabacum*, also made them good candidates for this assay. Interestingly, DsEcp20-3, like DsEcp32-3, was able to trigger a cell death response in all pine genotypes tested. Taken together, these results suggest that the DsEcp20-3 and DsEcp32-3 proteins could also be recognized by an immune receptor (possibly conserved with *N. benthamiana*) or, as described above, participate in membrane perturbation in *Pn. radiata*. Consistent with what was observed in the non-host plants, DsEcp20-4 was unable to elicit a cell death response in the pine shoots. This may suggest that it does not have the conserved epitope required to elicit cell death following recognition by an immune receptor. To identify the regions or amino acid residues involved in cell death induction, and thus potential epitopes for recognition by cognate immune receptors, region swaps between cell death-eliciting and non-eliciting members of the Ecp20 and Ecp32 families, based on information gained from the prediction of surface-exposed residues by AlphaFold2, can now be performed in *Nicotiana* species or pine, similar to that described for the Avr4 effector protein of *D. septosporum* and *F. fulva* in *N. benthamiana* (Mesarich et al., 2016). This, in turn, might help to explain the differences in cell death-inducing capacity between, for example, CfEcp32-1 and DsEcp32-1, as well as DsEcp20-3 and DsEcp20-4.

As it is well known that plant cell death is a strategy exploited by necrotrophic and hemibiotrophic pathogens to acquire nutrients from their hosts during their necrotrophic stage or to facilitate a biotrophic–necrotrophic switch (Shao et al., 2021). It is possible that the DsEcp20-3 and DsEcp32-3 proteins from *D. septosporum* are involved in these processes. In addition to their cell death-eliciting activities, support for this hypothesis is provided by the fact that the expression of both *DsEcp20-3* and *DsEcp32-3* is highly induced during the Mid and Late stages of pine infection by *D. septosporum*. Another possibility is that DsEcp20-3 may be acting as an Avr factor of *D. septosporum*

recognized by the host, similar to that already proposed for the core effector DsEcp2-1 (Guo et al., 2020), while other family members, such as DsEcp20-4, which is also highly expressed during pine infection, might be virulence factors that interact with pine PR5 proteins to suppress host defences. One way to approach these research questions is to generate mutants of *D. septosporum* deleted for a *DsEcp20* or *DsEcp32* gene (e.g., using CRISPR/Cas9 technology) to determine what impact their deletion or disruption has on necrotrophic infection by this fungus. The problem with this method is that because these genes form part of multigene families, functional redundancy may mask the role of the individual effectors (i.e., the deletion mutants may not show a reduction in virulence). However, this does not seem to be case for the FfEcp20 family, since *FfEcp20-2* was recently deleted from *F. fulva* strain 0WU, with mutants showing reduced virulence to tomato compared to the wild-type (Karimi-Jashni et al., 2022). Among the FfEcp20 family, FfEcp20-2 is also the most highly expressed *in planta* (**Table 2.1**) and elicited the most consistent cell death in *N. benthamiana*. Together these results suggest that certain effector family members may have key roles in virulence. Therefore, it will be interesting to create strains in which genes corresponding to several or all family members are deleted to assess whether fungal virulence, particularly in the necrotrophic infection stage, is compromised. Recently, the first successful application of CRISPR/Cas9 genome editing to *D. septosporum* was reported, in which a gene encoding a secreted protein that elicited cell death in *N. benthamiana* and pine was disrupted (McCarthy et al., 2022) (**Chapter 3** and **Chapter 4**), which will ultimately help with creating such strains.

In conclusion, two new candidate effector families with cell death-eliciting activity were identified from *D. septosporum* and *F. fulva*. Evidence was obtained to suggest that members of these families are recognized as IPs by immune receptors of phylogenetically distinct plant species (angiosperms and a gymnosperm). By characterizing the virulence (and potential avirulence) functions of such 'core' effectors, the knowledge gained could ultimately be used in durable disease resistance breeding programs active against these two, and other, important plant pathogens.



GRADUATE
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STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Mariana Massoco Tarallo
Name/title of Primary Supervisor:	Rosie E. Bradshaw
In which chapter is the manuscript /published work:	
Please select one of the following three options: ☒ The manuscript/published work is published or in press Please provide the full reference of the Research Output: Tarallo, M., McDougal, R. L., Chen, Z., Wang, Y., Bradshaw, R. E. & Mesarich, C. H. (2022). Characterization of two conserved cell death elicitor families from the Dothideomycete fungal pathogens <i>Dothistroma septosporum</i> and <i>Fulvia fulva</i> (syn. <i>Cladosporium fulvum</i>). <i>Frontiers in Microbiology</i>, 13, 964851 https://doi.org/10.3389/fmicb.2022.964851 ☐ The manuscript is currently under review for publication – please indicate The name of the journal: The percentage of the manuscript/published work that was contributed by the candidate: 92% Describe the contribution that the candidate has made to the manuscript/published work: The supervisors helped in planning the project and proofreading the manuscript. Overseas collaborators performed the VIGS experiments (Sections 2.2.10, 2.2.11 and 2.3.7). Everything else was done by the candidate. ☐ It is intended that the manuscript will be published, but it has not yet been submitted to a journal	
Candidate's Signature:	Mariana Tarallo Digitally signed by Mariana Tarallo Date: 2022.12.05 14:35:54 +13'00'
Date:	12-Dec-2022
Primary Supervisor's Signature:	Bradshaw, Rosie Digitally signed by Bradshaw, Rosie Date: 2022.12.12 11:31:25 +13'00'
Date:	12-Dec-2022

3 Characterization of candidate effector proteins from the foliar pine pathogens *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* in non-host and host species

3.1 Introduction

Plants are constantly exposed to a wide array of microorganisms, many of which are pathogenic. To combat these pathogens, plants have a simple, yet efficient, immune system that is built on the recognition of non-self. More specifically, after penetration by a pathogen, plants can detect microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) through extracellular pattern recognition receptors (PRRs). This recognition triggers a signal transduction cascade, activating defence responses that prevent or slow further pathogen ingress (Hogenhout et al., 2009; Jones & Dangl, 2006). Successful pathogens, however, can deliver a collection of small proteins, termed effectors, into and around plant cells to suppress these defences. If the plant has the ability to recognize such effectors, through its repertoire of extracellular or intracellular resistance (R) proteins, immune responses are again activated that can restrict pathogen spread.

Pathogen effectors can be predicted using a combination of bioinformatic tools and functional characterization studies, a process known as effectoromics. With the ability to rapidly acquire genomic and transcriptomic information, and since many effector proteins possess recognisable features, such as secretion signals, small size, and a high proportion of cysteine residues, it has become increasingly easy to employ computational methods to predict a set of candidate effectors (CEs) for a pathogen. These candidate proteins can then be heterologously expressed in host and non-host plants, with those triggering a plant cell death response candidates for recognition by a plant R protein.

Transformation of plant tissue with *Agrobacterium tumefaciens* (i.e., using a method called agroinfiltration) is an established way of heterologously expressing genes of interest *in planta* to study their function (Opabode, 2006; Van der Hoorn et al., 2000;

Ziemienowicz, 2014). In this case, the bacteria can act as vectors by carrying a gene of interest, such as an effector gene, into plant cells. The method is based on the fact that *A. tumefaciens*, a tumour-inducing plant pathogen, naturally transfers its DNA to the plant cell. The transfer-DNA (T-DNA) is localized on the tumour-inducing (Ti) plasmid, that also contains genes involved in the transfer process – virulence (*vir*) genes (Tinland, 1996; Ziemienowicz, 2001). Since the T-DNA region has the genes that allow for disease formation in the host, disarmed laboratory strains of *A. tumefaciens* have been created in which those tumour-inducing genes were deleted. In these strains, the T-DNA and the *vir* genes are placed on separate plasmids. This is called a “binary vector” system, where the T-DNA plasmid carries the gene of interest that will be transferred into the plant; the other plasmid – the *vir* helper plasmid – contains the *vir* genes that encode for the proteins that will help cleave the T-DNA ready for integration of the gene of interest into the plant genome (Gelvin, 2003).

Unlike that for angiosperms, application of the *A. tumefaciens* transformation system to gymnosperms, such as *Pinus radiata*, is not straight forward (see **Section 1.6** and **Section 1.7 of Chapter 1**) (Maleki et al., 2018; Tang et al., 2014). As such, screening a large number of CE proteins for their ability to trigger cell death in gymnosperm plants using the *A. tumefaciens* transformation system is not currently possible. Studies using model angiosperm plants, such as *Nicotiana benthamiana* and *Nicotiana tabacum*, are a reliable and fast alternative to improve understanding of pathogens and their effector proteins in cases where their host plants are not easily amenable to *A. tumefaciens*-mediated transformation. These two *Nicotiana* species carry characterised and uncharacterised extracellular and intracellular immune receptors, which make them ideal for broad screening of pathogen effectors (Ma et al., 2012). Transient expression of genes of interest in *Nicotiana* species can be more useful than stable transformation, especially when considering the short time frame in which genes can be analysed and the fact there is no necessity for regeneration of the transformed cell into a transgenic plant (Kapila et al., 1997).

There are many examples in which the *A. tumefaciens* transformation system has been used to screen CEs, predicted through the use of bioinformatic tools (see **Section 1.6** and **Section 1.7 of Chapter 1**), in *Nicotiana* species. For these reasons, a

non-host approach was first used to investigate effectors from pine pathogens. For that, the *Dothistroma septosporum* genome was used to predict an initial set of effector candidates, as it had the most complete and well-annotated genomes. A set of *D. septosporum* CEs was determined based on protein characteristics, pre-existing *in planta* expression data and functional predictions, as well as tertiary structure predictions. Next, these *D. septosporum* CEs were used to identify likely orthologues in *Cyclaneusma minus* and *Phytophthora pluvialis*, thereby identifying a set of shared effector candidates between the three foliar pine pathogens. CE proteins that triggered cell death responses in one or both of the *Nicotiana* species and that were strongly up-regulated *in planta* (in the case of the *D. septosporum* orthologues), were selected to be tested in the pine assay that was developed in **Chapter 2**. Therefore, using this effectoromics approach, the aim of this Chapter was to identify common effector proteins from pine pathogens that potentially have an important role during infection.

3.2 Methods

3.2.1 Identification of shared candidate effector proteins from pine pathogens *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

To identify common CE proteins shared between the three foliar pathogens of pine, *D. septosporum*, *C. minus* and *P. pluvialis*, the genomic information of *D. septosporum* was used first because it has the more complete and well-annotated genome, as well as transcriptomic data (Bradshaw et al., 2016; de Wit et al., 2012).

D. septosporum effector prediction was based on a pipeline from Hunziker et al. (2021) with minor modifications:

1. *D. septosporum* NZE10 genome sequences, gene models and predicted protein sequences (de Wit et al., 2012) were downloaded from the Joint Genome Institute (JGI) (<http://genome.jgi.doe.gov/Dotse1.home.html>).
2. To identify putatively secreted proteins, it was important to identify the N-terminal secretion signal (signal peptide), a small sequence of 15 to 60 amino acids in length that functions to direct proteins through the endoplasmic reticulum–Golgi secretory pathway. Signal peptides were predicted using the SignalP v3.0 programme (Bendtsen et al., 2004).

3. Some non-secreted proteins can also contain a signal peptide (e.g., membrane-bound proteins) (Emanuelsson et al., 2007). To exclude these proteins, the following programmes were used: TMHMM v2.0 (Krogh et al., 2001), to ensure the absence of transmembrane domains and BIG-PI (Eisenhaber et al., 2004), to exclude glycosylphosphatidylinositol (GPI)-anchored proteins.
4. To help refine which candidate proteins may be biologically important effectors, the time course transcriptome data from Bradshaw et al. (2016) were used. The gene expression data corresponding to three *D. septosporum* infection stages in pine (Kabir et al., 2015) were used to select candidates with an RNA-seq value of >50 Reads Per Million per Kilobase (RPMK) during at least one infection stage.
5. CE proteins were submitted to homology searches using the BLASTp and tBLASTn tools from the National Centre for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) and JGI (E-value < 10⁻⁵). Proteins with known functions unrelated to virulence were excluded. For this reason, proteins that were predicted to be hypothetical and that had a broad distribution across other fungal species were selected, along with others that had a known role in fungal virulence.
6. To help narrow down the list of CE proteins, EffectorP v3.0 (Sperschneider & Dodds, 2021) was used. From a list of secreted protein sequences, EffectorP can identify secreted effectors that are potentially involved in plant-pathogen interactions, classifying them into apoplastic and cytoplasmic effectors, or non-effectors. Candidates that were classified as non-effectors were discarded.

To further characterize the CEs, comparisons with the characterised apoplastic secretome of *Fulvia fulva* (Mesarich et al., 2018) also helped further identify and characterize *D. septosporum* CEs.

The list of CE proteins from *D. septosporum* NZE10 was then used to identify likely orthologues in *C. minus* NZFS809 (McDougal, unpublished) and *P. pluvialis* NZFS3000 (Studholme et al., 2016), through reciprocal tBLASTn and BLASTp searches (E-value < 10⁻⁵).

The genomes of 18 other *D. septosporum* isolates (Bradshaw et al., 2019), *Dothistroma pini* CBS 116487 (GenBank assembly accession GCA_002116355.1) and *P. pluvialis* isolate LC9-1 (Studholme et al., 2016) were also used to search for

orthologues of the CE proteins, through reciprocal tBLASTn and BLASTp searches (E-value < 10⁻⁵).

tBLASTn and BLASTp (E-value < 10⁻²) were also used to search the genomes of other pine pathogens for homologues of the CEs. These were: *Gremmeniella abietina* (PRJNA347218), *Elytroderma deformans* (PRJNA537175), *Cronartium ribicola* (PRJNA190829), *Cronartium quercuum* f. sp. *fusiforme* (PRJNA67371), *Fusarium circinatum* (PRJNA565749), *Lecanosticta acicola* (PRJNA212329) and *Pseudocercospora pini-densiflorae* (PRJNA212512). The same searches were also performed in the pine endophyte *Lophodermium nitens* (PRJNA335148).

Using methods outlined in **Section 2.2.1** of **Chapter 2**, *D. septosporum*, *C. minus* and *P. pluvialis* CEs, without their signal peptide sequence, were assessed for possible structural similarities to characterised proteins using AlphaFold2 (Jumper et al., 2021; Mirdita et al., 2022). Here in this Chapter, two values were used to assess the confidence of the predictions: the predicted local-distance difference test (pLDDT) score, which ranges from 0 to 100, with values closer to 100 indicating a high confidence in prediction, and the global superposition metric template modelling score (TM-score), ranging from 0 to 1, with a value of ≥0.5 indicating a similar fold between structures. The predicted structures were then queried against those in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using the protein structure comparison server Dali (Holm, 2020). Here, a Dali Z-score of ≥2 was used to infer structural similarity. Protein tertiary structures were visualized and rendered in PyMOL v2.5, with alignments carried out using the CEalign tool (DeLano, 2002).

The Foldseek server (<https://search.foldseek.com/search>) (van Kempen et al., 2022) was used to identify similarities in tertiary structure between AlphaFold2-predicted structures of *D. septosporum* CEs and proteins of *Phytophthora* species present in the server AlphaFold/UniProt50 v3, AlphaFold/Swiss-Prot v2 and AlphaFold/Proteome v2 databases.

Surface residues were calculated using the PyMOL script “findSurfaceResidues” (<https://pymolwiki.org/index.php/FindSurfaceResidues>) with a 2.5 Å² cut-off for the residue to be considered exposed.

Intrinsically disordered regions (IDR) were predicted by querying protein sequences in the Predictor of Natural Disordered Regions (PONDR®) server (<http://www.pondr.com/>) (Molecular Kinetics, Inc., Washington State University, and the WSU Research Foundation). A prediction score ≥ 0.8 was considered a significant IDR prediction.

Protein sequence alignments were performed using Clustal Ω (Sievers et al., 2011) in Jalview (<https://www.jalview.org>). Phylogenetic trees were constructed with the neighbour-joining method based on 1,000 bootstrap replicates using Geneious Software v9.1.8 (Kearse et al., 2012) in conjunction with protein sequence alignments.

3.2.2 Effector protein expression for functional characterization in non-host and host plants

All methods referring to effector gene cloning to generate expression vectors to be used in *A. tumefaciens*-mediated transient transformation assays (ATTAs) and to transform the yeast *Pichia pastoris* GS115 for protein production are described in **Section 2.2.6 of Chapter 2**, along with the pine infiltration method with purified CE proteins, which is described in **Section 2.2.8 of Chapter 2**. For the ATTAs, the responses observed in the infiltration zones of *N. benthamiana* and *N. tabacum* were classified as no visible response, chlorosis, weak cell death or strong cell death. All biological material, primers and plasmids used in this Chapter are shown in **Appendix A.1, Appendix A.2** and **Appendix A.3**, respectively.

3.2.3 Expression of candidate defence marker genes in *Nicotiana benthamiana* to determine activation of immune responses

To determine if the cell death-eliciting activity of *D. septosporum* CE proteins was associated with plant immunity responses, the expression of *N. benthamiana* defence marker genes was analysed. First, the expression of two hypersensitive response (HR)-specific marker genes, *NbHIN1* (Takahashi et al., 2004) and *NbHSR203J* (Pontier et al., 1994), was examined. To determine if *D. septosporum* CEs-activated immunity is accompanied by alteration of hormone signalling pathways, the following genes were also analysed: *NbPR1a* and *NbPR2* (Dean et al., 2005) (salicylic acid (SA)-dependent immunity), *NbPR4* (Asai & Yoshioka, 2009) and *NbLOX* (Rodriguez et al., 2013) (jasmonic

acid (JA)-dependent immunity) and *NbERF1* (Asai & Yoshioka, 2009) (ethylene (ET)-dependent immunity). PAMP-triggered immunity (PTI) markers (*NbAcre31*, *NbWRKY7* and *NbWRKY8* (McLellan et al., 2013)) were also assessed. All primer sequences are listed in **Appendix Table A.2**.

To assess plant immunity responses, *A. tumefaciens* strains transformed with *D. septosporum* CE genes, were infiltrated into *N. benthamiana* leaves (as described in **Section 2.2.7 of Chapter 2**); those with empty pICH86988 vector were used as a negative control. Two time points were analysed: 24 and 48 h after infiltration (hai) with the expression vector for the CE or empty pICH86988 vector control. For each time point, four replicates were taken; each replicate consisted of two whole *N. benthamiana* leaves infiltrated with the appropriate *A. tumefaciens* suspension.

N. benthamiana leaves were harvested and ground to a fine powder with a mortar and pestle, using liquid nitrogen. RNA was extracted with a RNeasy Plant Mini Kit (Qiagen, Hilden Germany), following the manufacturer's instructions. Concentration and purity of RNA were estimated with a NanoDrop®. For cDNA synthesis, 1000 ng/μL of the total RNA from each sample were used. The synthesis was performed with the QuantiTect® Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions.

A standard curve was made for each primer pair used in real time-quantitative PCR (qPCR) to calculate their PCR efficiency (E). Six 5-fold serial dilutions of *N. benthamiana* cDNA, from a starting concentration of 1000 ng/μL, were used with three replicates for each dilution. Each 10 μL PCR was prepared with 1x SensiFAST SYBR® No-ROX mix (Meridian Bioscience, Ohio, USA), 0.5 μM of each primer and 5 ng of cDNA. PCR conditions were 1 cycle at 95°C for 3 min, 40 cycles of 95°C for 5 s, 60°C for 10 s and 72°C for 20 s, then 1 cycle at 40°C for 10 s. Each cDNA sample was analysed in triplicate. Reactions were performed using a LightCycler 480 III (Roche) and analysed using a LightCycler® 480 SW v1.5.1 (Roche).

The cycle threshold (Ct) value of each targeted gene, expressed as the mean of the three technical replicates, was converted into relative quantities (RQs) using the function $RQ = E^{\Delta Ct}$, where ΔCt is the difference between the lowest Ct value across all

samples for the targeted gene and the Ct value of a given sample of the same targeted gene. Normalized-relative quantity (NRQ) of each targeted gene was calculated as the ratio of the targeted gene RQ and the reference gene RQ. The *N. benthamiana* actin gene (*NbActin*) was used as the reference gene. Individual fold change values were determined by dividing the targeted gene NRQ by the reference NRQ (Leastro et al., 2020).

3.3 Results

3.3.1 Identification of common candidate effector proteins from *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

Effectors possess characteristics that are helpful for their identification. One of these features is the presence of an N-terminal signal peptide for secretion into the host and the absence of transmembrane domains. Another feature is that effectors are frequently small (≤ 300 amino acids) and cysteine-rich, although this is not true for all effectors. All of these characteristics were employed to identify *D. septosporum* CE, so they could be later compared with proteins from the other two pine pathogens, *C. minus* and *P. pluvialis*.

Firstly, the predicted secretome of *D. septosporum* NZE10 was narrowed down as follows (**Figure 3.1**): SignalP v3.0 predicted 1487 proteins containing a signal peptide. Of these, only 855 were predicted to be secreted based on the absence of a transmembrane domain and GPI anchor sites. Out of the 855 proteins, 397 had expression *in planta* above the threshold of 50 RPKM during at least one infection stage. Searches in NCBI and JGI databases narrowed down the list to 42 proteins, 37 of which exclusively had homology to proteins annotated as hypotheticals, including several with homology to *F. fulva* proteins detected in the tomato apoplastic environment during infection (Mesarich et al., 2018). The remaining five proteins had homology to characterized proteins with known virulence functions in other pathosystems. More specifically, Ds46236 is an orthologue of the Ecp6 effector protein from *F. fulva*, which has three carbohydrate-binding lysin motif (LysM) domains that sequester chitin oligomers (PAMPs) in the host apoplast to help the pathogen avoid detection by the plant (Bolton et al., 2008; de Jonge et al., 2010). Ds131290 is predicted to have one lysin LysM carbohydrate-binding domain, suggesting that this protein might also be involved in suppressing chitin-triggered plant defence

responses. Ds70155 is a cerato-platanin, a known virulence factor from the fungal tree pathogen *Ceratocystis platani*, which can trigger plant cell death responses (Baccelli et al., 2014). Ds69113 is an orthologue of FfPhiA-2 from *F. fulva*, a cell wall-associated protein involved in phialide development, cells that are responsible for the production and release of conidia (Melin et al., 2003). Ds75009 (DsHdp1) is a hydrophobin, also a known virulence factor (Bayry et al., 2012); the gene coding for this protein is one of the most highly expressed during *D. septosporum* infection of pine, especially in the Mid and Late stages (beginning and end of the necrotrophic phase) (**Appendix Table A.8**).

Next, EffectorP v3.0 was used to identify a total of 29 *D. septosporum* CE proteins from the set of 42, with 19 being classified as apoplastic effectors, four as cytoplasmic effectors, and six classified as both apoplastic and cytoplasmic effectors (dual) (**Figure 3.1** and **Appendix Table A.8**). Then, the tertiary structures of all *D. septosporum* CE proteins were predicted using AlphaFold2 (**Table 3.1** and **Appendix Figure A.12**). From the 29 proteins, five had a predicted TM-score <0.5, resulting in a predicted structure not reliable for comparison with other characterized structures in the RCSB PDB.

Most of the CE proteins had sequence similarity with hypothetical proteins of unknown functions from the NCBI and JGI databases (**Table 3.1**). This is consistent with the fact that most effectors have low or no similarity to proteins with a known function. However, when these proteins had their tertiary structures predicted with AlphaFold2, it was possible to identify possible roles/functions that sequence similarity was unable to uncover.

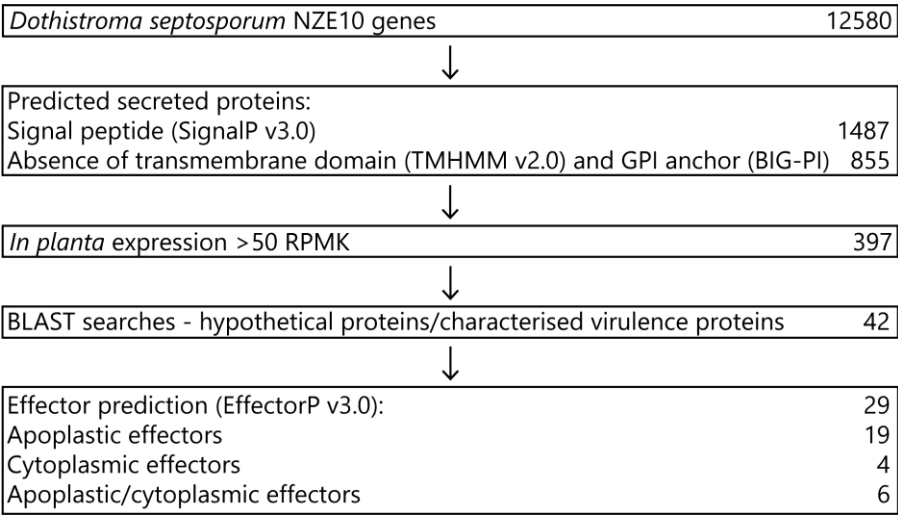


Figure 3.1. Pipeline results for *Dothistroma septosporum* NZE10 effector prediction. The number of proteins from each step is shown on the right. RPMK: Reads Per Million per Kilobase.

The 29 *D. septosporum* CEs were then compared with the predicted proteins from *C. minus* and *P. pluvialis* by tBLASTn and BLASTp, to find possible orthologues in these two foliar pine pathogens. **Table 3.1** and **Appendix Figure A.13** shows the *D. septosporum* CE proteins identified in this study and their orthologues, if present, in *C. minus* and *P. pluvialis*, as well as the top hit in the RCSB PDB to the predicted tertiary structure of each *D. septosporum* CE protein.

Table 3.1. *Dothistroma septosporum* NZE10 candidate effector proteins (DsCEs) and their orthologues in *Cyclaneusma minus* NZFS809 and *Phytophthora pluvialis* NZFS3000.

Pathogen protein ID ^a	Description ^b	EffectorP prediction ^c	Orthologue identity ^d	DsCE AlphaFold structural prediction RCSB PDB accession ID ^e
<i>D. septosporum</i> only				
Ds19040	HP	Apoplasic		-
Ds24695	HP	Dual		Microneme antigen – 2yil (10.3)
Ds42387	HP	Cytoplasmic		-
Ds43416	DsEcp20-4	Apoplasic		Root induced effector protein TSP1 – 7cwj (18.4)
Ds51158	DsEcp20-2	Dual		Major allergen Alt a 1 subunit – 4aud (9.0)
Ds51311	DsEcp20-1	Dual		Root induced effector protein TSP1 – 7cwj (9.3)
Ds69113*	Phialide A-like	Apoplasic		16.4 kDa salivary peptide – 7kc8 (10.1)
Ds70155*	Cerato-platanin-like	Apoplasic		Cerato-platanin-1 – 3m3g (23.2)
Ds70694**	DsEcp20-3	Apoplasic		Effector protein PevD1 – 5xmz (9.3)
Ds71565	HP	Dual		-
Ds74283	HP	Apoplasic		30 kDa protein – 4efq (12.6)
Ds74790	HP	Apoplasic		Alpha-galactosyl-binding lectin 4nds – (5.7)
Ds74875	HP	Apoplasic		TMV resistance protein – 5v6i (9.1)
Ds75009	Hydrophobin-like	Apoplasic		Hydrophobin – 2pl6 (6.2)
Ds129328	HP	Dual		14-3-3 protein – 6a5s (7.9)

Table 3.1. Continued.

Pathogen protein ID ^a	Description ^b	EffectorP prediction ^c	Orthologue identity ^d			DsCE AlphaFold structural prediction RCSB PDB accession ID ^e
Ds131088	HP	Cytoplasmic				-
Ds158381**	DsEcp2-1	Apoplastic				Kp4 toxin – 1kpt (6.8)
<i>D. septosporum</i> and <i>C. minus</i>			Ds and Cm			
Ds46236* – Cm7343	DsEcp6-like	Apoplastic	31.8	Extracellular protein–6 - 4b8v (27.5)		
Ds67456 – Cm3176	HP	Cytoplasmic	45.7	Glucuronyltransferase – 1v84 (9.0)		
Ds69845 – Cm7313	HP	Apoplastic	45.8	Pyocyanin demethylase – 5k21 (6.0)		
Ds70057** – Cm835	DsEcp32-like	Apoplastic	29.1	Trypsin inhibitor – 4j2k (9.1)		
Ds71487** – Cm2492	RcCD1 orthologue	Apoplastic	36.5	Serine proteinase inhibitor – 3e8l (11.9)		
Ds75860 – Cm835	DsEcp32-like	Dual	28.6	16.4 kDa salivary peptide – 7kc8 (9.7)		
Ds124808 – Cm8683	HP	Apoplastic	25.9	-		
Ds131290* – Cm4412	LysM CBD	Apoplastic	49.2	Chitinase A – 5bum (7.4)		
Ds153323 – Cm5304	HP	Cytoplasmic	34.9	Chaperone modulator protein – 3ucs (3.4)		
Ds174276* – Cm835	DsEcp32-like	Apoplastic	26.3	Trypsin inhibitor – 4j2k (9.7)		
<i>D. septosporum</i>, <i>C. minus</i> and <i>P. pluvialis</i>			Ds and Cm	Ds and Pp	Cm and Pp	
Ds69335* – Cm8840 – Pp7927	CfEcp57-1/CAP superfamily	Apoplastic	40.1	15.2	15.9	Pry1 protein – 5jys (19.1)
Ds131885** – Cm2721 – Pp10632	VmE02 orthologue	Apoplastic	64.2	65.8	64.9	EGF family domain containing protein – 4z81 (8.5)

^a *Dothistroma septosporum* NZE10 (Ds) JGI protein ID; *Cyclaneusma minus* NZFS809 (Cm) ID (McDougal, unpublished); *Phytophthora pluvialis* NZFS3000 (Pp) ID (Studholme et al., 2016).

^b Functional predictions based on tBLASTn and BLASTp searches (E-value < 10⁻⁵). HP: similarity with other proteins described as hypotheticals.

^c Protein sequences were queried at <https://github.com/JanaSperschneider/EffectorP-3.0>. Dual prediction: apoplasic/cytoplasmic prediction.

^d Percentage full-length pairwise amino acid identity between each orthologous pair from the three pine pathogens.

^e DsCE protein structural predictions were made using AlphaFold2 (Jumper et al., 2021) and the predicted structure was queried against those in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using the protein structure comparison server, Dali (Holm, 2020). The top hit is shown, next to the RCSB PDB accession ID, and in parenthesis the Z-score is shown. A significant hit was considered if the Z-score was ≥ 2. The Dash symbol indicates that the AlphaFold2 tertiary predicted structure was not reliable for comparisons due to low pLDDT and TM-scores.

* DsCE proteins that were previously expressed in *Nicotiana benthamiana* and *Nicotiana tabacum* using an *Agrobacterium tumefaciens*-mediated transient expression to assess their ability to elicit cell death (Guo et al., 2020; Hunziker et al., 2018). The second asterisk indicates the DsCE proteins that previously triggered cell death in *N. benthamiana* and/or *N. tabacum* in those studies.

From the 29 DsCEs identified, ten proteins have likely orthologues in the *C. minus* genome analysed, with their amino acid identities ranging from 25.9% to 64.2% (**Table 3.1**). From these ten proteins, only two are also present in *P. pluvialis*, therefore being shared between the three pine pathogens. The two sets of proteins are Ds69335, Cm8840 and Pp7927, and Ds131885, Cm2721 and Pp10632. This low number of shared proteins was expected, considering the evolutionary distance between fungi and oomycetes. Moreover, all 29 *D. septosporum* CEs, with the exception of two (Ds19040 and Ds74875) have homologues in other fungal pine pathogens analysed: *G. abietina*, *E. deformans*, *Cr. ribicola*, *Cr. quercuum* f. sp. *fusiforme*, *Fu. circinatum*, *L. acicola*, *Pc. pini-densiflorae* (**Appendix Table A.9**).

In summary, based on *D. septosporum* genomic and transcriptomic information, together with effector predictions and protein comparisons with *C. minus* and *P. pluvialis*, it was possible to identify two different CE proteins shared between the two fungal pathogens *D. septosporum* and *C. minus*, as well as the oomycete pathogen *P. pluvialis*. Furthermore, it was possible to identify a set of conserved (core) CEs among fungal species, including other pine pathogens (**Table 3.1** and **Appendix Table A.9**). The two CE proteins shared between *D. septosporum*, *C. minus* and *P. pluvialis* are also present in the genomes of the seven other forest pathogens analysed here, suggesting they might have important core virulence functions. Next, all *D. septosporum* CEs and selected *C. minus* and *P. pluvialis* proteins from **Table 3.1** were cloned for functional testing in non-host plants for their ability to trigger chlorosis or cell death (and thus to be potentially recognised by immune receptors). This is shown in the next section.

3.3.2 Candidate effector protein Ds74283 from *Dothistroma septosporum* NZE10 triggers cell death in *Nicotiana benthamiana*

To determine if all the 29 *D. septosporum* CE proteins identified in **Table 3.1** have the ability to trigger plant defence responses, the gene encoding each protein was cloned into a binary vector, then transformed into *A. tumefaciens* strain GV3101 for use in an ATTA of non-host species. Proteins were assessed for their ability to trigger chlorosis or cell death in *N. benthamiana* and *N. tabacum*. From these 29 CEs, 14 proteins have been tested in previous studies on these non-host species, as follows:

- In **Chapter 2**, proteins belonging to the DsEcp20 (Ds43416, Ds51158, Ds51311 and Ds70694) and DsEcp32 (Ds70057, Ds75860 and Ds174276) families were tested for cell death induction.
- Eight other CE proteins were previously tested in both *Nicotiana* species by Hunziker et al. (2021) or Guo et al. (2020). These were Ds46236, Ds69113, Ds69335, Ds70155, Ds71487, Ds131290, Ds131885 and Ds158381 (DsEcp2-1), of which Ds71487, Ds131885 and DsEcp2-1 triggering cell death responses. All of these CE proteins were re-tested in this Chapter and confirmed the results observed previously.

The remaining 14 new proteins identified in this Chapter and listed in **Table 3.1** were also tested for their ability trigger chlorosis or cell death in the non-host plants *N. benthamiana* and *N. tabacum*. From those, four CEs found only in *D. septosporum* (of the three foliar pine species investigated) triggered cell death responses in *N. benthamiana* but not in *N. tabacum* and are discussed below. CE proteins that are shared between *D. septosporum* and *C. minus* or shared between the three pine pathogens are discussed in **Section 3.3.3**.

Effector candidates found only in *Dothistroma septosporum*

As expected, the ATTA positive control INF1 triggered strong cell death, consistent with its recognition by the LRK1 immune receptor in *Nicotiana* species (Kamoun et al., 1997; Kanzaki et al., 2008), while the empty expression vector pICH86988 that was used as a negative control gave no chlorosis or cell death.

Ds74283 consistently triggered cell death and chlorosis in all infiltration zones of *N. benthamiana*. The degree of cell death varied between a strong response, indistinguishable from cell death elicited by positive control INF1, to cell death covering approx. 50% of the entire infiltration zone (and associated with weak chlorosis) or strong chlorosis (**Figure 3.2A** and **C**). The protein did not trigger chlorosis or cell death in *N. tabacum* (**Figure 3.2B** and **D**). Given how consistent Ds74283 was in triggering chlorosis/cell death in *N. benthamiana*, orthologous proteins from two other Dothideomycetes, *F. fulva* and *Venturia inaequalis*, were also tested for their ability to elicit chlorosis or cell death in both *Nicotiana* species. The orthologue from *F. fulva* (JGI

ID 189800) and the two homologues from *V. inaequalis* (JGI ID 17736 and 23981) also triggered cell death in *N. benthamiana*, but not *N. tabacum* (**Appendix Figure A.14**). Interestingly, the *D. septosporum* proteins appeared to trigger a more consistent, strong cell death response than the proteins from the other two other pathogens (**Appendix Figure A.14**).

The three other proteins found only in *D. septosporum* that were tested by ATTA triggered plant defence responses that were not consistent between replicates in *N. benthamiana* (**Figure 3.2A** and **C**). Ds19040 triggered either weak patchy cell death or chlorosis in 33% of infiltration zones. Ds24695 only triggered strong cell death in 8% of infiltration zones. Ds42387 triggered some degree of plant response in 25% of infiltration zones, but only 8% showed strong cell death. None of the proteins triggered visible chlorosis or cell death in *N. tabacum* (**Figure 3.2B** and **D**).

These results suggest that *D. septosporum* secretes apoplastic proteins that are likely being recognized by receptors in the non-host *N. benthamiana* to activate the plant's immune system.

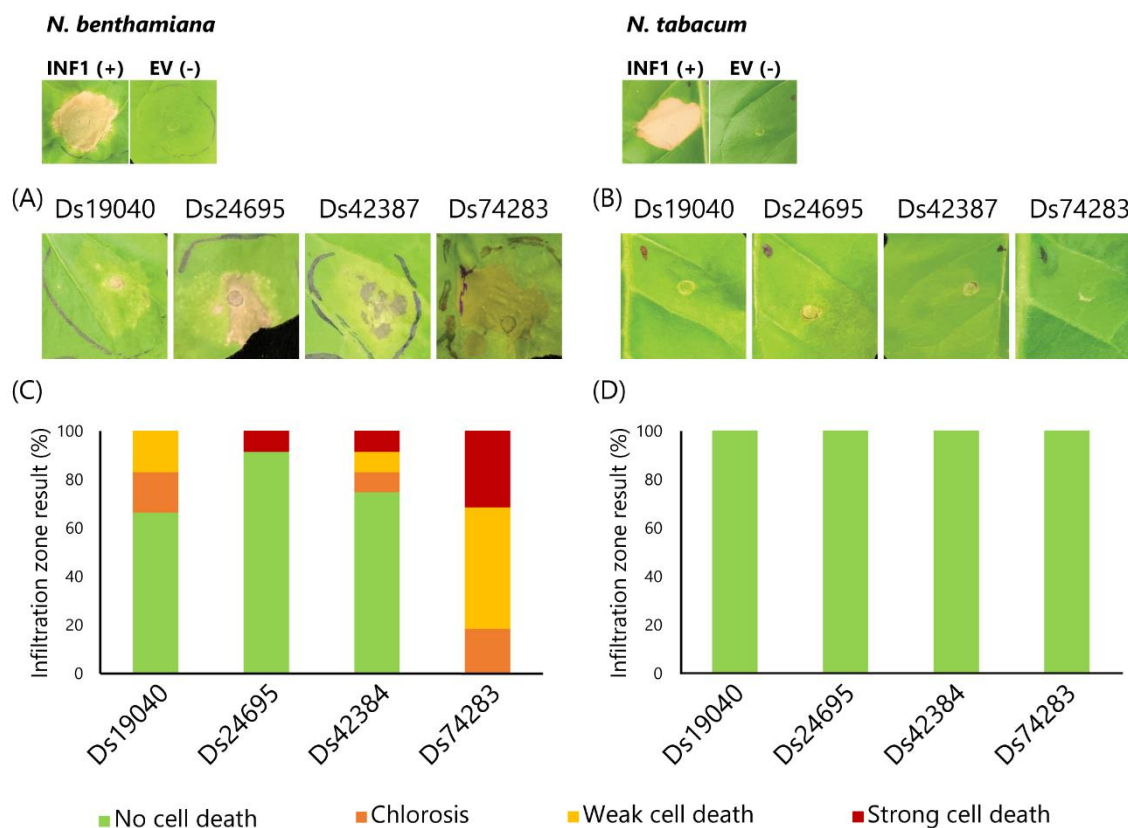


Figure 3.2. Candidate effectors (CEs) from the foliar pine pathogen *Dothistroma septosporum* NZE10 trigger chlorosis or cell death in non-host plants. Here, CE proteins from *D. septosporum* that had no homologues in the other two pine pathogens were expressed in *Nicotiana benthamiana* (A) and *Nicotiana tabacum* (B) using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit chlorosis or cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. Graphs display the percentages of infiltration zones in *N. benthamiana* (C) and *N. tabacum* (D) that showed chlorosis or cell death in response to each protein, divided into four categories: strong cell death, weak cell death, chlorosis, and no cell death (no visible response). INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Ds: *D. septosporum*. Photos were taken 7 days after infiltration.

3.3.2.1 Apoplastic localization is required for Ds74283 from *Dothistroma septosporum* to trigger plant chlorosis or cell death

Since Ds74283 was the only effector candidate of those in **Figure 3.2** to trigger a consistent chlorosis/cell death response, it was determined whether its secretion is required for the induction of these responses. For that, a version of the protein lacking the PR1 α signal peptide for secretion to the apoplast was transiently expressed in

N. benthamiana. Results show that Ds74283 without a signal peptide lost its ability to trigger cell death (**Figure 3.3A**). To confirm this result was not due to the absence of the protein, this protein was detected from agroinfiltrated plants via Western blotting; the predicted molecular weight of the protein without the signal peptide is 22.6 kDa (**Figure 3.3B**). Taken together, these results indicate that secretion to the apoplastic environment is essential for the chlorosis/cell death-inducing activity of Ds74283 in *N. benthamiana*.

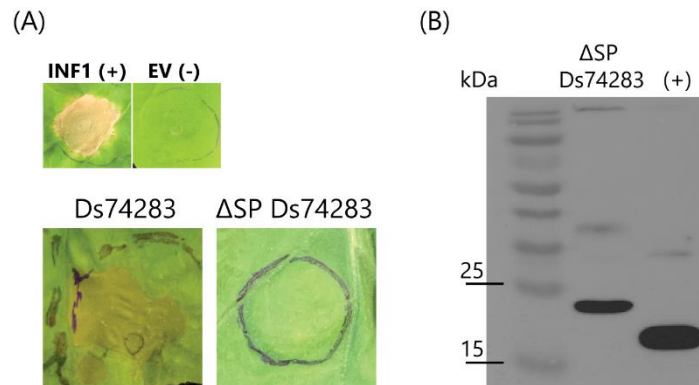


Figure 3.3. Apoplastic localization is required for chlorosis/cell death elicitation by Ds74283 from *Dothistroma septosporum* in *Nicotiana benthamiana*. **(A)** Ds74283 was expressed without a signal peptide in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess its ability to elicit chlorosis or cell death. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Ds: *D. septosporum*; ΔSP: no signal peptide. Photos were taken 7 days after infiltration. **(B)** Western blot detection of Ds74283 lacking the PR1α signal peptide (ΔSP) in *N. benthamiana*; expected size: 23.0 kDa. The positive control is *D. septosporum* candidate effector DsEcp20-3 (17.4 kDa). Anti-FLAG antibody was used for immuno-detection. The marker on the left of the membrane was the PageRuler™ Prestained Protein Ladder (ThermoScientific).

3.3.2.2 Ds74283 is predicted to have a tertiary structure that is similar to proteins with characterized tertiary structures from other pathogens

The tertiary structure of Ds74283 was predicted using AlphaFold2 (**Figure 3.4**). The programme predicted a structure with a pLDDT score of 89.5 and a predicted TM-score of 0.869. The predicted structure has six β-sheets with two antiparallel β-strands each, one β-strand, a single turn α-helix, and various loops. Even though not predicted, two cysteine residues at positions 157 and 184 are likely to form a disulphide bond, as these residues are close to each other in the predicted structure (**Figure 3.4A**).

The top hit when comparing the predicted structure of Ds74283 with solved tertiary structures present in the RCSB PDB was a lipoprotein from the silk moth *Bombyx mori*, Bmlp7 (RSCB PDB ID 4EFQ) and, in particular the C-terminal domain of this protein, which has a β -trefoil carbohydrate-binding fold (Pietrzyk et al., 2012). This protein is part of the 30K protein superfamily, present in the hemolymph of insects (Ujita et al., 2002). Even though the biological function of these proteins is unknown, it is thought that they might be involved in insect defence against fungal pathogens (Ujita et al., 2002). Other structural hits of Ds74283 include a cytolytic pore-forming toxin (PFT) with a β -trefoil lectin domain from the pathogenic bacterium *Vibrio vulnificus* (RSCB PDB ID 4OWK) (Kaus et al., 2014).

Even though these proteins all have a β -trefoil fold, their α -helix topology varies and, in the case of Ds74283 and the cytolsin, their disulphide bonds are not structurally aligned (**Figure 3.4**). The structures of Ff189800, Vi17736 and Vi23981 were also predicted using AlphaFold2 (with a respective pLDDT and TM-score of 89.4 and 0.873 for Ff189800, 85.6 and 0.802 for Vi17736 and, 84.7 and 0.796 for Vi23981) (**Appendix Figure A.14**). All three proteins are predicted to form one disulphide bond that is conserved with the predicted Ds74283 structure.

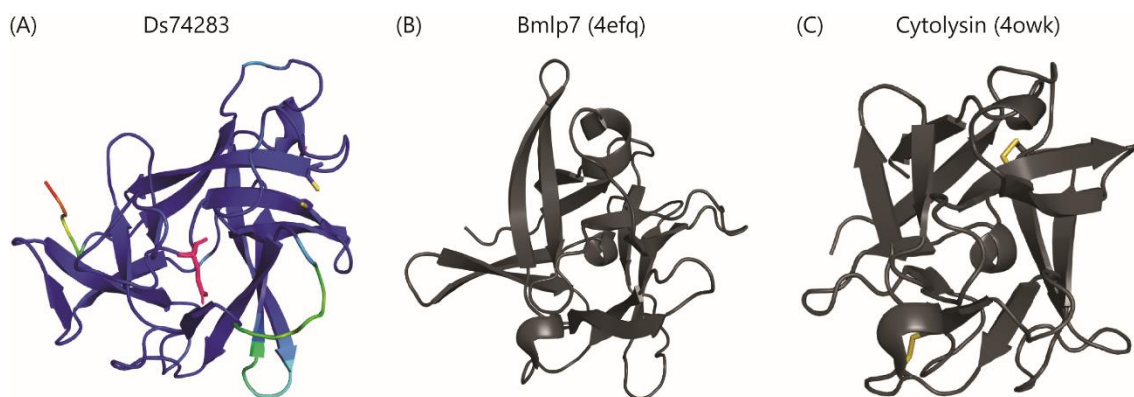


Figure 3.4. Predicted protein tertiary structure of Ds74283 from *Dothistroma septosporum* and related proteins from other fungal species. **(A)** Structure of Ds74283 predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Cysteines are shown as yellow sticks. The position of a non-synonymous change in a surface-exposed amino acid (E150Q) in *D. septosporum* NZE10 when compared with 18 other *D. septosporum* isolates is shown as a magenta stick at the centre of the structure. **(B)** C-terminus of characterized structure of Bmlp7 from *Bombyx mori* (Research Collaboratory for Structural Bioinformatics Protein Data Bank protein data bank [RCSB PDB] ID: 4efq) (Pietrzyk et al., 2012). **(C)** Characterized structure of cytolysin from *Vibrio vulnificus* (RCSB PDB ID: 4owk) (Kaus et al., 2014). Ds: *D. septosporum*.

3.3.2.3 *Ds74283* is expressed during pine infection and is present in other fungal plant pathogens, including other *Dothistroma septosporum* isolates and *Dothistroma pini*

The chlorosis/cell death elicitor Ds74283 is a small, secreted protein of 195 amino acids with two cysteine residues from *D. septosporum* (**Figure 3.5**). The gene coding for this protein has two exons and it is highly expressed during the Mid and Late *in planta* stages of pine infection, when compared with the Early stage and *in vitro* (Bradshaw et al., 2016) (**Appendix Table A.8**).

Ds74283 is widely conserved in other fungal species, including other isolates of *D. septosporum* and *D. pini*, the other causal agent of DNB. The gene encoding this protein is present in all other 18 *D. septosporum* genomes available, with the Colombian (COLN) isolate being the most diverse, but with only one of 16 polymorphisms (6%)

resulting in an amino acid difference (E150Q) compared to the NZE10 sequence (**Figure 3.5** and **Appendix Figure A.15**). The glutamate residue at position 150 is predicted to be surface-exposed (**Figure 3.4A**); therefore, changes in this position might alter the affinity of the protein for its target. However, a glutamate to glutamine substitution is unlikely to cause a major change in the protein conformation (Betts & Russell, 2003). Between the two *Dothistroma* species, there is 93.8% full-length amino acid identity of Ds74238, with seven non-synonymous modifications (**Figure 3.6** and **Appendix Figure A.15**). However, none of those amino acid changes are predicted to affect the structure or function of the proteins.

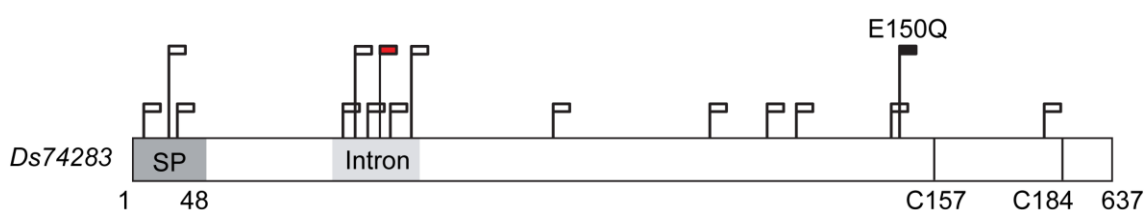


Figure 3.5. Schematic diagram showing sequence variation in *Dothistroma septosporum* NZE10 candidate effector protein Ds74283 across 18 other *D. septosporum* isolates. The dark grey box indicates the signal peptide, and the light grey box indicates the intron position. The black flag shows the position of a nucleotide variation that led to a non-synonymous difference, with the specific amino acid difference indicated above the flag. White flags show the positions of synonymous nucleotide variations. The red flag shows the position of a nucleotide deletion. Vertical black lines show the positions of cysteine residues with the respective amino acid positions indicated. Nucleotide positions of the signal peptide are shown under the gene. SP: signal peptide; Ds: *D. septosporum*.

Ds74283 was also assessed for its presence in other species of fungi (JGI MycoCosm database) to determine its level of conservation, specifically among other plant pathogens. Ds74283 had significant BLASTp hits with proteins exclusively annotated as hypothetical proteins according to searches in NCBI and JGI using BLASTp and tBLASTn. Ds74283 had approx. 600 hits in the JGI MycoCosm protein database, however only 56 of these hits were with fungal plant pathogens, most of them belonging to the Dothideomycetes class. From these JGI hits, a homologue of Ds74283 was shown to be present in 35 species of plant pathogens and, in certain species, this protein appears to be part of a multigene family, which is not the case for *D. septosporum*. For example, *Zymoseptoria tritici* and *V. inaequalis* have two paralogues and

Pseudocercospora musae has three. As mentioned before, homologues of Ds74283 are not found in the genome of *C. minus*. Interestingly, however, the protein is present in *E. deformans* (with 33% full-length pairwise amino acid identity), which belongs to the same family as *C. minus*. In this case, the protein is also not encoded by a multigene family. Besides *E. deformans*, homologues of this protein are also found in pine pathogens, *G. abietina*, *L. acicola* and *Pc. pini-densiflorae* and the pine endophyte *Lo. nitens* (**Appendix Table A.9**). **Figure 3.6** shows the protein alignment of some homologues of Ds74283 present in other fungal pathogens from different classes, including the most diverse *D. septosporum* isolate (COLN) and *D. pini*.

3.3.3 *Dothistroma septosporum* candidate effector protein orthologues from *Cyclaneusma minus* NZFS809 and *Phytophthora pluvialis* NZFS3000 also trigger cell death in *Nicotiana* species

Based on the *D. septosporum* CEs previously identified in **Section 3.3.1 (Table 3.1)**, it was possible to identify orthologues of these CEs in the genomes of *C. minus* and *P. pluvialis*. As mentioned above, from the 29 CEs of *D. septosporum*, 12 of these had orthologues in *C. minus*, and from these, only two were also present in *P. pluvialis*. The orthologues in *C. minus* of *D. septosporum* CEs that were known to trigger non-host cell death responses were tested for their ability to trigger chlorosis or cell death in both non-host *Nicotiana* species (**Figure 3.7** for *N. benthamiana* and **Figure 3.8** for *N. tabacum*). The same was done for the CEs shared between *D. septosporum*, *C. minus* and *P. pluvialis* (**Figure 3.7** and **Figure 3.8**).

Orthologues between *Dothistroma septosporum* and *Cyclaneusma minus*

As mentioned in **Section 3.3.2**, Ds71487 was known to trigger chlorosis in *N. benthamiana* and consistent strong cell death in *N. tabacum* (Hunziker et al., 2021). The orthologue in *C. minus*, Cm2492, triggered either chlorosis or weak cell death in 72% of infiltration zones, in *N. benthamiana* (**Figure 3.7A** and **C**). Interestingly, in *N. tabacum*, the *C. minus* protein only triggered chlorosis or weak cell death in 33% of infiltration zones, while the *D. septosporum* protein triggered strong cell death in 83% of infiltration zones (**Figure 3.8A** and **C**). There is no orthologue of these proteins in *P. pluvialis*.

Ds75860 is part of the DsEcp32 family described in **Chapter 2**, renamed as DsEcp32-1. This protein consistently triggered cell death in *N. benthamiana*, varying between a weak or strong response in almost all infiltration zones (**Figure 3.7A** and **C**). In contrast, Cm835, the orthologue of DsEcp32-1 in *C. minus*, only triggered a strong cell death response in approx. 4% of infiltration zones in *N. benthamiana*, and a weak cell death response in 9%. The remaining infiltration zones gave either weak chlorosis (27%) or, in most cases, no cell death (59%). In *N. tabacum*, neither Ds75860 or Cm835 triggered cell death consistently, with Ds75860 eliciting a response in only 14% of infiltration zones, while Cm835 triggered a weak cell death response in 8% of infiltration zones, and chlorosis in 25% (**Figure 3.8A** and **C**).

Orthologues between *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

Next, the two common CEs shared between all three pine pathogens *D. septosporum*, *C. minus* and *P. pluvialis* were assessed to determine whether they triggered chlorosis or cell death in *N. benthamiana* and *N. tabacum*.

From the first set of cross-kingdom CEs, Ds69335 was previously tested (Hunziker et al., 2021) and did not trigger chlorosis or cell death in *N. benthamiana* or *N. tabacum*, even though it could be detected via Western blot, indicating the protein was being expressed in these non-host plants (**Appendix Figure A.16**). Orthologues in the two other pine pathogens, *C. minus* and *P. pluvialis*, were identified and tested for their ability to trigger chlorosis or cell death. However, just as was observed for the *D. septosporum* protein, Cm8840 and Pp7927 did not trigger chlorosis or cell death in either *Nicotiana* species (**Figure 3.7** and **Figure 3.8**); however, neither of these proteins could be detected via Western blot despite several attempts. The orthologue from *F. fulva* (JGI ID 192236) was also tested in both *N. benthamiana* and *N. tabacum*, and it did not elicit any plant response (**Appendix Figure A.17**). These results suggest that these proteins are not recognized by an *Nicotiana* immune receptor and may instead have a role in suppressing the plant immune responses. Or that they are being recognized, but the recognition does not result in a visible response, or that there is a low affinity between these proteins and the respective receptor or even that they are unstable and therefore are degraded in the plant apoplast before they can be recognized.

From the second set of cross-kingdom proteins, Ds131885 was previously shown to trigger strong chlorosis in *N. benthamiana* and consistent strong cell death in *N. tabacum* (Hunziker et al., 2021). Orthologues of this protein were also found in *C. minus* and *P. pluvialis*, and similar responses were observed for these two other proteins. In *N. benthamiana*, Cm2721 and Pp10632 consistently triggered strong chlorosis in all infiltration zones (**Figure 3.7B** and **C**). In *N. tabacum*, Pp10632 triggered strong cell death in all infiltration zones analysed, while Cm2721 triggered strong cell death across 20% of infiltration sites, weak cell death with some chlorosis across 40% of infiltration sites and strong chlorosis across 40% of infiltration sites (**Figure 3.8B** and **C**). The orthologue from *V. inaequalis* (JGI ID 13495) was also tested in both non-host plants

and consistently triggered chlorosis in *N. benthamiana* and cell death in *N. tabacum* (**Appendix Figure A.17**).

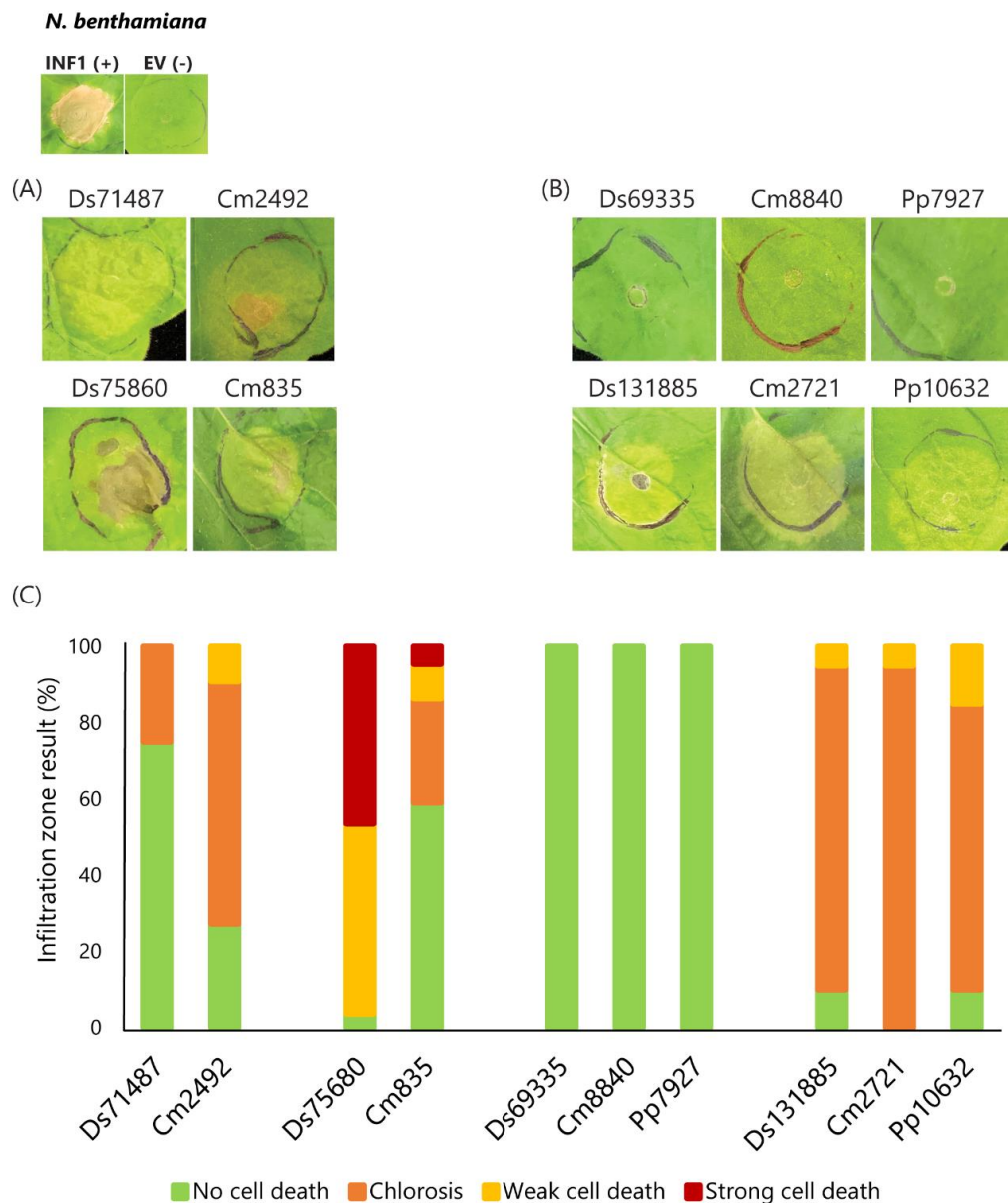


Figure 3.7. Candidate effectors from foliar pine pathogens *Dothistroma septosporum* NZE10, *Cyclaneusma minus* NZFS809 and *Phytophthora pluvialis* NZFS3000 trigger chlorosis or cell death in the non-host plant *Nicotiana benthamiana*. **(A)** Orthologues between *D. septosporum* and *C. minus*. **(B)** Orthologues between *D. septosporum*, *C. minus* and *P. pluvialis*. Proteins were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit chlorosis or cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. **(C)** Graphs display the percentage of infiltration zones that showed chlorosis or cell death in response to each different protein, divided into four categories: strong cell death, weak cell death, chlorosis, and no cell death (no visible response). INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*. Photos were taken 7 days after infiltration.

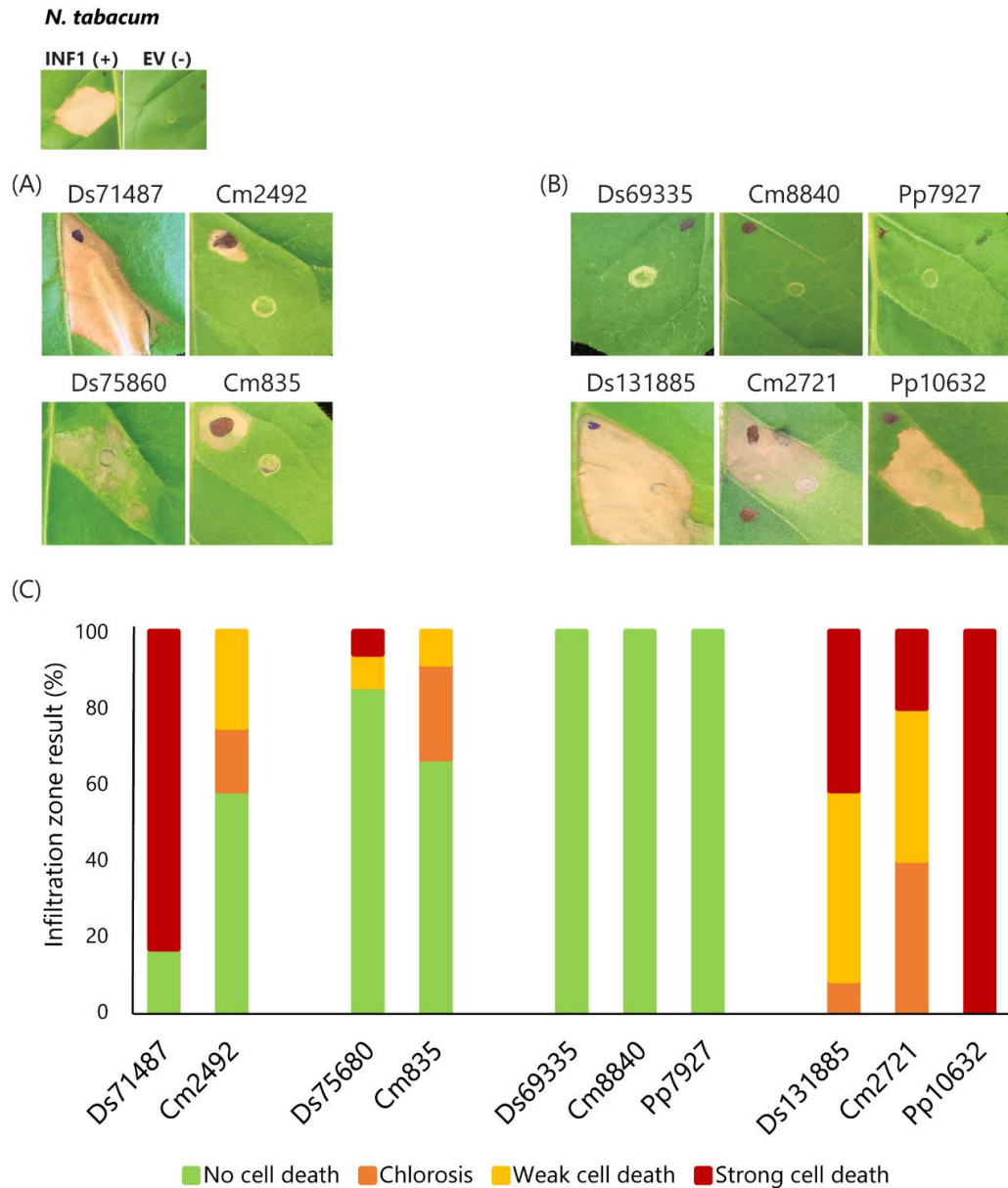


Figure 3.8. Candidate effectors from foliar pine pathogens *Dothistroma septosporum* NZE10, *Cyclaneusma minus* NZFS809 and *Phytophthora pluvialis* NZFS3000 trigger chlorosis or cell death in the non-host plant *Nicotiana tabacum*. **(A)** Orthologues between *D. septosporum* and *C. minus*. **(B)** Orthologues between *D. septosporum*, *C. minus* and *P. pluvialis*. Proteins were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit chlorosis or cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. **(C)** Graphs display the percentage of infiltration zones that showed chlorosis or cell death in response to each different protein, divided into four categories: strong cell death, weak cell death, chlorosis, and no cell death (no visible response). INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*. Photos were taken 7 days after infiltration.

3.3.3.1 Apoplastic localization is required for orthologous candidate effector proteins of *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* to trigger plant cell death

To determine if secretion of CEs from the three pine pathogens is required for induction of cell death, all proteins that previously elicited strong and consistent cell death, but now lacking the PR1 α signal peptide for secretion to the apoplast, were transiently expressed in *N. benthamiana* or *N. tabacum*.

The results show that all proteins without a signal peptide lost their ability to trigger cell death (**Figure 3.9A**). To confirm this result was not due to the absence of the protein, each of these proteins were detected from agroinfiltrated plants via Western blotting (**Figure 3.9B**). This was done for Cm2492, Cm835, Cm2721 and Pp1032, since the remaining Ds75860, Ds71487 and Ds131885, without their signal peptide, were previously detected via Western blotting (Ds75860 - **Appendix Figure A.8**) (Ds71487 and Ds131885 - Hunziker et al. (2021)); the predicted molecular weight of the proteins without their signal peptide is 23 kDa for Cm2492 (Ds71487 orthologue), 22.6 kDa for Cm835 (Ds75860 orthologue), 16.3 kDa for Cm2721 and 16.2 kDa for Pp10632 (Ds131885 orthologues). Taken together, these results indicate that secretion into the apoplastic environment is essential for the cell death-inducing activity these proteins have in *Nicotiana* species. The difference in sizes observed could be explained by post-translational modifications, such as N-linked glycosylation, or multimer formation.

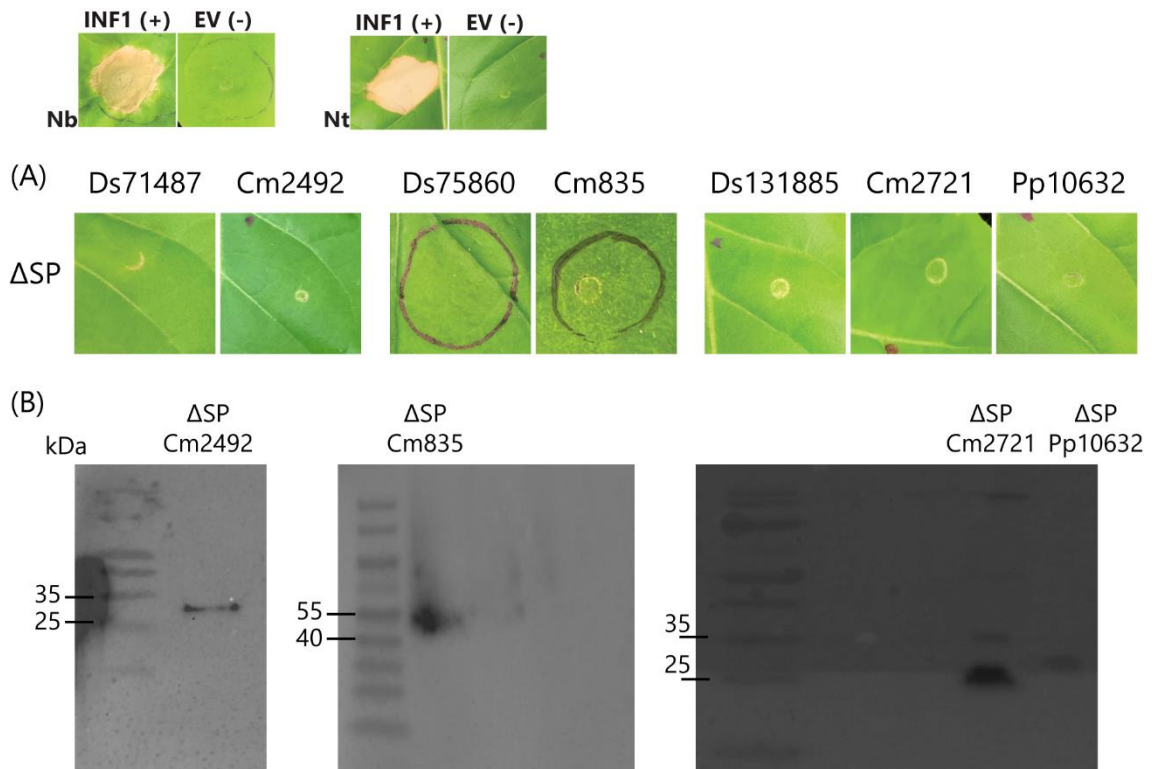


Figure 3.9. Apoplastic localization is required for cell death elicitation by *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* candidate effector (CE) proteins. **(A)** Proteins that previously triggered consistent cell death in non-host *Nicotiana benthamiana* and/or *Nicotiana tabacum* were expressed without a signal peptide (Δ SP) using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. INF1, *Phytophthora infestans* elicitin positive cell death control; EV, empty vector negative no-cell death control; Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*; Nb: *N. benthamiana*; Nt: *N. tabacum*. Photos were taken 7 days after infiltration. **(B)** Western blot detection of CE proteins that previously triggered a cell death response in *N. benthamiana* and/or *N. tabacum*, but that now lack the PR1 α signal peptide (Δ SP). Expected sizes (kDa): Δ SP Cm2492: 23.0; Δ SP Cm835: 22.6; Δ SP Cm2721: 16.3; Δ SP Pp10632: 16.2. Anti-FLAG antibody was used for immuno-detection. Marker on the left of each membrane was the PageRuler™ Prestained Protein Ladder (ThermoScientific).

3.3.3.2 Orthologues between *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* have structural similarity to characterized proteins from other pathogens

The finding that some of the CE proteins from the three pine pathogens triggered chlorosis/cell death in non-host species, suggests they are being recognized as effectors

by non-host plant receptors. On this basis, they were evaluated further to determine their structures and expression patterns *in planta*.

Orthologues between *Dothistroma septosporum* and *Cyclaneusma minus*

Ds71487 and Cm2492 are orthologues of the fungal PAMP RcCDI1 (JGI ID 10140) from the barley pathogen *Rhynchosporium commune* (Franco-Orozco et al., 2017). Homologues of this protein from different fungal species, like *D. septosporum* and *C. minus*, have the ability to trigger cell death in non-host species (Franco-Orozco et al., 2017). RcCDI1 has 40.7% full-length pairwise amino acid identity with Ds71487 and 39.7% with Cm2492. Alignment between the three proteins shows conservation of four cysteine residues (**Figure 3.18**). *Ds71487* has three exons and encodes a 200-amino acid protein, with five cysteine residues. The gene is strongly up-regulated throughout the three pine infection stages, reaching its highest expression at the late stage (Bradshaw et al., 2016) (**Appendix Table A.8**). *Cm2492* does not have any introns, with a protein of 202 amino acids and five cysteine residues.

The structures of Ds71487 and Cm2492 were predicted using AlphaFold2 (**Figure 3.10**). For Ds71487, AlphaFold2 predicted a tertiary structure with a pLDDT score of 91.3 and a predicted TM-score of 0.88, while Cm2492 had a pLDDT score of 90.8 and a predicted TM-score of 0.87. Both structures are made of four sets of two-antiparallel-strand β -sheets and two three-strand β -sheets and possess two disulphide bonds each that align in the structures (**Figure 3.10A and B**). Cm2492 has an extra single-turn α -helix.

Searches against solved tertiary structures in the RCSB PDB showed that both proteins had significant hits with serine proteinase inhibitors, botulinum neurotoxin and ricin-b lectins, all of which have a β -trefoil fold. However, the topology of these structures differs from those of Ds71487 and Cm2492, and the disulphide bonds do not align (**Figure 3.10**).

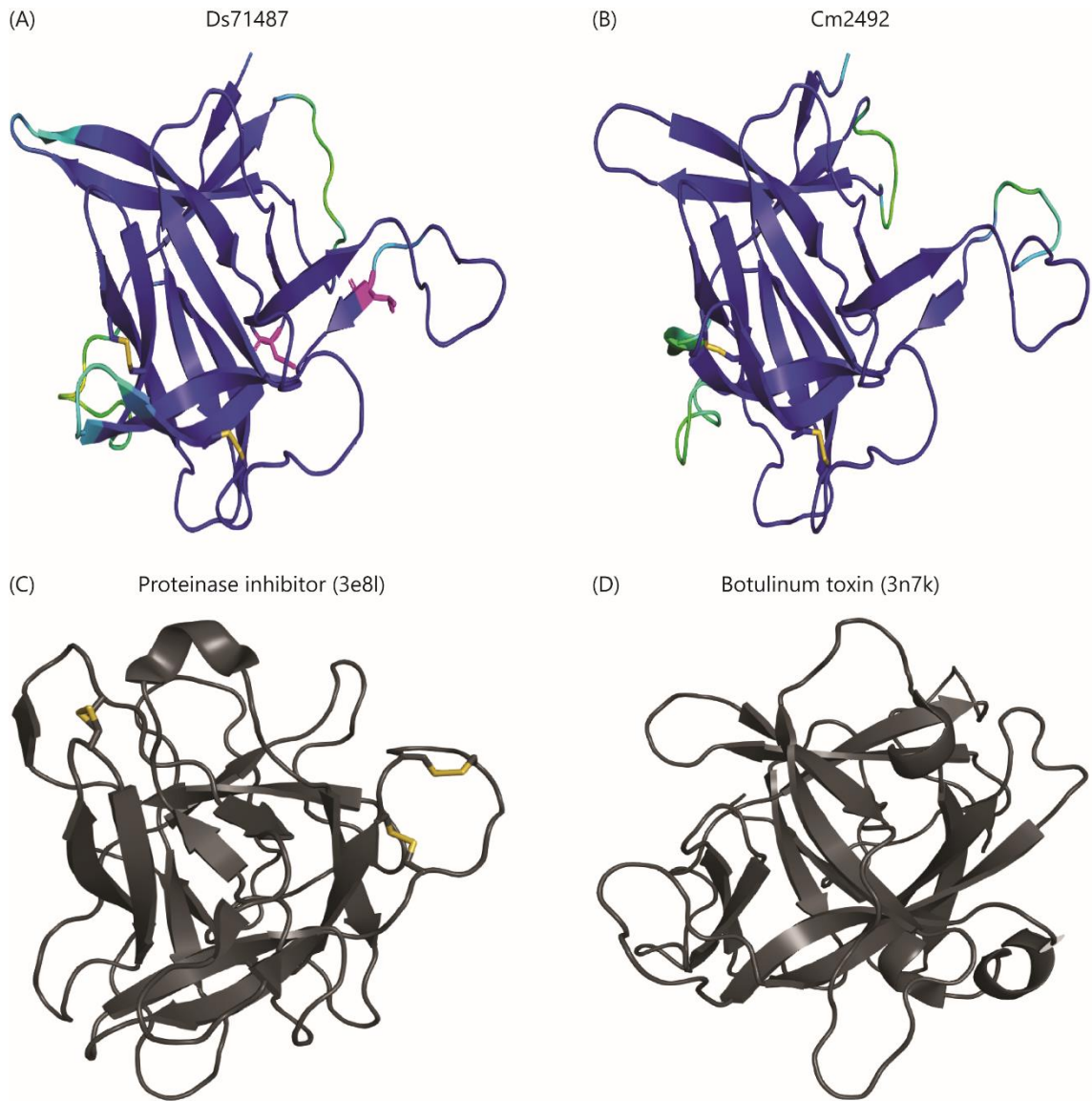


Figure 3.10. Predicted protein tertiary structures of Ds71487 from *Dothistroma septosporum* and Cm2492 from *Cyclaneusma minus*. **(A)** Predicted structure of Ds71487. The positions of two non-synonymous changes in surface-exposed amino acids (D135E and L156V) in *D. septosporum* NZE10 when compared with Guatemalan isolate (GUA1) are shown as magenta sticks. **(B)** Predicted structure of Cm2492. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Disulphide bonds are shown as yellow sticks. **(C)** Characterized structure of a proteinase inhibitor from *Sagittaria sagittifolia* (Research Collaboratory for Structural Bioinformatics Protein Data Bank [RCSB PDB] ID: 3e8l) (Bao et al., 2009). **(D)** N-terminus of a characterized structure of botulinum toxin from *Clostridium botulinum* (RCSB PDB ID: 3n7k) (Karalewitz et al., 2010). Ds: *D. septosporum*; Cm: *C. minus*.

The effector protein pair Ds75860 (DsEcp32-1) and Cm835 are part of the Ecp32 family. Both proteins have a full-length pairwise amino acid identity of 28.6% (**Table 3.1**). As described in **Section 2.3.1** of **Chapter 2**, Ds75860 encodes a 199-amino acid protein, with four cysteine residues and no introns. The gene that codes for this protein is only expressed at a low level *in planta* (**Appendix Table A.8**). Cm835 also does not have any introns, with a protein of 203 amino acids and five cysteine residues.

AlphaFold2 was previously used to predict the structure of Ds75860 and now was used to predict the structure of Cm835, showing a tertiary structure with a pLDDT score of 89.6 and a predicted TM-score of 0.869 (**Figure 3.11**). As expected for members of the Ecp32 family, the predicted tertiary structures of both Ds75860 and Cm835 had similarity with the solved tertiary structures of trypsin inhibitors and lectins; proteins that share a β -trefoil fold and are known virulence factors in plant-pathogenic fungi (Renko et al., 2012; Sabotič et al., 2019; Varrot et al., 2013). Ds75860 has five β -sheets composed of two antiparallel strands and one β -strand, while Cm835 has six β -sheets composed of two antiparallel strands, and both proteins differ in their α -helix topology. Cm835 has two predicted disulphide bonds and Ds75860 has one predicted and, due to residue proximity, cysteines at positions 66 and 77 are likely to also form a bond (**Figure 3.11**).

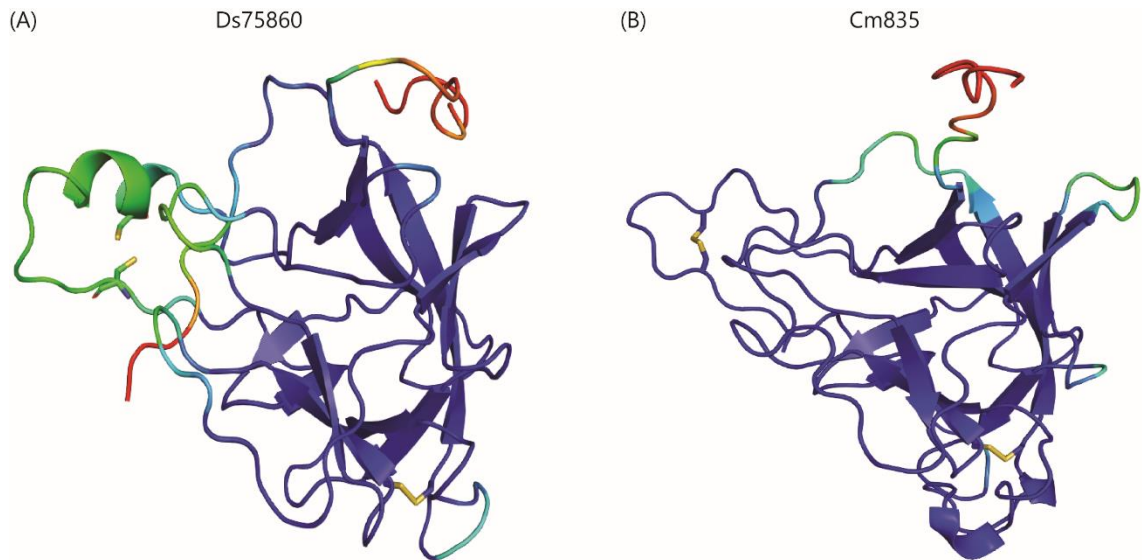


Figure 3.11. Predicted protein tertiary structures of Ds75860 from *Dothistroma septosporum* and Cm835 from *Cyclaneusma minus*. **(A)** Predicted structure of Ds75860. **(B)** Predicted structure of Cm835. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Disulphide bonds and/or cysteine residues are shown as yellow sticks. Ds: *D. septosporum*; Cm: *C. minus*.

Orthologues between *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

Even though neither Ds69335, Cm8840 or Pp7927 triggered chlorosis or cell death in non-host species, they were of interest on the basis of being identified in all three foliar pine pathogens, with Ds69335 being strongly up-regulated at the Early (biotrophic) stage of pine infection by *D. septosporum* (**Appendix Table A.8**). Ds69335, Cm8840 and Pp7927 are orthologous proteins that are also homologues of CfEcp57-1 (JGI ID 192236), an apoplastic protein from *C. fulvum*. CfEcp57-1 has 78%, 41% and 15% full-length pairwise amino acid identity with Ds69335, Cm8840 and Pp7927, respectively (**Figure 3.19**).

All four proteins contain a conserved domain of the 'cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins' (CAP) superfamily. CAP proteins are virulence factors in pathogenic fungi (Schneider & Di Pietro, 2013).

Ds69335 consists of two exons that encode a 281-amino acid protein, with five cysteine residues (**Figure 3.12**). *Cm8840*, with three exons, encodes a 297-amino acid protein, also with five cysteine residues, whilst *Pp7927* has no introns and encodes a protein with 473 amino acids including six cysteine residues (**Figure 3.12**).

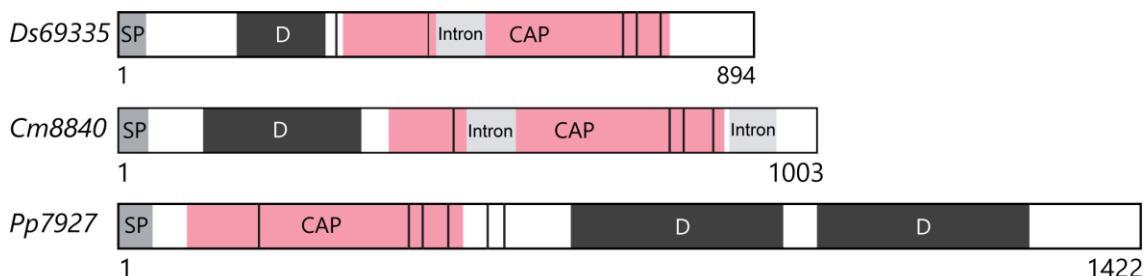


Figure 3.12. Schematic representation of genes *Ds69335* from *Dothistroma septosporum* NZE10, *Cm8840* from *Cyclaneusma minus* NZFS809 and *Pp7927* from *Phytophthora pluvialis* NZFS3000. Dark grey boxes indicate the signal peptide positions and light grey boxes indicate intron positions. The predicted intrinsically disordered regions (IDRs) are indicated by black boxes, while red boxes indicate the predicted 'cysteine-rich secretory proteins, antigen 5, and pathogenesis-related' 1' (CAP) domain. Vertical black lines show the positions of cysteine residues. SP: signal peptide; D: IDR; Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*.

Tertiary structure prediction of the three proteins from the pine pathogens using AlphaFold2 revealed that all three proteins have strong predicted structural homology to pathogen-related yeast 1 (Pry1) (RCSB PDB ID 5jys), a sterol and lipid-binding CAP protein from *Saccharomyces cerevisiae* (Darwiche et al., 2016) (**Figure 3.13**). For *Ds69335*, AlphaFold2 predicted a tertiary structure with a pLDDT score of 74.9 and a predicted TM-score of 0.683, while *Cm8840* had a pLDDT score of 74.1 and a predicted TM-score of 0.651, and *Pp7927* had a pLDDT score of 68.1 and a predicted TM-score of 0.545.

The structure of the Pry1 protein, like those reported for other CAP proteins, has an approx. 150-amino acid domain that adopts an α - β - α sandwich conformation, with two disulphide bonds, all of which is conserved in the predicted structures of *Ds69335*, *Cm8841* and *Pp7927* (**Figure 3.13**). *Ds69335* cysteines residues at positions 150 and 230, and at positions 225 and 248 likely form disulphide bonds. The same is also likely for cysteine residues at positions 103 and 183 of *Cm8840*.

The sterol-binding region of Pry proteins comprises a loop of aromatic amino acids, known as caveolin-binding motif (CBM) (Choudhary et al., 2014). A key feature of the CBM is the presence of aromatic amino acids at positions 1, 3 and 8 (**Figure 3.13F**), which seem to be essential for binding to occur. This was also highlighted by comparisons between the CBM of MpPR-1 members from *Moniliophthora perniciosa*, the hemibiotrophic fungal pathogen of cacao. It was shown that members with non-aromatic amino acids at position 3 did not have the ability to bind to sterol. Substitution of a non-aromatic residue in this position for an aromatic residue converts the protein into a sterol-binding protein (Darwiche et al., 2017a). Even though Ds69335 and Cm8840 have conserved aromatic amino acids in positions 1 and 8, they have a glycine and a glutamic acid in position 3, respectively (**Figure 3.13F**). Glycine can be substituted by other small amino acids, such as alanine, which was shown to be a functional substitution for sterol binding in one of the PR-1 proteins from *M. perniciosa* (Darwiche et al., 2017a). This could suggest that Cm8840 might have a functional CBM, while Ds69335 does not. Pp7927 is atypical, as it does not have any conserved aromatic amino acids at positions 1, 3 or 8 (**Figure 3.13F**). It remains to be determined if the CAP proteins from *D. septosporum*, *C. minus* and *P. pluvialis* have the ability to bind to sterol, and even if they do not, they can still have the ability to bind fatty acids and other small hydrophobic molecules, which occurs in the parallel helix from the CAP domain.

All three proteins are also predicted to have IDRs, with Ds69335 and Cm8840 having putative N-terminal IDRs and Pp7927 putatively having a long C-terminal IDR (**Figure 3.12**). **Figure 3.13** shows the predicted tertiary structures of Ds69335, Cm8840 and Pp7927 without the putative IDRs; the complete structure of these proteins is shown in **Appendix Figure A.18**.

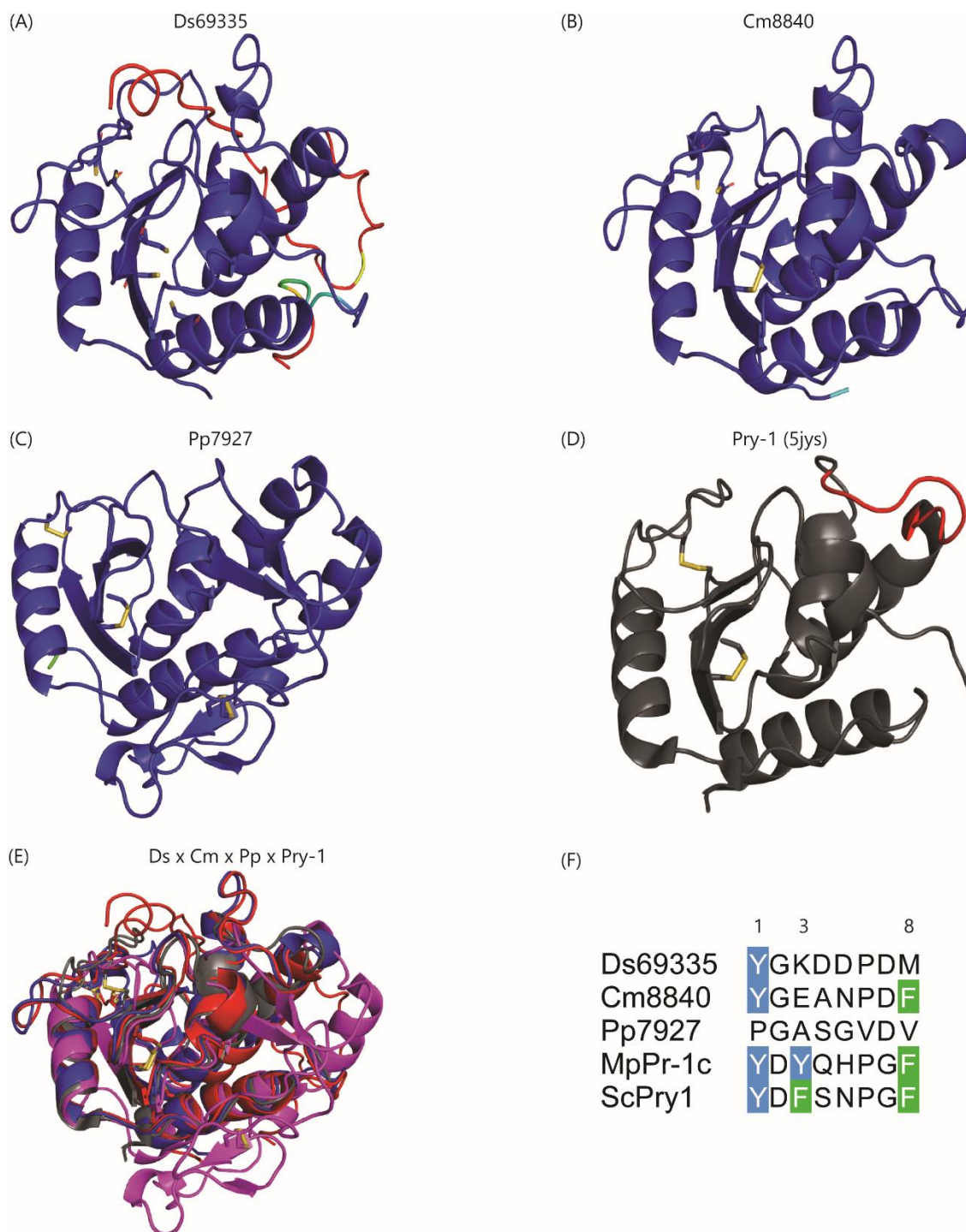


Figure 3.13. Predicted protein tertiary structure of Ds69335 from *Dothistroma septosporum*, Cm8840 from *Cyclaneusma minus* and Pp7927 from *Phytophthora pluvialis* without the predicted intrinsically disordered region. **(A)** Predicted structure of Ds69335. **(B)** Predicted structure of Cm8840. **(C)** Predicted structure of Pp7927. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions.

Disulphide bonds and/or cysteine residues are shown as yellow sticks. **(D)** Characterized structure of Pry1 from *Saccharomyces cerevisiae* (Research Collaboratory for Structural Bioinformatics Protein Data Bank [RCSB PDB] ID: 5jys) (Darwiche et al., 2016). The caveolin-binding motif (CBM) is highlighted in red. **(E)** Alignment of predicted Ds69335 (red), Cm8840 (blue), Pp7927 (magenta) and Pry1 (grey). **(F)** Alignment of the caveolin-binding motif from *D. septosporum*, *C. minus*, *P. pluvialis*, *Moniliophthora perniciosa* (GenBank accession AEZ63362.1) and *S. cerevisiae* (GenBank accession PTN40155.1). Aromatic amino acids tyrosine (Y) and phenylalanine (F) are highlighted in blue and green, respectively. Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*; Sc: *S. cerevisiae*; Mp: *M. perniciosa*.

The second cross-kingdom CE was identified by similarity between proteins Ds131885, Cm2721 and Pp10632. All three proteins are orthologues of VmE02 (GenBank accession KUI69068.1) (Nie et al., 2019), a PAMP from the apple-pathogenic fungus *Valsa mali*, which has 60% full-length amino acid identity with Ds131885, 63% with Cm2721 and 68% with Pp10632 (**Figure 3.20**).

Ds131885 is a 144-amino acid protein encoded by a gene with three exons. *Ds131885* is highly expressed during pine infection, especially during the Mid and Late necrotrophic stages when compared with Early stage and *in vitro* (**Appendix Table A.8**). *Cm2721* also has three exons and encodes a protein of 148 amino acids. Both proteins have ten cysteine residues, which are conserved between them. *Pp10632*, with two exons, encodes a 146-amino acid protein, with 11 cysteine residues, ten of them being conserved between the three proteins. A schematic of the genes coding for each of these proteins is shown in **Figure 3.14**.

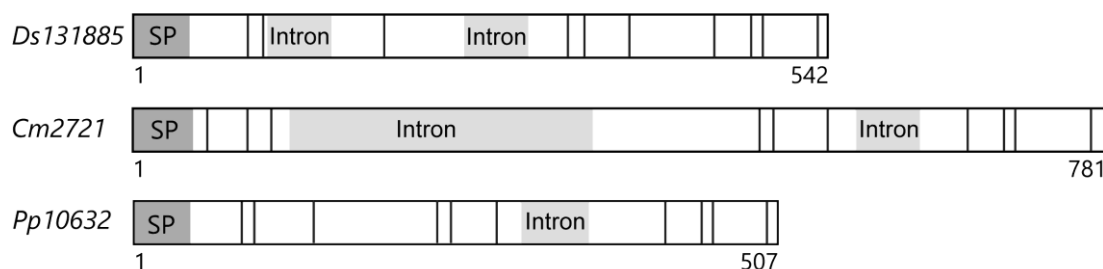


Figure 3.14. Schematic representation of genes *Ds131885* from *Dothistroma septosporum* NZE10, *Cm2721* from *Cyclaneusma minus* NZFS809 and *Pp10632* from *Phytophthora pluvialis* NZFS3000. Dark grey boxes indicate the signal peptide positions and light grey boxes indicate intron positions. Vertical black lines show the positions of cysteine residues. SP: signal peptide; Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*.

The tertiary structures of Ds131885, Cm2721, Pp10632 and the characterised *Va. mali* PAMP VmE02 were also predicted using AlphaFold2 (**Figure 3.15**). For Ds131885, AlphaFold2 predicted a tertiary structure with a pLDDT score of 94.3 and a predicted TM-score of 0.874, while Cm2721 had a pLDDT score of 94.3 and a predicted TM-score of 0.877, Pp10632 had a pLDDT score of 94.7 and a predicted TM-score of 0.886 and VmE02 had a pLDDT score of 95.4 and a predicted TM-score of 0.882. Both Ds131885 and Cm2721 have two β -sheets composed of three antiparallel strands and two β -sheets composed of two antiparallel strands. Pp7927 has two β -sheets composed of three antiparallel strands and another composed of two antiparallel strands. All three structures have five aligned disulphide bonds (**Figure 3.15**).

The top structural hit for all three proteins in the RCSB PDB was domain II (DII) of apical membrane antigen 4 (AMA4) from *Toxoplasma gondii* (Parker et al., 2016). The protein has one internal disulphide bond that is conserved with Ds131885, Cm2721 and Pp10632 (**Figure 3.15**).

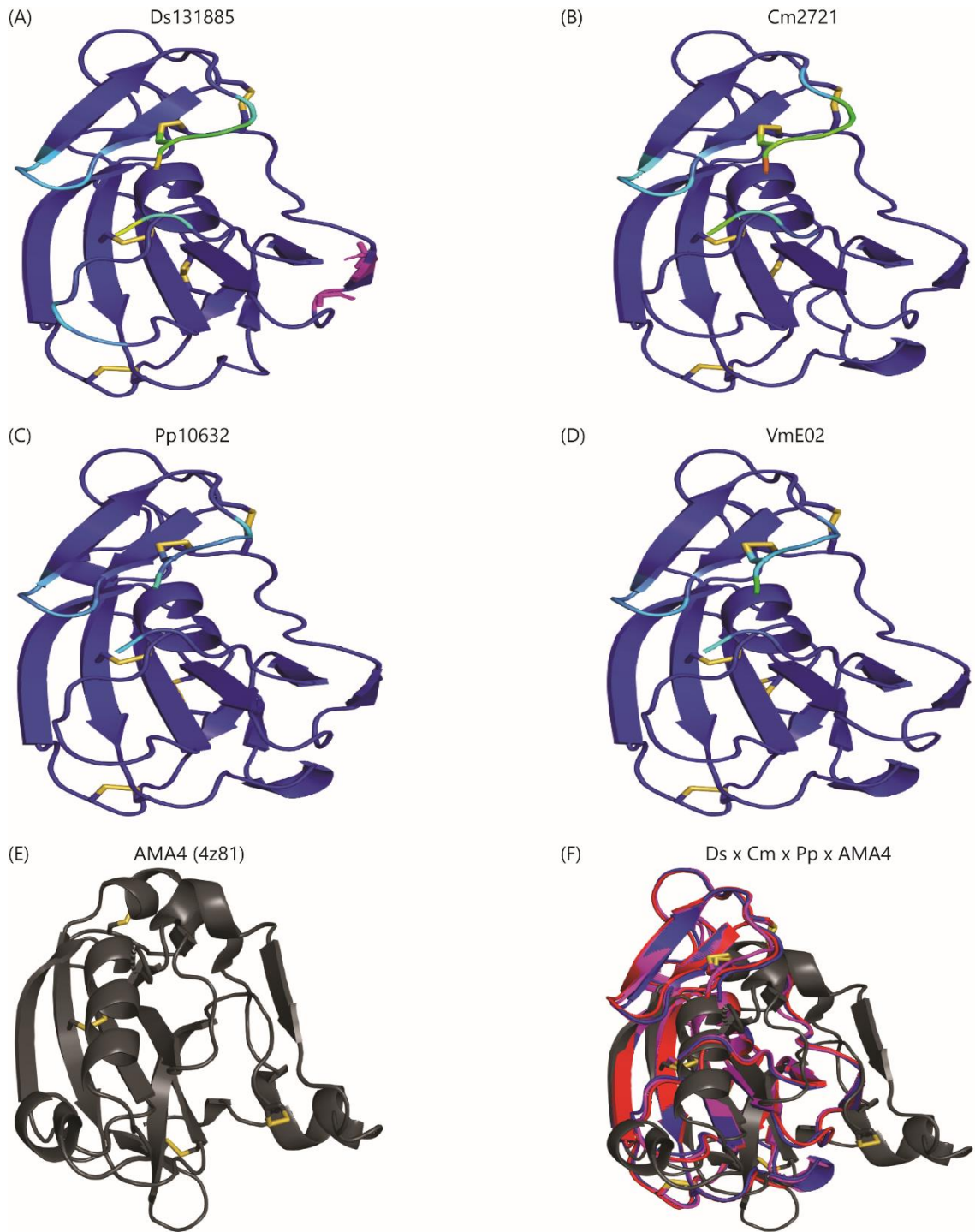


Figure 3.15. Predicted protein tertiary structure of Ds131885 from *Dothistroma septosporum*, Cm2721 from *Cyclaneusma minus* and Pp10632 from *Phytophthora pluvialis*. **(A)** Predicted structure of Ds131885. The position of two non-synonymous changes in surface-exposed amino acids (G109A and A111E) in *D. septosporum* NZE10 when compared with 18 other *D. septosporum* isolates are shown as magenta sticks. **(B)** Predicted structure of Cm2721. **(C)** Predicted structure of Pp10632. **(D)** Predicted structure of VmE02 from *Valsa mali*. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022),

and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Disulphide bonds are shown as yellow sticks. **(E)** Domain II of characterized structure of AMA4 from *Toxoplasma gondii* (Research Collaboratory for Structural Bioinformatics Protein Data Bank [RCSB PDB] ID: 4z81) (Parker et al., 2016). **(F)** Alignment of predicted Ds131885 (red), Cm2492 (blue), Pp10632 (magenta) and AMA4 (grey). Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*; Vm: *Va. mali*.

3.3.3.3 A conserved fold is present in sequence-unrelated proteins from *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

Following structure prediction of CE proteins, it was determined that *D. septosporum* proteins Ds69113 and Ds74283 (present only in *D. septosporum*), and Ds71487 and Ds75860 (both present in *C. minus* as well – Cm2492 and Cm835, respectively), had structural similarity with characterized proteins that have a β -trefoil fold. This was already known for Ds75860 (DsEcp32-1), as discussed in **Section 2.3.2 of Chapter 2**. The β -trefoil fold is characterized by a pseudo-three-fold symmetry aligned vertically through a six-stranded β -barrel, and the threefold repeating motif, typically comprising 40–50 amino acids in length, is comprised of four antiparallel β -strands (Murzin & Chothia, 1992).

According to the Structural Classification Of Proteins (SCOP - <https://scop.mrc-lmb.cam.ac.uk/>) database, the β -trefoil fold can be divided into ten superfamilies, and sequence similarity between proteins from different superfamilies is low. However, conserved hydrophobic residues are believed to stabilize the fold (Feng et al., 2010; Kirioka et al., 2017; Murzin & Chothia, 1992). Full-length pairwise amino acid alignments were performed between all proteins and indicates conservation of hydrophobic residues (**Appendix Figure A.19**). Alignment of the four structures indicates a conserved disulphide bond that could not be identified by sequence alignment (**Figure 3.16A**). These results suggests that, as observed for other β -trefoil proteins, Ds69113, Ds71487, Ds74283 and Ds75860 are structurally related despite having low amino acid similarity.

Ds69113, Ds71487 and Ds75860 all have structural similarities with β -trefoil proteins that belong to the proteinase inhibitor 1-like superfamily, while Ds74283 has

structural similarities with proteins from the 30K lipoprotein C-terminal domain-like superfamily, based on searches of their predicted tertiary structures against those characterized in the RCSB PDB.

Given the similarity in tertiary structure between these sequence-unrelated *D. septosporum* proteins (and *C. minus* orthologues), it was assessed whether the β -trefoil fold was also present in proteins of *P. pluvialis*. For this purpose, the Foldseek server (van Kempen et al., 2022) was used to detect similarities between the predicted structures of the β -trefoil proteins from *D. septosporum* and the predicted structures of *Phytophthora* proteins present in the AlphaFold/UniProt50 v3, AlphaFold/Swiss-Prot v2 and AlphaFold/Proteome v2 databases.

First, the predicted tertiary structures of Ds69113, Ds74283, Ds71487 and Ds75860 were queried against the predicted protein structures of *Phytophthora* species present in the server database. The top structural hit for each of the *D. septosporum* proteins queried was then used to search the RCSB PDB for structural similarity, and it was confirmed that all the *Phytophthora* structures identified based on Foldseek searches had a predicted β -trefoil fold (**Appendix Table A.10**). The amino acid sequence for each of these structures was then compared with the predicted proteins of *P. pluvialis* NZFS3000 (BLASTp, E-value < 10^{-5}). Two proteins were identified (best BLASTp hit); Pp12312 is a 329-amino acid protein and Pp7483 is a 1044-amino acid protein, however none of them are predicted to have a signal peptide. Their tertiary structures were then predicted with AlphaFold2 (**Appendix Figure A.20**) and used to identify proteins with structural similarity in the RCSB PDB. Both *P. pluvialis* proteins had structural similarity with β -trefoil proteins (**Figure 3.16B** and **Appendix Table A.10**).

These results shows that despite being sequence-unrelated, *D. septosporum*, *C. minus* and *P. pluvialis* all have proteins that adopt a β -trefoil fold. It remains to be determined if *P. pluvialis* has other proteins with this fold and if the identified *P. pluvialis* proteins also have the ability to trigger non-host and host cell death responses in the same manner as the fungal ones. Since these proteins do not have a putative signal peptide, testing them in ATTAs would only be possible if they are secreted by a non-classical pathway.

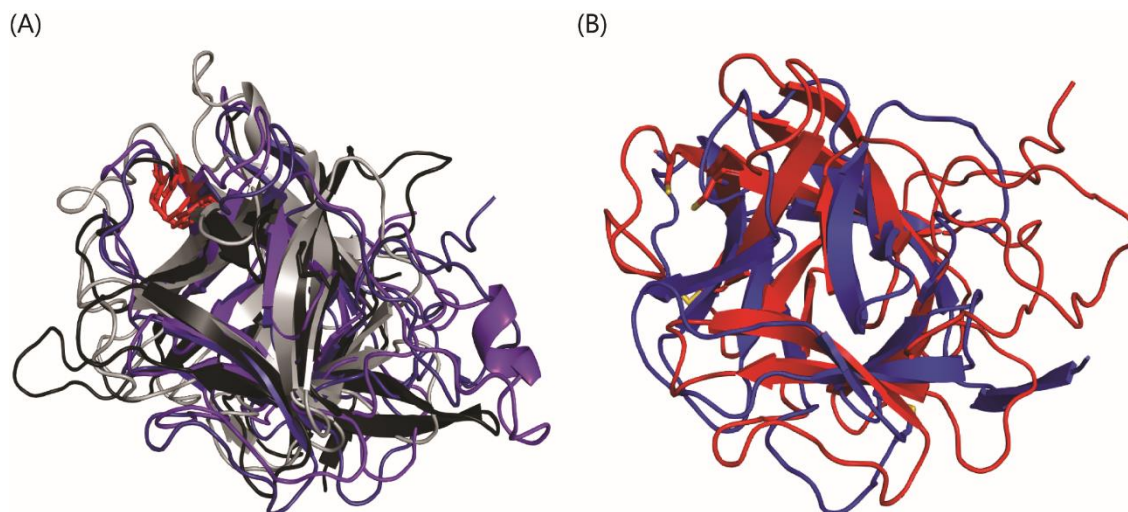


Figure 3.16. Predicted protein tertiary structures of Ds69113, Ds71487, Ds74283 and Ds75860 from *Dothistroma septosporum* have a common β -trefoil fold. **(A)** Alignment of predicted structures of Ds69113 (blue), Ds71487 (black), Ds74283 (grey) and Ds75860 (purple). The conserved disulphide bond is shown as red sticks. **(B)** Alignment of the predicted structure of Ds69113 from *D. septosporum* (red) with the β -trefoil domain of the predicted structure of Pp12323 from *P. pluvialis* (blue). The full predicted structure for Pp12323 is shown in **Appendix Figure A.20**. Protein structures were predicted with AlphaFold2, rendered, and coloured in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022).

3.3.3.4 Candidate effectors from *Dothistroma septosporum* NZE10, *Cyclaneusma minus* NZFS809 and *Phytophthora pluvialis* NZFS3000 are conserved in other fungal and oomycete pathogens

Core effectors are conserved amongst a diverse number of pathogens, with some of them present in bacteria, fungi, and oomycetes. The fact that CE proteins shared between *D. septosporum* NZE10, *C. minus* NZFS809 and *P. pluvialis* NZFS3000 trigger chlorosis/cell death in non-host species, suggests they could be recognized as effectors, and might be conserved in other species of plant pathogens. For this reason, other fungal and oomycete plant pathogens, including other *D. septosporum* isolates, *D. pini* and additional pine pathogens, were assessed for the presence of the CE proteins triggering cell death, as was done for the *D. septosporum* CE Ds74283 (**Section 3.3.2.3**). Sequences from orthologues of the CE proteins Ds69335, Ds71487 and Ds131885 in the most diverse *D. septosporum* isolate and *D. pini* are shown in **Appendix Figure A.15**.

The protein sequences of *D. septosporum* CEs Ds71487 (also present in *C. minus*), Ds69335 and Ds131885 (both present in *C. minus* and *P. pluvialis*) are all very similar to their orthologues from *D. pini* (**Figure 3.18**, **Figure 3.19** and **Figure 3.20**), with 91.6%, 79.1% and 92.4% full-length amino acid identity, respectively, between the two *Dothistroma* species. Regarding different *D. septosporum* isolates, the Colombian (COLN and COLS) isolates showed the greatest amino acid sequence diversity, when compared to strain NZE10, for most of the *D. septosporum* CEs. In fact, for Ds69335 and Ds131885, COLN was the most diverse isolate, while the Guatemalan isolate (GUA1) had the most sequence variation in Ds71487. The three *D. septosporum* NZE10 CE genes, Ds69335, Ds71487 and Ds131885, had similar numbers of DNA polymorphisms when compared to the other 18 *D. septosporum* isolates, with most of those being synonymous nucleotide changes (**Figure 3.17**). Ds69335 and Ds131885 had the highest number of non-synonymous modifications, with 43% (6 out of 14) and 37.5% (6 out of 16), respectively, while Ds71487 only had 19% (3 out of 16) (**Figure 3.17**). Most modifications in Ds69335 were in the IDR (**Appendix Figure A.18**). In Ds69335, a substitution of proline to serine at position 91 is interesting, since the proline residue is considered to be surface-exposed, and substitutions of this residue can alter protein folds (Betts & Russell, 2003) (**Appendix Figure A.18**). The other substitutions in Ds69335, even though in residues predicted to be surface-exposed (N38, V67, A82 and Q157) (**Appendix Figure A.18**) are not likely to cause major changes in protein conformation (Betts & Russell, 2003). The protein Ds69335 from Colombian isolate COLN was tested for chlorosis or cell death-eliciting activity in *Nicotiana* species, along with the orthologues from *D. pini* and *F. fulva* (JGI ID 192236). However, none of the three proteins triggered chlorosis or cell death (**Appendix Figure A.17**). For Ds71487, the two substitutions (D135E and L156V) occur in surface-exposed residues (**Figure 3.10**), but these are also unlikely to change the protein conformation (Betts & Russell, 2003). In Ds131885, there is a substitution of glycine to alanine at position 109 (surface-exposed; **Figure 3.15**), and to aspartate at position 113. Substitutions in glycine to any other amino acids are known to, in many cases, highly impact the structure of proteins (Betts & Russell, 2003), which could alter recognition of the protein by a host immune receptor or other plant target. It would be interesting to determine if these substitutions will lead to differences in their ability to trigger plant cell death.

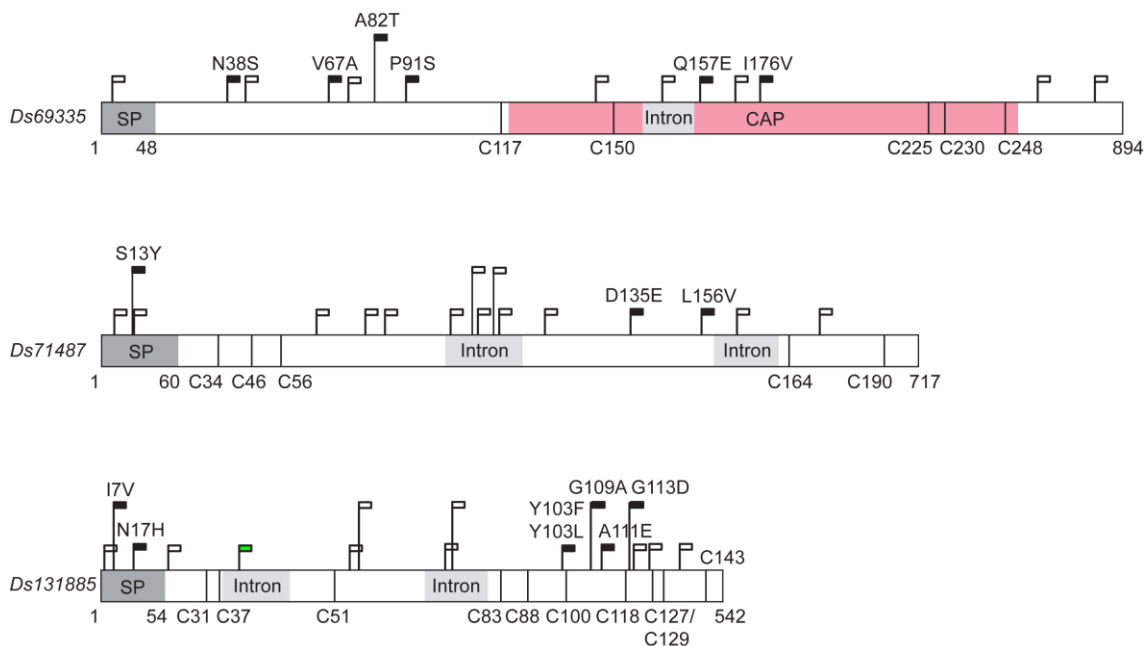


Figure 3.17. Schematic diagram showing sequence variation in *Dothistroma septosporum* NZE10 candidate effector proteins Ds69335, Ds71487 and Ds131885 across 18 other *D. septosporum* isolates. Dark grey boxes indicate signal peptide nucleotide positions and light grey boxes indicate intron positions. Red boxes indicate the predicted 'cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1' (CAP) domain for Ds69335. Black flags show the positions of nucleotide variations that led to non-synonymous changes, with the specific amino acid differences indicated above the flags. White flags show the position of synonymous nucleotide variations. The green flag shows the position of a nucleotide insertion. Vertical black lines show the positions of cysteine residues with the respective amino acid number indicated. SP: signal peptide.

These proteins were also assessed for their presence in other species of fungi (JGI MycoCosm database – BLASTp) and oomycetes (NCBI database – BLASTp and tBLASTn).

Ds71487 had a total of 628 significant hits in the JGI MycoCosm protein database. The protein is present in 97 different species of fungal plant pathogens (different isolates from the same species were excluded); 53 from the Dothideomycetes class, 34 Sordariomycetes class and 10 Leotiomyces. In most of these species, the protein seems to be encoded by a single-copy gene. However, a few species have two copies of the *Ds71847* gene, which is the case for *F. fulva* and *Parastagonospora nodorum*. Homologues of this protein were also found in the other pine pathogens, *G. abietina*, *E. deformans* and *L. acicola*, and the pine endophyte *Lo. nitens* (**Appendix Table A.9**). **Figure 3.18** shows the protein alignment of selected homologues of Ds71487 and Cm2492 present in other

fungal pathogens from different classes, including the most diverse *D. septosporum* isolate (GUA1) and *D. pini*.

<i>D. septosporum</i> NZE10	1 - MKTVAAASAALLSLASVA--TAQTVQSKPFYLVLDCA-SKYNGSALVSCHEGAGIES	54
<i>D. septosporum</i> GUA1	1 - MKTVAAASAALLSLASVA--TAQTVQSKPFYLVLDCA-SKYNGSALVSCHEGAGIES	54
<i>D. pini</i>	1 - MKTFIAASAALLSLASVA--TAQTTQSKPFHLVLDCA-GDYNGYALVACHEGAGIEG	54
<i>C. minus</i>	1 - MKSAVFASIFAFAASSALAQY-DTQSPSFYLLQLISDNSTINGSYLTSCHSAGAAIES	55
<i>Z. tritici</i>	1 - MKSTIAGTAAAL-LALSSSVAAQTQSAFFRLVISSSVKAVDGKVLASACHEGAAIEG	56
<i>E. deformans</i>	1 - MIKSALFNLFLLLVAATGVTAQY-LNQSRIFSLFLVSNSSVNGSTLVPCHEGAAIEG	57
<i>G. abietina</i>	1 - MRSSFLA-GVVAFAATQVAAQYANQSAFFNLVLESKNSTVNGTYLSACHEGAAIEG	56
<i>R. commune</i>	1 - MHLSILT-SCLA-LATGISAQYNNVTSKPFQLILQSSNRTLNGKGLFACHEGAGIEG	55
<i>Co. melonis</i>	1 - MKAHV--IALLGATLASAQF-TNQTRPFWLKFTPCAGSGSETYLSCHSGAGQAG	53
<i>Cy. parasitica</i>	1 - MKTTPLL--LAASAASLASAQT-YNTSAPFTLELSSSNTTIDGQFLGACHSGAAIEA	54
<i>D. septosporum</i> NZE10	55 LCVAATLGSN----VKGSTYMLNTTTGEV--GDKNAGTPTGLVYKLPISGGVGESE	104
<i>D. septosporum</i> GUA1	55 LCVAATLGSN----VKGSTYMLNTTTGEV--GDKNAGTPTGLVYKLPISGGVGESE	104
<i>D. pini</i>	55 LCVSATPGTN----LNGSTYMLNTTSGQV--ADKNAGLPGLVYNLVL-SGGQTESE	104
<i>C. minus</i>	56 LCLYGPEAAD----PDMAETFKLNTTSPY-----VANATGLITWVLQG--SNFNESE	101
<i>Z. tritici</i>	57 LCISDPAGS-----TFQFNNTTGGQS-----ESNVGTITYKLDA--GTVQPSY	96
<i>E. deformans</i>	58 LCVTNNAASP-----STAYYYFNTTGDDVETEPNPGATGILSYILEI--SNDNVTSE	107
<i>G. abietina</i>	57 LCLAGKSPPG-----SYEVYYLNYSSDAT--PDPELGITGLLTWLLQGVNGSFNVSS	106
<i>R. commune</i>	56 LCVGTSGPSS-----TSDTYNFNTTYQQQ-ANQGLPGQGLVTLWLRG--GNFNVSS	104
<i>Co. melonis</i>	54 LCPETTEPVQEANRPTHTNYFLNETQSDY-----NGHLLGALTWNQPI-GNGDPVSS	105
<i>Cy. parasitica</i>	55 LCLDGTNGTA-----TDYNTFTLNVSTSS--TGNAVETGILVWVLET--GDFNVSS	101
<i>D. septosporum</i> NZE10	105 PMQLSYNEASNVALPLFEPSTTATL-VAFDKDNHNMISQSYIDD-----TVVPAKT--	153
<i>D. septosporum</i> GUA1	105 PMQLSYNEASNVALPLFEPSTTATL-VAFDKDNHNMISQSYIDD-----TVVPAKT--	153
<i>D. pini</i>	105 PMQLSYNPASNVALALFEPSTYATQ-VAFDKDNHNMISQSYVDD-----TAAPAKT--	153
<i>C. minus</i>	102 ILNFYFNPTSNIAMPLFEPSETGQA-IAFDCEDNAYISAYLNY-----STSPSPSYG	152
<i>Z. tritici</i>	97 GLTFFYSPTSLAHAQGGIGQDQQ-FGFDKDNKLVHPT-YYDE-----SVYPPKSLG	147
<i>E. deformans</i>	108 PMALSGSYTSNVVPIIFTPGDDGLY-VAFDSGKMNISQSYIDD-----TVYPPQVY--	156
<i>G. abietina</i>	107 ALQLGVQATTNVATPLFYPGDETYNTLGFDDKDNKLVFVS-YFDD-----TVVPGVY--	156
<i>R. commune</i>	105 SLQLTPSPTSDVAVPLFFPGDQGSFGYGFDDKDKLVFAG-YLDN-----TVSPPVY--	154
<i>Co. melonis</i>	106 GMMLNLQIVSNGAIPISFGNTALFTLGFDENEKLFISYRADDKAVAGS--QAPY--	159
<i>Cy. parasitica</i>	102 GLVFTPPLTSNVVAPEFEPGSEIDLVGFDNDDNLFYSSYVDDKTFDGTFTPEI--	157
<i>D. septosporum</i> NZE10	154 ---GELAYYRWYVCKSNYEGTQYSTLNNWAGKYPPQNPSCEKVEVVRKFA*---	201
<i>D. septosporum</i> GUA1	154 ---GEVAYYRWYVCKSNYEGTQYSTLNNWAGKYPPQNPSCEKVEVVRKFA*---	201
<i>D. pini</i>	154 ---GTVAYYRWYVCKSYEGYQYALNNWAGKYPPENPTCEKVEVVRKFV*---	201
<i>C. minus</i>	153 EDGASVQAFYNWYLCRTNDEGYTYETVWVTA-GEVTPDQSAVDLHRVVF*---	203
<i>Z. tritici</i>	148 ---SSKSEYHWYFCNYNYAGYQSLNNAVGDVKKKNTCAKVDVVRYSAA*---	198
<i>E. deformans</i>	157 ---KTIAYYRWYACITNYLGYTYQTLAWVIGTGEPENPTCVKVDVVRVFA*---	204
<i>G. abietina</i>	157 ---QSKPLYRWYACITN-VGYTYNTLAWVIGSHSPENPSQKQVSVKRVFV*---	203
<i>R. commune</i>	155 ---KAQAYYRWYACITN-AGTYQTLAWAVGSGKPNPSCEKVDVVRVFI*---	201
<i>Co. melonis</i>	160 ---GDEAYYRWYCYTFVGNYYRSLVWITA-GPASNPTCEAVDVYREWA*---	206
<i>Cy. parasitica</i>	158 ---TPYPLQWYACWTFDLAYYQTLAWVIG-GVNNPTCAVAVNVRNF*---	204

Figure 3.18. Candidate effector Ds71487 from *Dothistroma septosporum* NZE10 and Cm2492 from *Cyclaneusma minus* NZFS809 have sequence similarity with proteins from other fungal plant pathogens. Ds71487 and Cm2492 were aligned with proteins from plant pathogens *Zymoseptoria tritici* (*Mycosphaerella graminicola* – JGI ID 111505), *Dothistroma pini* and *D. septosporum* GUA1 (the most polymorphic isolate compared to Ds71487 NZE10) (Dothideomycete), as well as *Elytroderma deformans* (JGI ID 657969), *Gremmeniella abietina* (JGI ID 384930) and *Rhynchosporium commune* (GenBank accession KX499545) (Leotiomyces), and *Colletotrichum melonis* (JGI ID 2049) and *Cryphonectria parasitica* (JGI ID 354167) (Sordariomycetes). The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined.

Ds69335 is widely conserved among other fungal and oomycete species. There are two other *D. septosporum* NZE10 proteins with predicted CAP domains, Ds54921 and

Ds138776. Both Ds54921 and Ds138776 are only expressed at a low level *in planta*, and while Ds54921 is predicted to be secreted, Ds138776 does not have a predicted signal peptide. The Ds69335 protein had more than 5000 significant BLASTp hits in the JGI MycoCosm protein database. Of these, approx. 700 were with other fungal plant pathogens, most with more than one gene copy. Hits with fungal pathogens were spread outside the Dothideomycetes class, including hits with species from Leotiomycetes, Sordariomycetes and Ustilaginomycetes. Homologues of these proteins were also found in other pine pathogens, *G. abietina*, *E. deformans*, *Cr. ribicola*, *Fu. circinatum*, *L. acicola* and *Pc. pini-densiflorae*, and the pine endophyte *Lo. nitens* (**Appendix Table A.9**). In oomycetes, homologues of Pp7927 are present in three oomycete families: Peronosporaceae, Pythiaceae and Saprolegniaceae, according to BLASTp searches in the NCBI database. Most hits are with species from the Peronosporaceae family, more specifically with species from the genus *Phytophthora*. The protein is also present in *P. pluvialis* isolate LC9-1, with an identical amino acid sequence as that from *P. pluvialis* isolate NZFS3000. Interestingly, amino acid sequences from Pp7927 (473 amino acids) and homologues in other *Phytophthora* species are much larger in size when compared with the fungal homologues (Ds69335 has 281 amino acids and Cm8840 has 297) (**Appendix Figure A.13**). This has been shown to be true for other members of the CAP superfamily, and it is suggested that changes outside the conserved domain region might mediate protein interactions (Gibbs et al., 2008). The CAP domain, however, seems to be highly conserved between fungi and oomycetes. **Figure 3.19** shows the protein alignment of the predicted CAP domain sequence from some homologues of Ds69335, Cm8840 and Pp7927 present in other fungal and oomycete pathogens from different classes, including the most diverse *D. septosporum* isolate (COLN) and *D. pini*.

<i>D. septosporum</i> NZE10	118	-----VYHHNLHRAN-----	HSVSDIAWDDGLASIAQTIAESC	VYAHNVQEG	159
<i>D. septosporum</i> COLN	118	-----VYHHNLHRAN-----	HSVSDIAWDDGLASIAQTIAESC	VYAHNVEEG	159
<i>D. pini</i>	122	-----VYHHNLHRAN-----	HSVSDIAWDDGLASIAQTIAESC	VYAHNTEEQ	163
<i>C. minus</i>	133	-----YHHNLHRAN-----	HSVSDMTYNSTLAGYAATVAATCV	FAHDLSEG	173
<i>P. pluvialis</i>	31	-----VDRHNYFRTTGLPWSAGDMRR	VGWSSDLATTAATAAKCSATTAA--	G	76
<i>F. fulva</i>	123	-----VYHHNLHRAN-----	HSVGLLEWDLGLASIAQTIAESC	VYAHNTEEG	164
<i>E. deformans</i>	190	-----LYHHNVHRTN-----	HSAPAVDWNGTLASYAATVAASC	VYAHDLTPG	231
<i>G. abietina</i>	171	-----LQYHNYHRAN-----	HSAPALVWEQELADYALITAKTC	VMVHDMTEG	212
<i>Co. gramineum</i>	134	DLESTCINTHNLHRAN-----	HSAPALSWDSQLASYALVTAKTC	VFEDHMSeg	181
<i>P. cactorum</i>	31	-----IDRHNYFRTTGLPWSAGDMRR	IGWSSDLATSAASTASKCSATSAA--	G	76
<i>P. infestans</i>	31	-----IDRHNYFRTTGLPWSAGDMRR	IGWSSDLATSAATTASKCSATSAA--	G	76
<i>P. megakarya</i>	25	--QAIWVDRHNYFRTTGLPWSAGDMRR	IGWGAELATSAASTAEKCSAVTAA--	G	74
<i>D. septosporum</i> NZE10	160	GGGYGQNI AAGVD-----	AANISAIITDLFYNGEEPYFA--	DQY GKDDP	201
<i>D. septosporum</i> COLN	160	GGGYGQNI AAGVD-----	AANVSAITDLFYNGEEPYFA--	DQY GKDDP	201
<i>D. pini</i>	164	GGGYGQNI AAGVD-----	AANISAIITDLFYNGEEPYFA--	DQY GKDDP	205
<i>C. minus</i>	174	GGGYGQNI ASFGETGDVEALGASSMLAGA	ITDMWYNGELGLFL-P-EYYGEANP		225
<i>P. pluvialis</i>	77	VNAYQSA-----	ASN-SSAVVDEAIQQWVVETSMITL	KTLAQPGASGV	118
<i>F. fulva</i>	165	GGGYGQNI AAGVD-----	AANVSAITDLFYNGEEPYFS--	GEYGRDDP	206
<i>E. deformans</i>	232	NGGYGQNI AVYGETGDVEALGEAHQVAQAST	DMWYNGEINSFL-P-EYYGEDNP		283
<i>G. abietina</i>	213	TGGYGQNL AAYGTTGD IASLNTATAVADS	ISNQWVNGEMDNVP----	YQGNP	261
<i>Co. gramineum</i>	182	GGGYGQNL AMWGATGSE-SLGEAKAAQRA	ITEQWYNGEINLYP----	GYGLSSL	230
<i>P. cactorum</i>	77	VNAYESS-----	SAN-SSSIIDEAIQQWVVETSVTTL	KTLQPQASGI	118
<i>P. infestans</i>	77	VNAYQSS-----	SAN-SSSIIDEAIQQWVVETSMITL	KTLSPQASGI	118
<i>P. megakarya</i>	75	VNVFQSS-----	SAN-SSAVIDEAIQQWVVETSMITL	KTLAQPGANGV	116
<i>D. septosporum</i> NZE10	202	DMTNFELFGHFTQIVWKDTISVGCATVQCP	PNGLANTGDGVEPVFTVCNY-----		250
<i>D. septosporum</i> COLN	202	DMTNFELFGHFTQIVWKDTISVGCATVQCP	PNGLANTGDGVEPVFTVCNY-----		250
<i>D. pini</i>	206	DMTNFELFGHFTQIVWKDTISVGCATVQCP	PNGLANTGNDVEPVFTVCNY-----		254
<i>C. minus</i>	226	DFTNFEWGHFSQLVWKNSNQVGCASFQCP	QSTGLF--DFDGFVTCNYG----		273
<i>P. pluvialis</i>	119	-DVGTTGTYNAYSQVLWASTTSVGCATASCS	GGDL-----VVCEYSPAGN		161
<i>F. fulva</i>	207	DMTNFELWGHFSQIVWKGTHVGCATVECP	PNGLANTGDGVEPVFTVCNY-----		255
<i>E. deformans</i>	284	DFSNDFAWGHFSQMVKWSTEEIGCASQYCD	AGTIFS--GFGSWFTVCNY-----		330
<i>G. abietina</i>	262	ATSGVPEYLHFTQVWVVGSSVGCATVQCG	AGI-LP--YHSLYTVVCNY-----		306
<i>Co. gramineum</i>	231	DMSNFLSWGHFSQLVWVDSTTVGCAVN	YCAAGT-LS--SIGSWYTVVCNY-----		276
<i>P. cactorum</i>	119	-DVGTTGTYSYSQVLWASTTSVGCATASCP	SGE I-----VVCEY-----		156
<i>P. infestans</i>	119	-DVGTTGTYSYSQVLWASTTSVGCATASCP	SGGDF-----VVCEY-----		156
<i>P. megakarya</i>	117	-DVGTTGTYSYSQVLWAATTSGCATASCP	SGQ I-----VVCEYSP----		156

Figure 3.19. 'Cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1' protein (CAP) domains from candidate effector Ds69335 from *Dothistroma septosporum* NZE10, Cm8840 from *Cyclaneusma minus* NZFS809 and Pp7927 from *Phytophthora pluvialis* NZFS3000 have sequence similarity with CAP domains from other fungal and oomycete plant pathogens. Ds69335, Cm8840 and Pp10632 CAP domains were aligned with the CAP domain from plant pathogens *Fulvia fulva* (JGI ID 192236), *Dothistroma pini* and *D. septosporum* COLN (the most diverse in sequence from Ds69335 NZE10) (Dothideomycetes), as well as *Elytroderma deformans* (JGI ID 169805) and *Gremmeniella abietina* (JGI ID 523856) (Leotiomyces), *Colletotrichum graminicola* (JGI ID 6113) (Sordariomycetes), and oomycete plant pathogens, *Phytophthora cactorum* (KAG3029317.1), *Phytophthora megakarya* (OWZ11629.1) and *Phytophthora infestans* (XP_002898012.1). CAP domains were predicted through the Conserved Domain Database (CDD) from NCBI. The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black.

The other cross-kingdom protein, Ds131885, had 751 significant BLASTp hits in the JGI MycoCosm protein database. From JGI hits, Ds131885 is present in 134 species of plant pathogens. Ds131885 is encoded by a single-copy gene in *D. septosporum* and

most other fungal species, however, some species have a higher number of Ds131885 homologues, which is the case for *Cr. quercuum* f. sp. *fusiforme* (six), *Macrophomina phaseolina* (four) and *Puccinia graminis* f. sp. *tritici* (seven). The *P. pluvialis* Pp10632 protein has sequence similarity with proteins from two oomycete families, Peronosporaceae and Pythiaceae, according to BLASTp searches in the NCBI database (queried only against oomycete species). In Peronosporaceae, the family to which *P. pluvialis* belongs, the protein seems to be widely distributed in other *Phytophthora* species, with *Phytophthora sojae* and *Phytophthora infestans* having two copies of the gene. The protein is also present in *P. pluvialis* isolate LC9-1, with identical amino acid sequence as the one from isolate NZFS3000. Homologues of these proteins are also present in the other fungal pine pathogens *G. abietina*, *E. deformans*, *Cr. ribicola*, *Cr. quercuum* f. sp. *fusiforme*, *Fu. circinatum*, *L. acicola* and *Pc. pini-densiflorae*, and the pine endophyte *Lo. nitens* (**Appendix Table A.9**). **Figure 3.20** shows the protein alignment of some homologues of Ds131885, Cm2721 and Pp10632 present in other fungal and oomycete pathogens from different classes, including the most diverse *D. septosporum* isolate and *D. pini*.

<i>D. septosporum</i> NZE10	1	---	<u>MQFRTF</u>	<u>IGLAITLAYANATLDPATNSKKGKCPKT</u>	<u>IACSASDVSTAIQ</u>	47	
<i>D. septosporum</i> COLN	1	---	<u>MQFRTF</u>	<u>IGLAITLAYAHATLDPATNSKKGKCPKT</u>	<u>IACSASDVSTAIQ</u>	47	
<i>D. pini</i>	1	---	<u>MQFRTFVGLA</u>	<u>ITLACANATLDPATNSKKGKCPKS</u>	<u>IACSATDVSTAIQ</u>	47	
<i>C. minus</i>	1	MASLLKSL	---	LLLAATLVSANVDSCSSNTKGKCPGTLKCSASASSSAIQ		48	
<i>P. pluvialis</i>	1	---	<u>MHFSVFVAL</u>	<u>VAAAPALVSATLDPASSNSKKGKCPSTYSCSATKVS</u>	<u>KAIQ</u>	47	
<i>E. deformans</i>	1	MVHF	KTVASFLMAIGSLATVNATLDPASSNTQGGKCPGTYSCSASKT	STAIQ		51	
<i>G. abietina</i>	1	---	<u>MQFPNVL</u>	<u>SLLALATMATATLSPASSNTKGKIPSTYNCSATKVS</u>	<u>KAIQ</u>	47	
<i>Co. graminicola</i>	1	---	<u>MNFSSLLLVVLVS</u>	<u>ALADATLEPATSNKKGKCPSKYACSATKVS</u>	<u>KAIQ</u>	47	
<i>Va. mali</i>	1	---	<u>MHITSLFA</u>	<u>ILAAPVMVLATLDPATSNTKGSCPSTYSCDASKVS</u>	<u>NSIQ</u>	47	
<i>P. nicotianae</i>	1	---	<u>MRFTLAFALLAVPALVS</u>	<u>ATLDPCCSNSKKGKCPSTYNCSVTKVS</u>	<u>KAIQ</u>	47	
<i>P. parasitica</i>	1	---	<u>MRFTLAFALLAVPALVS</u>	<u>ATLDPCCSNSKKGKCPSTYNCSVTKVS</u>	<u>KAIQ</u>	47	
<i>P. sojae</i>	1	---	<u>MRFSFALVLLVAPALVS</u>	<u>ATLDPATSNTKGKCPSVYNCSATKVSTAIQ</u>		47	
<i>D. septosporum</i> NZE10	48	AAEC	SHNTRTPGTQTFAVFETDHSYDSSKGAPYGTCSAYTCAVPT	--TFTS		96	
<i>D. septosporum</i> COLN	48	AAEC	SHNTRTPGTQTFAVFETDHSYDSSKGAPYGTCSAYTCAVPT	--TFTS		96	
<i>D. pini</i>	48	AAEC	SHNTRTPGTQTFAVFETDHSYDSSKGAPYGTCSAYTCAVPT	--SFTS		96	
<i>C. minus</i>	49	AAEC	SHNTRTSETQTFAVFETDHEYDSSHGAPYGTCEAYTCTPPT	EDEM	TD	99	
<i>P. pluvialis</i>	48	AAEC	SHNTRTSTTQTFAVFETDHYDGNNGYPYGTCSAYTCDPPT	SAEM	ED	98	
<i>E. deformans</i>	52	AAEC	SHNTRVSGTQTFAVFETDHYDSSHGAPYGTCSAYTCTAPT	ASEM	TD	102	
<i>G. abietina</i>	48	AAEC	SHNTRTSTTQTFAVFETDHYDANNGAPYGTCSAYTCTSP	SSQM	KT	98	
<i>Co. graminicola</i>	48	AAEC	SHNTRTGKAQTFAVFETDHYDASHGAPYGTCSAYTCDAPT	--QMLA		96	
<i>Va. mali</i>	48	AAEC	AYNTRTSETETFAVFETEHKYDDVEGAPYGTCSAYTCTAPT	SSSEM	ET	98	
<i>P. nicotianae</i>	48	AAEC	SHNTRTSTTQTFAVFETDHYDGNNGYPYGTCSAYTCDPPT	SDEM	ED	98	
<i>P. parasitica</i>	48	AAEC	SHNTRTSTTQTFAVFETDHYDGNNGYPYGTCSAYTCDPPT	SDEM	ED	98	
<i>P. sojae</i>	48	AAEC	SHNTRTSETQTFAVFETDHYDGNNGYPYGTCSAYTCE	SPTADV	MTA	98	
<i>D. septosporum</i> NZE10	97	-DSD	CWTFWWSGSGTASGVGTGCI	RSPTTGECGCENSSGQFI	AGSSSCT*	145	
<i>D. septosporum</i> COLN	97	-DSD	CWTFWWSGSGTESGVGTGCI	RSPTTGECGCENSSGQFI	AGSSSCT*	145	
<i>D. pini</i>	97	-DSD	CWTFWWSGSGTSGVGTGCI	RSPTTGECGCENSSGQFI	AGSDSCT*	145	
<i>C. minus</i>	100	VD	EDCWTFWWSGEGEESGVGTNCI	EDPTTGECGCEDSSGTFI	AGSDSCT*	149	
<i>P. pluvialis</i>	99	-NDD	CWTFWWSGNGTDSGEGAGCI	KDPTTGDCGCENSDGTFI	VGSSSCT*	147	
<i>E. deformans</i>	103	VN	ADCWTFWWSDDGEAEGFGTNCI	ESPDGTCGCEDSDGTFVYGGTDCT	*	152	
<i>G. abietina</i>	99	-D	ADAWTFWWSGEGESTGYGTTCI	KSPSTGICGCENSDGTFVPGSSSCK*		147	
<i>Co. graminicola</i>	97	-D	ADCWTFWWSGTGEQSGVGTGCI	RDPTTGDCGCENSDGTFI	PGSDSCK*	145	
<i>Va. mali</i>	99	-N	SDCWTFWWSDDGESGVGTDCI	KDPSSGECGCEDSDGTFI	VGSSSCT*	147	
<i>P. nicotianae</i>	99	-N	DDCWTFWWSGNGTDSGVGTGCI	KDPTTGDCGCENSDGTFVADSSS	CV*	147	
<i>P. parasitica</i>	99	-N	DDCWTFWWSGNGTDSGVGTGCI	KDPTTGDCGCENSDGTFVADSSS	CV*	147	
<i>P. sojae</i>	99	-N	DDCWTFWWSGNGTDSGVGTGCI	KDPTTGNCGCENSDGQFI	ADSSS	CV*	147

Figure 3.20. Candidate effector Ds131885 from *Dothistroma septosporum* NZE10, Cm2721 from *Cyclaneusma minus* NZFS809 and Pp10632 from *Phytophthora pluvialis* NZFS3000 have sequence similarity with proteins from other fungal and oomycete plant pathogens. Ds131885, Cm2721 and Pp10632 were aligned with proteins from fungal plant pathogens, *Dothistroma pini* and *D. septosporum* COLN (the most polymorphic isolate from Ds131885 NZE10) (Dothideomycetes), as well as *Elytroderma deformans* (JGI ID 653320) and *Gremmeniella abietina* (JGI ID 401586) (Leotiomyces), *Valsa mali* (KUI69068.1) and *Colletotrichum graminicola* (JGI ID 10636) (Sordariomycetes), and oomycete plant pathogens, *Phytophthora nicotianae* (KUF82351.1), *Phytophthora parasitica* (XP_008909123.1) and *Phytophthora sojae* (XP_009515305.1). The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined.

3.3.4 *Dothistroma septosporum* candidate effector proteins induce the expression of *Nicotiana benthamiana* defence related genes

To determine if the transient expression of some *D. septosporum* CEs triggers plant immune responses, the expression of HR genes and some well-characterised *N. benthamiana* defence-related marker genes were analysed in *D. septosporum* CE-expressing plants through ATTAs. *N. benthamiana* plants were agroinfiltrated with constructs expressing Ds69335, Ds74283 and Ds131885, as well as the empty pICH86988 vector, used as a negative control. Both Ds74283 and Ds131885 were selected due to their consistent ability to trigger non-host cell death, and Ds69335 was chosen due to its high up-regulation during *D. septosporum* infection of pine, and also for having orthologues in *C. minus* and *P. pluvialis*. Samples were analysed 24 and 48 hai. For plants expressing Ds69335 or Ds131885, the *N. benthamiana* genes analysed were differentially expressed at 48 hai, while for plants expressing Ds74283, 24 hai was enough to determine differential expression of the defence genes.

For *N. benthamiana* plants expressing Ds69335, none of the two HR-specific marker genes, *NbHIN1* and *NbHSR203J*, were activated at 24 or 48 hai (**Figure 3.21** and **Appendix Figure A.21**). This was expected since the proteins did not trigger any visible cell death in the non-host plant. There was also no alteration in the transcript levels of the genes related to hormone signalling pathways (SA, JA or ET) in any of the time points evaluated. However, Ds69335 seems to activate the expression of the three PTI markers (*NbACRE31*, *NbWRKY7* and *NbWRKY8*) analysed at 48 hai (**Figure 3.21** and **Appendix Figure A.21**). PTI is considered the first line of defence in plants and is activated by highly conserved PAMPs. Because Ds69335 (and the orthologues in *C. minus* and *P. pluvialis*) did not trigger chlorosis or cell death in *N. benthamiana*, this could indicate that activation of PTI was enough to prevent a stronger response (e.g., HR), which is seen by the recognition of an effector by an R protein, for example.

To determine if Ds131885-activated cell death was associated with plant defence responses, the same *N. benthamiana* genes were analysed upon agroinfiltration at 24 and 48 hai (**Figure 3.22** and **Appendix Figure A.23**). Expression of both HR markers was activated at 48 hai, consistent with the observation that the protein triggers consistent

chlorosis and cell death in non-host plants. The defence responses triggered by Ds131885 seemed to affect both the SA and JA signalling pathways, although of these the SA-dependent immunity gene markers were more highly activated. All PTI marker genes were activated in *N. benthamiana* leaves expressing Ds131885. These results suggest that cell death elicitor Ds131885 might be a PAMP that triggers plant immune responses by activating the SA- and JA-mediated signalling pathways.

Next, *N. benthamiana* genes following expression of Ds74283 were analysed. Here, it was possible to detect significant differential expression of all the tested *N. benthamiana* genes at 24 hai, which continued at 48 hai (**Figure 3.23** and **Appendix Figure A.22**). Consistent with the fact that the protein was able to elicit cell death, both HR-specific marker genes, *NbHIN1* and *NbHSR203J* were highly activated at 24 and 48 hai, along with PTI markers. Also, both the SA and JA signalling pathways seemed to be greatly affected by the expression of Ds74283 in *N. benthamiana*. One explanation for these results is that Ds74283 might have a high affinity for a *N. benthamiana* receptor or another plant target responsible for activating the immune system, triggering strong and rapid defence responses.

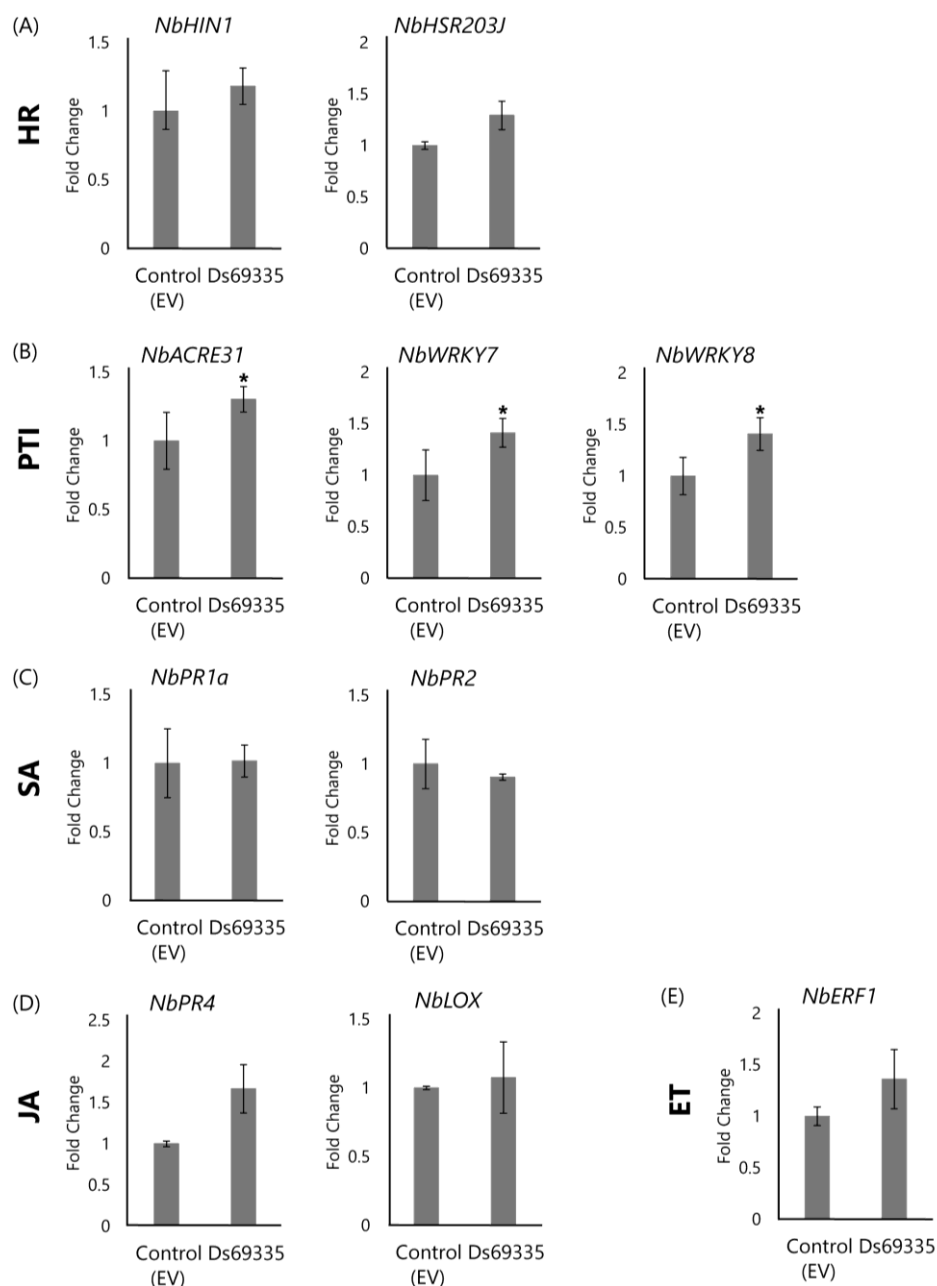


Figure 3.21. *Dothistroma septosporum* candidate effector Ds69335 triggers the expression of pathogen-associated molecular pattern (PAMP) immunity (PTI) genes in *Nicotiana benthamiana*. Expression of **(A)** hypersensitive-response (HR)-specific marker genes (*NbHIN1* and *NbHSR203J*), **(B)** PTI marker genes (*NbACRE31*, *NbWRKY7* and *WRKY8*) and defence-related marker genes **(C)** *NbPR1a* and *NbPR2*, salicylic acid (SA)-dependent immunity; **(D)** *NbPR4* and *NbLOX*, jasmonic acid (JA)-dependent immunity; **(E)** *ERF1*, ethylene-dependent immunity) in *N. benthamiana*. Ds69335 and empty pICH86988 vector (EV) were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay and leaves were sampled after 48 h. Transcript levels were normalized to the reference gene *NbActin* and compared to the level of the control (set as 1). Means and standard errors were calculated from at least three biological replicates. *, $P < 0.05$ using Student's *t*-test. Ds: *D. septosporum*.

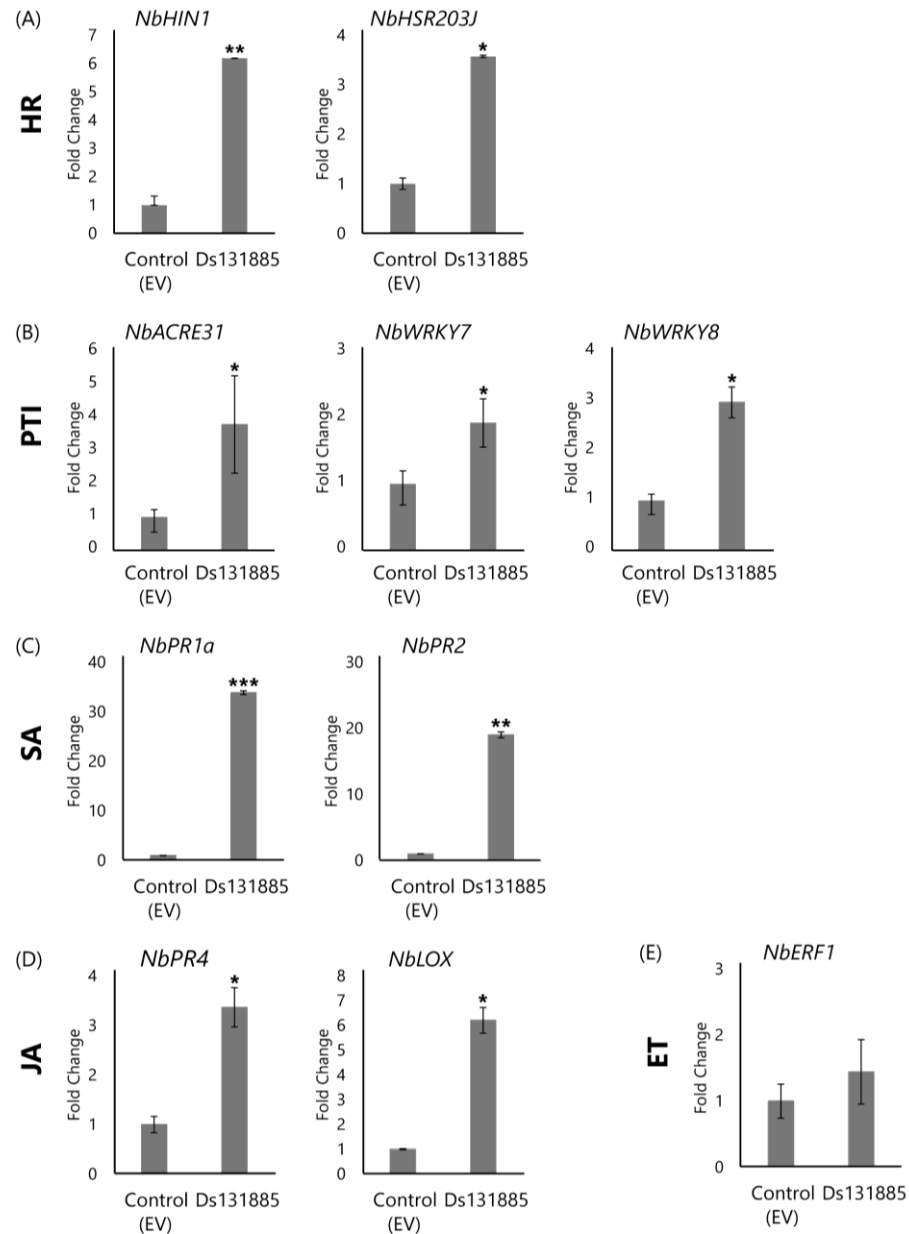


Figure 3.22. *Dothistroma septosporum* candidate effector Ds131885 triggers the expression of plant defence response genes in *Nicotiana benthamiana*. Expression of **(A)** hypersensitive-response (HR)-specific marker genes (*NbHIN1* and *NbHSR203J*), **(B)** pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes (*NbACRE31*, *NbWRKY7* and *WRKY8*) and defence-related marker genes **(C)** *NbPR1a* and *NbPR2*, salicylic acid (SA)-dependent immunity; **(D)** *NbPR4* and *NbLOX*, jasmonic acid (JA)- dependent immunity; **(E)** *ERF1*, ethylene-dependent immunity) in *N. benthamiana*. Ds131885 and empty pICH86988 vector (EV) were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay and leaves were sampled after 48 h. Transcript levels were normalized to the reference gene *NbActin* and compared to the level of the control (set as 1). Means and standard errors were calculated from at least three biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using Student's *t*-test. Ds: *D. septosporum*.

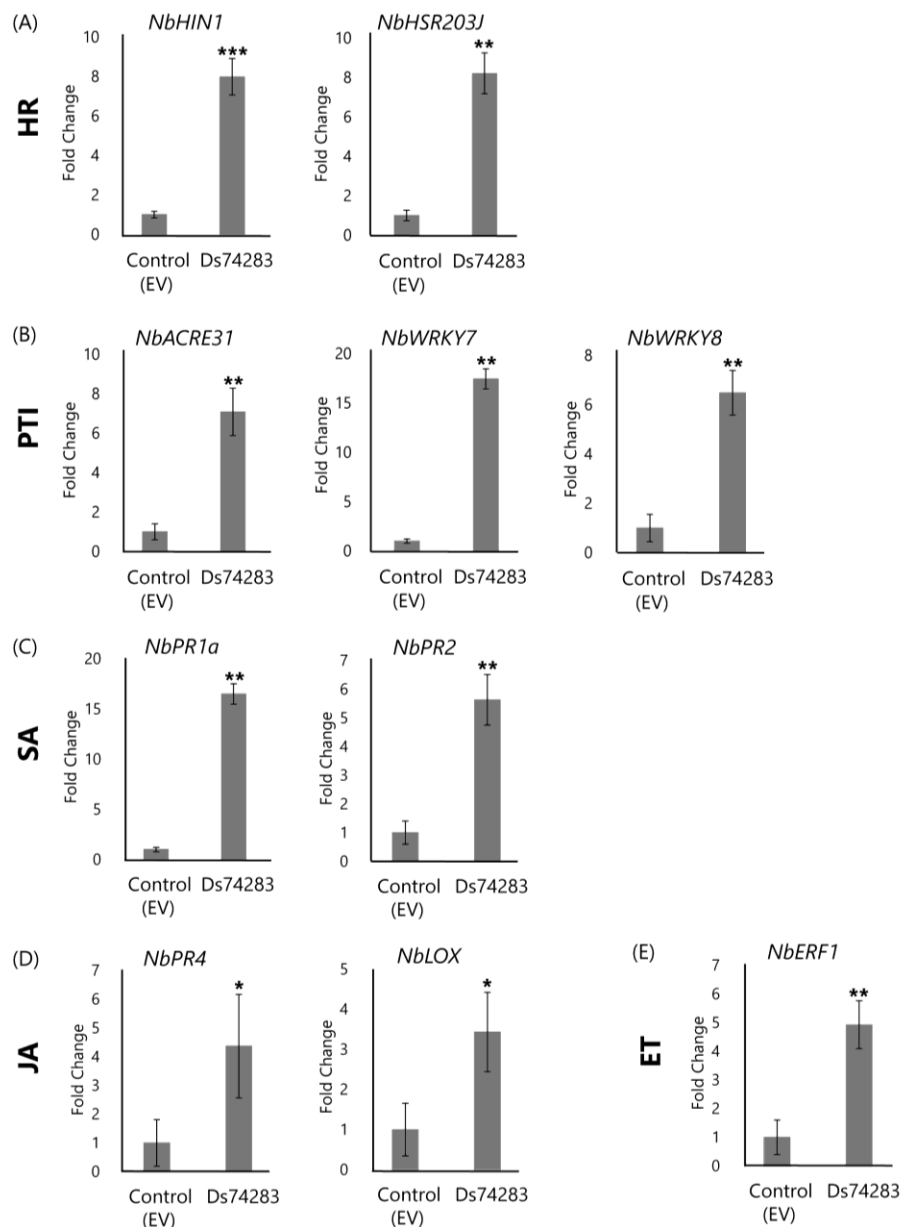


Figure 3.23. *Dothistroma septosporum* candidate effector Ds74283 triggers the expression of plant defence response genes in *Nicotiana benthamiana*. Expression of **(A)** hypersensitive-response (HR)-specific marker genes (*NbHIN1* and *NbHSR203J*), **(B)** pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes (*NbACRE31*, *NbWRKY7* and *WRKY8*) and defence-related marker genes **(C)** *NbPR1a* and *NbPR2*, salicylic acid (SA)-dependent immunity; **(D)** *NbPR4* and *NbLOX*, jasmonic acid (JA)- dependent immunity; **(E)** *ERF1*, ethylene-dependent immunity) in *N. benthamiana*. Ds74283 and empty pICH86988 vector (EV) were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay and leaves were sampled after 24 h. Transcript levels were normalized to the reference gene *NbActin* and compared to the level of the control (set as 1). Means and standard errors were calculated from at least three biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using Student's *t*-test. Ds: *D. septosporum*.

3.3.5 Candidate effector proteins from *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* trigger cell death in the host *Pinus radiata*

Transient expression of CE proteins from the three pine pathogens led to the identification of elicitors of chlorosis and cell death in non-host plants *N. benthamiana* and *N. tabacum*. Following the development of a method to infiltrate purified proteins from *D. septosporum* into *Pn. radiata* clonal shoots, it was determined that proteins DsEcp20-3 and DsEcp32-3 triggered cell death-like responses in these pine shoots (see **Section 2.3.6 of Chapter 2**). For this reason, other proteins were tested to determine whether they could also induce such a response in pine. The proteins tested were Ds74283 (present only in *D. septosporum*) and, Ds71487 and DsEcp2-1 (Ds158381) (also present in *C. minus*) from *D. septosporum*, and the cross-kingdom orthologues Ds131885 from *D. septosporum*, Cm2721 from *C. minus* and Pp10632 from *P. pluvialis*, all of which consistently triggered cell death in *N. benthamiana* and/or *N. tabacum* (**Figure 3.2**, **Figure 3.7** and **Figure 3.8**) (Guo et al., 2020; Hunziker et al., 2021). Candidate proteins were expressed in *Pi. pastoris*, purified and vacuum-infiltrated into pine shoots. The same three pine genotypes used previously were infiltrated here: two relatively susceptible to *D. septosporum* infection (S6 and S11) and one relatively tolerant (R4). To try to better assess the differences in the degree of cell death elicited between proteins, but most importantly between genotypes, a scale with examples of four different responses observed in each individual pine shoot was created (**Figure 3.24**); 4: strong response in more than 50% of the pine shoot; 3: strong response in less than 50% of the pine shoot; 2: weak cell death response; 1: no cell death response, when the pine shoot had the same response as the one observed in the elution buffer (negative control).

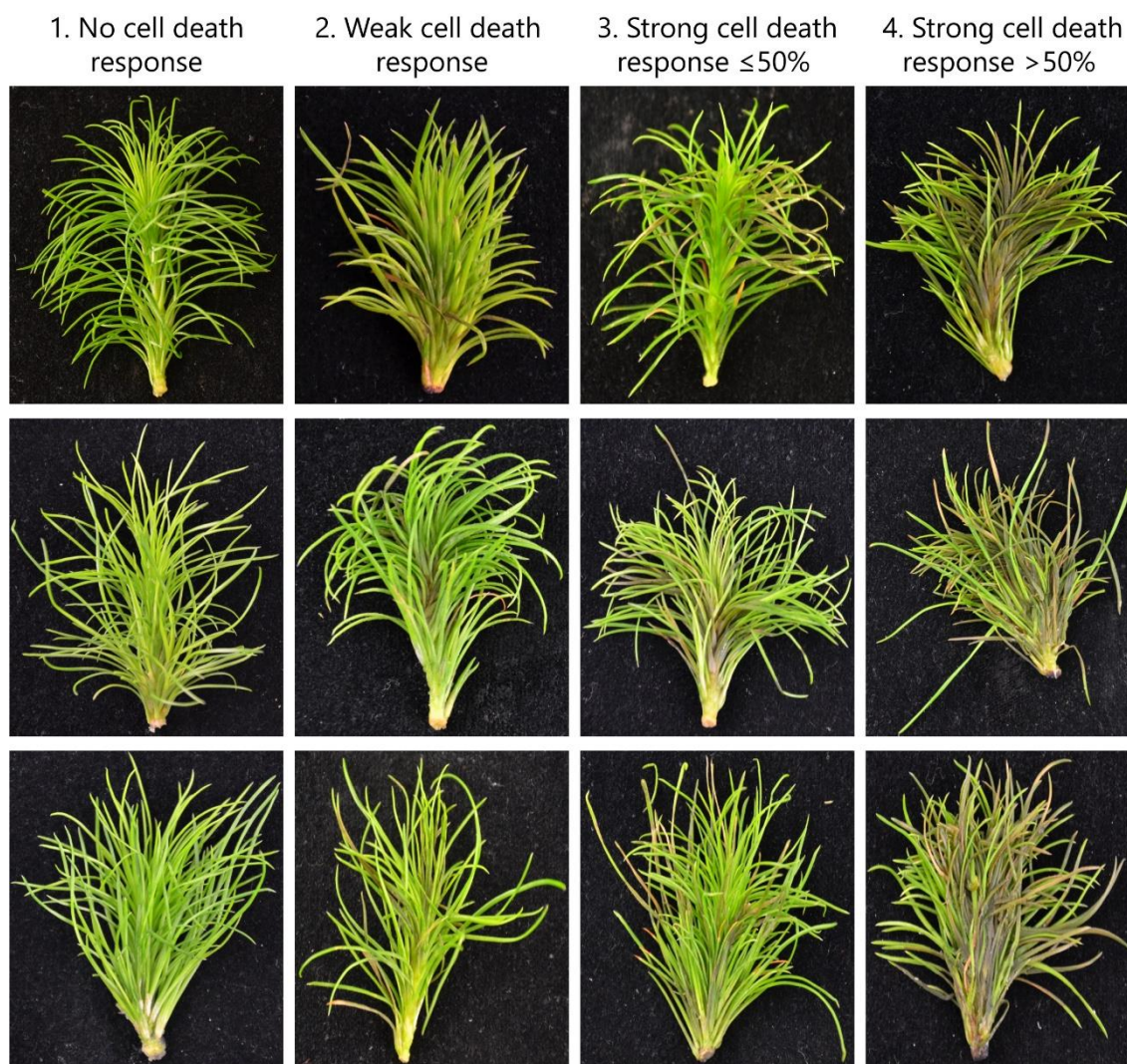


Figure 3.24. Classification of different responses observed in *Pinus radiata* following infiltration with candidate effector proteins. Responses were classified in four different categories: 1: no-cell death response, 2: weak cell death response, 3: strong response in less than 50% of the pine shoot and 4: strong response in more than 50% of the pine shoot.

3.3.5.1 *Dothistroma septosporum* candidate effectors triggers cell death in *Pinus radiata*

Three different candidate proteins identified in *D. septosporum* were tested for their ability to trigger cell death in *Pn. radiata*. All three proteins, Ds71487, Ds74283 and DsEcp2-1, triggered some degree of cell death in all pine genotypes used (**Figure 3.25**), consistent with their ability to trigger cell death in non-host plants (**Figure 3.2**, **Figure 3.7** and **Figure 3.8**) (Guo et al., 2020; Hunziker et al., 2021). The elution buffer used as a negative control did not trigger any visible damage to the shoots.

Both Ds71487 and Ds74283 triggered a strong response over the majority of the pine shoot area in genotype S11, possibly indicating a higher susceptibility than in genotype S6 (**Figure 3.26**). Interestingly, from all proteins tested, Ds74283 seemed to cause less damage to genotype R4, which could suggest a lower affinity of the protein for the respective pine immune receptor (if indeed an immune receptor-dependent response) in this genotype, when compared with others. DsEcp2-1 triggered a similar degree of cell death responses across all pine genotypes tested (**Figure 3.26**).

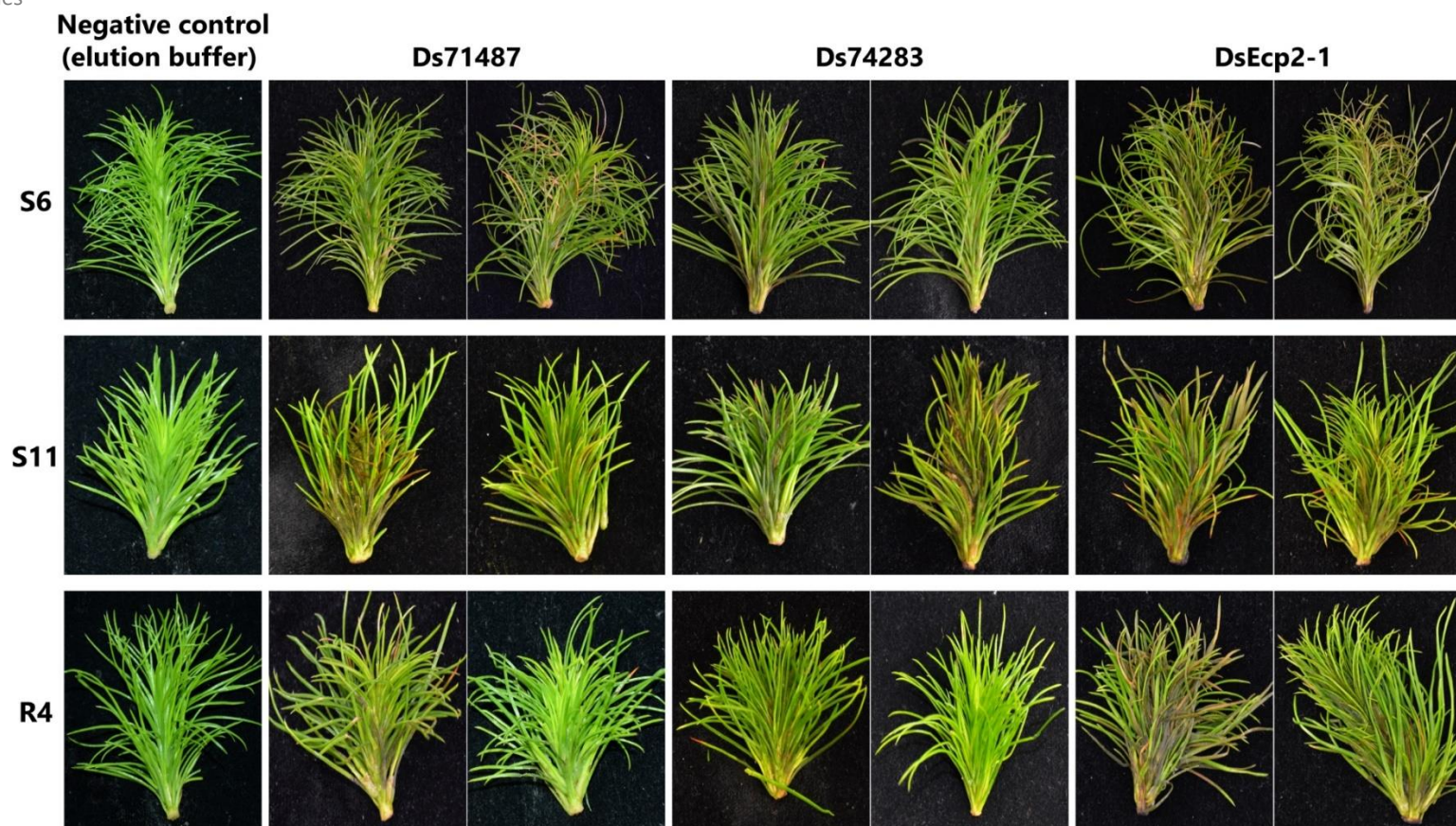


Figure 3.25. *Dothistroma septosporum* candidate effectors Ds71487, Ds74283 and DsEcp2-1 trigger cell death in *Pinus radiata* shoot tissues. Whole shoots of *Pn. radiata* derived from family seedlots that are relatively susceptible (S6 and S11) or tolerant (R4) to *D. septosporum* infection were infiltrated with purified Ds71487, Ds74283 and DsEcp2-1 proteins produced by heterologous expression in *Pichia pastoris*. Representative photos (from 12 to 24 pine shoots for each treatment) were taken 7 days after infiltration, with further imagens shown in **Appendix Figure A.24**. Ds: *D. septosporum*.

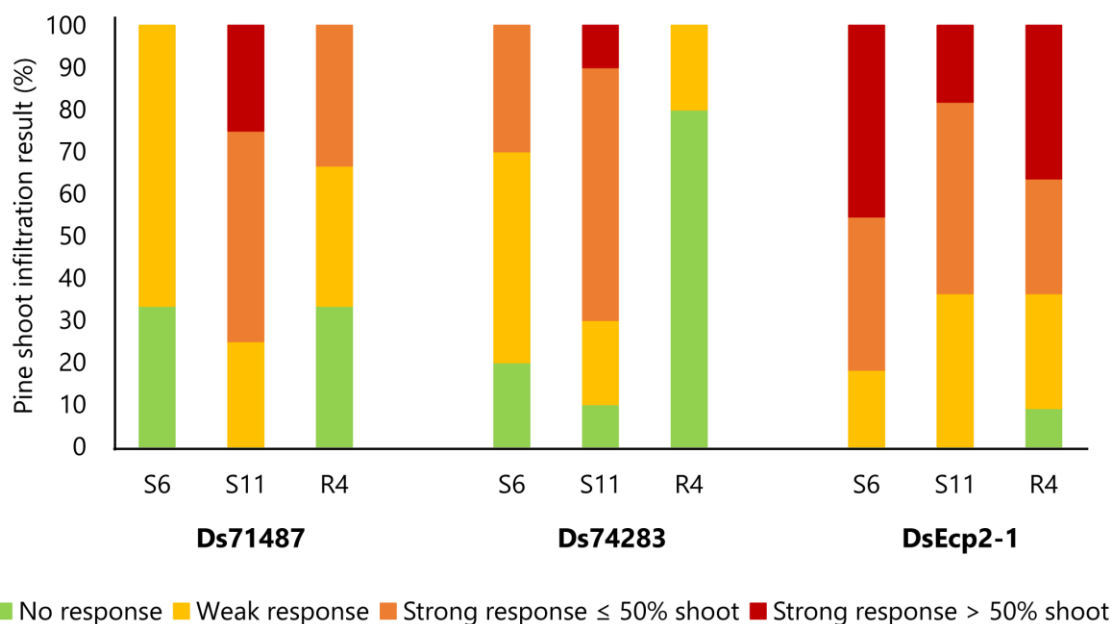


Figure 3.26. Degree of cell death in *Pinus radiata* shoots following infiltration with candidate effector (CE) proteins from *Dothistroma septosporum*. Graphs display the percentage of pine shoots that showed cell death in response to infiltration of purified CE proteins Ds71487, Ds74283 and DsEcp2-1, divided into four categories: 1: green, no cell death response; 2: yellow, weak cell death response; 3: orange, strong response in less than 50% of the pine shoot and 4: red, strong response in more than 50% of the pine shoot. Ds: *D. septosporum*.

3.3.5.2 A conserved protein between *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* triggers cell death in *Pinus radiata*

One of the highly conserved proteins identified in the three foliar pine pathogens elicited cell death in non-host plants. For this reason, the Ds131885, Cm2721 and Pp10632 proteins were screened in the pine host.

In all cases, as expected, the infiltration of elution buffer, used as a negative control, did not trigger any visible damage to the shoots. And, as observed for other the other proteins tested, Ds131885, Cm2721 and Pp10632 triggered some degree of cell death in the same three pine genotypes used previously (**Figure 3.27**). Of the three proteins, Ds131885 consistently triggered stronger cell death in all genotypes (**Figure 3.28**).

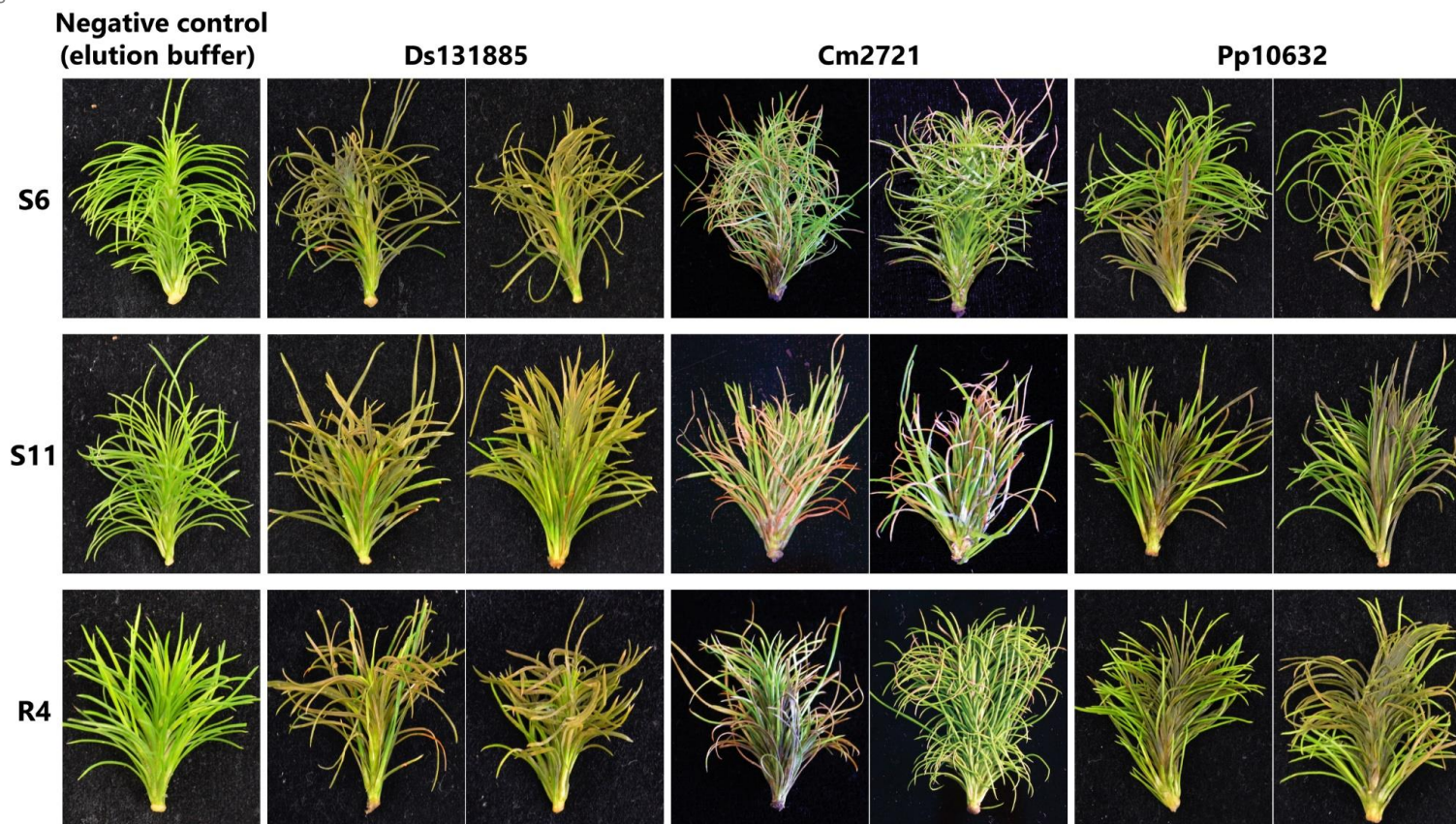


Figure 3.27. Conserved candidate effectors from *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis* trigger cell death in *Pinus radiata* shoot tissues. Whole shoots of *Pn. radiata* derived from family seedlots that are relatively susceptible (S6 and S11) or tolerant (R4) to *D. septosporum* infection were infiltrated with purified Ds131885, Cm2721 and Pp10632 proteins produced by heterologous expression in *Pichia pastoris*. Representative photos (from 12 to 24 pine shoots for each treatment) were taken 7 days after infiltration, with further images show in **Appendix Figure A.24**. Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*.

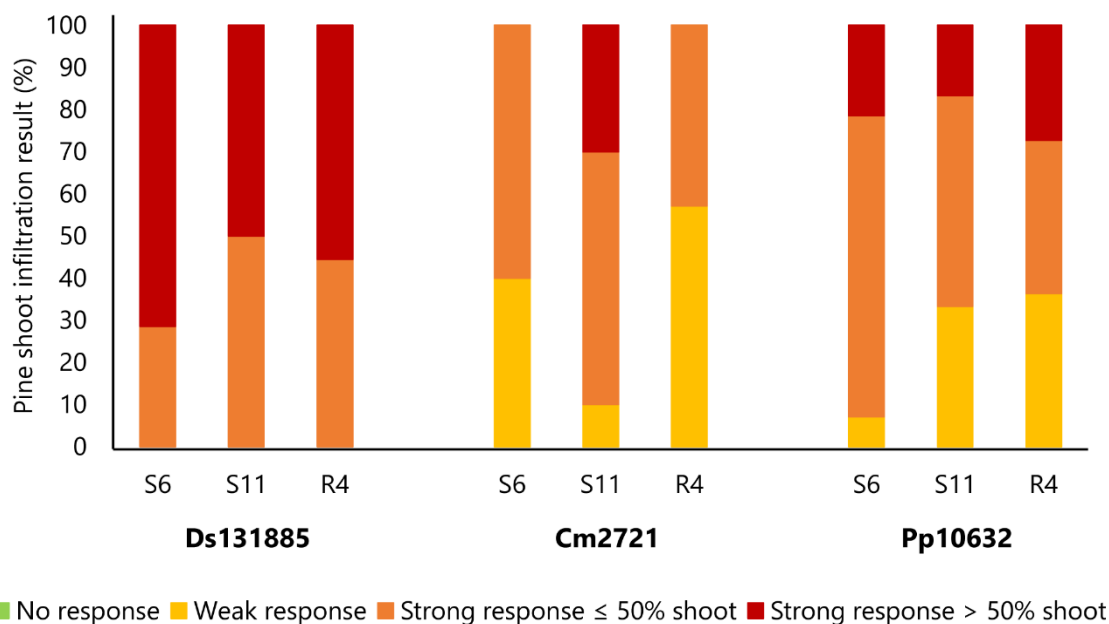


Figure 3.28. Degree of cell death in *Pinus radiata* following infiltration with a conserved candidate effector (CE) protein from *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*. Graphs display the percentage of pine shoots that showed cell death in response to infiltration of purified CE proteins Ds131885, Cm2721 and Pp7927, divided into four categories: 1: green, no cell death response; 2: yellow, weak cell death response; 3: orange, strong response in less than 50% of the pine shoot and 4: red, strong response in more than 50% of the pine shoot. Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*.

3.4 Discussion

Pathogens communicate with their hosts to promote host colonization by secreting proteinaceous molecules, termed effectors, that typically suppress the host immune system. In resistant host plants, however, one or more of these effectors can be recognized as invasion patterns by corresponding cell-surface-localized immune receptors to activate the plant immune system. *D. septosporum*, *C. minus* and *P. pluvialis* are foliar pathogens of *Pn. radiata*. *D. septosporum* and *C. minus* are fungi, while *P. pluvialis* is an oomycete. As in other pathosystems, these pathogens are also thought to secrete effectors when interacting with their host *Pn. radiata*. Using genomic and transcriptomic information, plus a combination of bioinformatic tools, a total of 29 *D. septosporum* CEs were identified, and then compared with protein sequences from *C. minus* and *P. pluvialis*. Thirteen of these proteins were also present in *C. minus* and, of these, two were present in *P. pluvialis* as well, therefore being shared between the three

pine pathogens. Even though there are some examples of conserved effectors between fungal and oomycete species, like NLPs and XEG1 (Fellbrich et al., 2002; Ma et al., 2015), a small number of shared CEs between the three pine pathogens was expected, considering the evolutionary divergence between fungi and oomycetes.

Most of the CEs were small proteins with four or more cysteine residues, which is consistent with other known (typically fungal) effectors (Lo Presti et al., 2015). Also, most of them had no sequence similarity with other characterized proteins, a feature that is also common to this type of protein. However, some had similarity to characterized proteins with known virulence functions in other pathosystems, like orthologues of the Ecp6 protein from *F. fulva*, present in both *D. septosporum* and *C. minus* (Ds46236 and Cm7346). Ecp6 has three carbohydrate-binding LysM domains, that can sequester chitin oligosaccharides released from the cell wall, suppressing elicitation of host immune responses (de Jonge et al., 2010; Sánchez-Vallet et al., 2013). Others included a *D. septosporum* protein (Ds70155) with homology to a cerato-platanin protein from the fungal tree pathogen *C. platani*, which can trigger plant cell death responses (Baccelli et al., 2014), and a hydrophobin (Ds75009), also a known virulence factor (Bayry et al., 2012). Interestingly, when deleted, *Ds75009* did not affect *D. septosporum* virulence in pine (Bradshaw et al., 2016). There are, however, three other hydrophobin genes in the *D. septosporum* genome, which could indicate functional redundancy.

CEs were transiently expressed in non-host plants *N. benthamiana* and *N. tabacum*, or directly infiltrated into pine shoots, to determine if they triggered chlorosis or cell death, indicative of possible recognition by a matching R protein. Pathogen CE proteins identified as having the ability to trigger non-host chlorosis or cell death were Ds74283 (present only in *D. septosporum*), orthologues between *D. septosporum* and *C. minus* Ds71487/Cm2492 and Ds75860/Cm835 and proteins shared between the three pine pathogens, Ds131885/Cm2721/Pp10632. The other set of shared cross-kingdom proteins, Ds69335, Cm8840 and Pp7927 did not chlorosis or trigger cell death. It is important to note that only Ds69335 could be detected via Western blot, which suggests the protein is not being recognized by plant receptors or other plant targets (in cases of visible responses, like cell death) while the other two proteins in this set could not be detected.

The chlorosis or cell death activity observed by the proteins mentioned above seems to be dependent on their secretion to the apoplast, as seen by the lack of chlorosis or cell death when the signal peptide was removed. This is consistent with the fact that *D. septosporum* and *P. pluvialis* colonize the intercellular environment around cells (Gómez-Gallego et al., 2019; Kabir et al., 2015), secreting effector proteins, and suggesting a common mode of colonization for *C. minus*.

Following this non-host approach, some of the CE proteins were tested for their ability to trigger cell death in the host *Pn. radiata*, using the protein infiltration method developed in **Section 2.3.6** of **Chapter 2**. Interestingly all proteins tested triggered a cell death response in pine, as observed in *Nicotiana* species, suggesting that these proteins could be recognized by an immune receptor (possibly conserved with *N. benthamiana*). Among the proteins tested, Ds74283 elicited a different degree of cell death between a relatively susceptible (S11) and a relatively tolerant (R4) genotype. This could be explained by divergence of R proteins or downstream signalling partners in the two genotypes. However, even though the infiltration seemed efficient (by infiltration with a dye), it remains possible that these different responses were due to varying efficiency of the infiltration method. For this reason, a broader screening with other pine genotypes should be performed.

Moreover, it is important to note that these cell death responses cannot be promptly classified as immune responses due to recognition by an R protein. The cell death observed could be due to direct interaction of these proteins with the host membrane, disrupting it and leading to necrotic lesions. Experiments such as virus-induced gene silencing (VIGS), which was performed to characterize the type of cell death triggered by DsEcp20-3 and DsEcp32-3 (see **Section 2.3.7** of **Chapter 2**), could be performed. Likewise, the expression of defence-related gene markers could be investigated. From these proteins, Ds74283 and Ds131885 were transiently expressed in *N. benthamiana*, and the expression of some well-known defence marker genes was analysed. When compared to the control, both Ds74283 and Ds131885 induced the expression of specific HR marker genes, consistent with the protein cell death eliciting ability, and also the upregulation of PTI marker genes, which could suggest that these proteins act as PAMPs, triggering strong HR-like responses; another example of the

blurred dichotomy of PTI and ETI responses (Thomma et al., 2011). Other experiments could be performed to confirm these results, and to further characterize the responses observed by the other cell death-inducing proteins, such as reactive oxygen species (ROS) or ethylene measurements.

AlphaFold2 was used to predict tertiary structures of key CE proteins from *D. septosporum*, *C. minus* and *P. pluvialis*. These structures were then compared with characterized structures in the RCSB PDB, to identify similarities with known virulence factors in other pathogens. Structural similarities between effectors can help to identify their potential roles in plant-pathogen interactions even when there is limited conservation of primary sequence. For example, Ds69845 has structural similarity with a pyocyanin demethylase. Pyocyanin is a secreted virulence factor from *Pseudomonas aeruginosa*, a gram-negative bacterium that causes diseases in plants and animals (Hall et al., 2016). Ds74875 was structurally similar to Y3, a cytotoxic protein from the mushroom *Coprinus comatus* that has the ability to bind glycan (Zhang et al., 2017). Ds129328 has structural similarity with 14-3-3, a eukaryotic-specific protein family with a primary role in signalling cascades; these proteins have been shown to be involved in virulence in fungi (Garcia-Rodas & Nosanchuk, 2016; Jingbo et al., 2016; Lozano-Durán & Robatzek, 2015). None of the proteins mentioned above triggered chlorosis or cell death in *Nicotiana* species, however, due to their predicted roles based on structural similarity with other virulence factors, they still might have important roles in promoting host colonization, for example by suppressing plant defences.

Proteinaceous effectors can share similar structural folds despite no or little sequence identity, which suggests either divergent evolution and/or selection has occurred to maintain an important structure even in the face of accumulated mutations (Andrie et al., 2008; de Guillen et al., 2015; Franceschetti et al., 2017; Seong & Krasileva, 2021, 2022). This was observed with members of the Ecp20 family from *D. septosporum* and *F. fulva* (see **Section 2.3.2** of **Chapter 2**), which are structurally similar to Alt a 1 proteins, but have no or low amino acid sequence identity to other members from this family.

Among the cell death elicitors discussed in this Chapter, Ds74283, Ds71487 (and orthologue Cm2492) and Cm835 were predicted to be structurally similar to proteins from diverse organisms with a β -trefoil fold. In addition, Ds75860, which is part of the Ecp32 family (see **Section 2.3.2** of **Chapter 2**) and CE Ds69113, which did not trigger chlorosis or cell death, are also structurally similar to proteins with a β -trefoil fold. Ds69113 is an orthologue of FfPhiA-2, a phialide A protein from *F. fulva*. These cell wall-associated proteins are involved in phialide development, cells responsible for the production and release of conidia (Melin et al., 2003). Moreover, using the Foldseek server, it was possible to determine that the β -trefoil fold is adopted by a subset of proteins from *Phytophthora* species, including *P. pluvialis*.

The β -trefoil fold is present throughout different kingdoms and is involved in many different processes and interactions between organisms (Žurga et al., 2015). The most common types of proteins that share this fold are trypsin inhibitors and lectins, many being virulence factors in plant-pathogenic fungi (discussed in **Section 2.4** of **Chapter 2**). Another example of β -trefoil fold-containing proteins are pore-forming toxins (PFTs), or cytolysins, part of the membrane-damaging toxins family. They function by inserting themselves into the host cell membrane, forming channels (pores), which increases membrane permeability, leading to cell lysis (Alouf, 2003). Since they function by binding to the host membrane, cytolysins also have domains that enable binding, such as carbohydrate-binding lectins. Candidalysin, secreted by *Candida albicans*, was the first fungal PFT identified (Moyes et al., 2016), and has the ability to disrupt the plasma membrane, leading to host cell death (Jonathan et al., 2022).

Proteins with a β -trefoil fold have low or no sequence similarity, with conservation observed only for some hydrophobic residues (Feng et al., 2010; Kirioka et al., 2017). These features are also present in the *D. septosporum* proteins analysed, and the prediction of tertiary structures also identified a conserved disulphide bond between Ds69113, Ds71487, Ds74283 and Ds75860 that seems to be important for their overall structure. However, the identified conserved disulphide bond is not present in the *P. pluvialis* (Pp13223) structure analysed.

The shared folds between these sequence-unrelated effector proteins suggests their importance for virulence, and also demonstrates how tertiary structure predictions can greatly contribute to better understanding the roles these proteins have in phytopathogenic interactions. For example, MAX effectors from *M. oryzae* share a common fold that could not be detected by sequence homology (Seong & Krasileva, 2021). Indeed, there is recent evidence that many pathogen effectors can be grouped into large structurally similar families (Rocafort et al., 2022; Seong & Krasileva, 2022; Yu et al., 2021). All of this is consistent with the β -trefoil proteins of *D. septosporum*, *C. minus* and *P. pluvialis* having a common ancestral origin, and that, during the course of evolution, these proteins have lost their sequence similarity, while maintaining a similar structure. It remains to be determined if, even having similar folds, these proteins also have the same virulence functions during pine infection. Interestingly, all CE proteins from *D. septosporum* that are predicted to have a β -trefoil fold are encoded by genes that are upregulated during the Late stage of pine infection (when compared with *in vitro*, Early, and Mid stages), which could suggest their role in the necrotrophic stage of the pathogen (**Appendix Table A.8**), especially considering this possible role of disrupting the host membrane.

Ds69335, Cm8840 and Pp7927 are orthologous proteins from the three pine pathogens, *D. septosporum*, *C. minus* and *P. pluvialis*, respectively. All three proteins have sequence similarity with proteins belonging to the CAP superfamily. Proteins from this superfamily are present not only in fungi and oomycetes, but also in bacteria, plants, and animals, being present in more than 2500 species (Schneider & Di Pietro, 2013). The CAP protein superfamily denomination comes from sequence similarity between the 'cysteine-rich secretory proteins' (CRISPs), from the mammalian reproductive tract, the 'antigen 5' (Ag5) present in the venom secretory ducts of insects, and 'plant pathogenesis-related protein 1' (PR-1), first identified in tobacco upon viral infection (Gibbs et al., 2008). All three proteins from *D. septosporum*, *C. minus* and *P. pluvialis* also have a strong structural similarity with Pry1, a *S. cerevisiae* protein which is part of the CAP superfamily. Pry1 proteins are sequence-related to PR-1 proteins from plants.

Structurally, the CAP domain has a conserved α - β - α sandwich domain of approx. 150 amino acids, with two highly conserved disulphide bonds, features that are also

present in the predicted structures of Ds69335, Cm8840 and Pp7927 (**Figure 3.13**). This level of structural conservation of the CAP domain suggests these proteins have essentially the same function, while the diversity observed outside this conserved region (N- or C-terminal extensions) might result in alteration of target specificity (Schneider & Di Pietro, 2013). All of the pine pathogen proteins have predicted IDRs outside of the CAP domain (**Figure 3.12** and **Appendix Figure A.18**). In *Ustilago maydis*, proteins with a Tin2 fold were shown to have long IDRs, with the expansion and contraction of these regions being related to diversification (Seong & Krasileva, 2022). IDRs evolve more quickly than ordered regions of a protein sequence (Brown et al., 2002), which could be a strategy for effectors to avoid recognition by the host immune system. Interestingly, most of the amino acid modifications between Ds69335 from *D. septosporum* NZE10 and other isolates occurred in the IDR.

In plants, PR-1 proteins are known to be up-regulated and accumulate in the apoplast during pathogen infection, which suggests they have antimicrobial activity (Breen et al., 2017). CAP proteins are also known virulence factors in fungi, even though there are not many studies characterizing CAP proteins from plant-pathogenic fungi, or oomycetes for that matter. The human pathogen *C. albicans* encodes RBT4 and RBE1, predicted secreted CAP proteins. Mutants lacking *Rbt4* or *Rbe1* had reduced virulence during infection of mouse (Braun et al., 2000; Röhm et al., 2013). FPR1 is another CAP protein, from the ascomycete plant and mammalian pathogen *Fusarium oxysporum*, and deletion of the gene encoding this protein also resulted in reduced virulence in the pathogen when infecting mice but had no effect in tomato (Prados-Rosales et al., 2012).

The Pry1 protein from *S. cerevisiae* is known to bind to lipids and sterols, which seems to be a feature conserved in several CAP family members (Choudhary & Schneider, 2012; Darwiche et al., 2017a; Darwiche et al., 2017b). Moreover, deletions of the yeast *Pry* genes resulted in mutants with eugenol hypersensitivity. Eugenol is an antimicrobial oil present in different plant species, including *Pinus*, that directly competes with cholesterol binding in Pry1, suggesting a detoxification role for CAP proteins by possibly neutralizing these hydrophobic compounds (Choudhary & Schneider, 2012; Darwiche et al., 2018). The hemibiotrophic plant pathogen *M. perniciosus* is predicted to secrete at least 11 PR-1-like proteins, many of which are upregulated during the biotrophic phase when infecting its

host cacao. Many of these also have the ability to bind to small hydrophobic molecules such as sterol or fatty acids (Darwiche et al., 2017a). Interestingly, Ds69335 is also highly up-regulated at the Early infection stage of *D. septosporum* in pine, which could suggest this protein, and perhaps its orthologues Cm8840 and Pp7927, might act as biotrophic effectors suppressing host defence responses by detoxification of host hydrophobic compounds. Interestingly, in nematodes, apoplast allergen-like proteins, also part of the CAP superfamily, are known to suppress plant immunity responses (Lozano-Torres et al., 2014). Considering the role of Pry1 in sterol binding, and their strong structural similarity, the CAP proteins from the three pine pathogens might be acting in the same way.

Alternatively, the pathogen might sequester sterol from the host plasma membrane for its own benefit during the biotrophic phase, which would be particularly beneficial for oomycetes, such as *P. pluvialis*, which cannot synthesize their own sterol. Sterols are involved in cell membrane structure, another indication that the secretion of CAP proteins helps the pathogen to survive the harsh apoplastic environment.

The second set of conserved cross-kingdom proteins, Ds131885, Cm82721 and Pp10632, are orthologues of VmE02, a cross-kingdom PAMP from the apple pathogen *Va. mali* (Nie et al., 2019). VmE02 triggers cell death in multiple plant species, including *N. benthamiana*, tomato, pepper, and apple, and is highly conserved in different *Phytophthora* and fungal species, including saprophytes and endophytes. VmE02 is suggested to be recognized by an extracellular immune receptor, and the transduction of defence response signals requires the extracellular co-receptors BAK1 and SOBIR1 (Nie et al., 2019; Nie et al., 2020). During apple infection, VmE02 is induced at early stages of pathogen infection and deletion of VmE02 generated mutants with a considerably reduced number of pycnidia but did not affect virulence (Nie et al., 2019).

In *N. benthamiana*, a receptor-like protein (RLP) RE02, that complexes with coreceptors BAK1 or SOBIR1 was identified as indispensable for VmE02-triggered cell death (Nie et al., 2020). RE02 is identical in sequence to NbCSPR, which mediates recognition of bacterial cold-shock protein (csp22) (Saur et al., 2016), which suggests this receptor-like protein is involved in the recognition of PAMPs from both eukaryotic and prokaryotic species. A small cysteine-rich protein from *Sclerotinia sclerotiorum* is also recognized by RE02, as well as by RLP30 from *Arabidopsis thaliana*, even though RLP30

and RE02 have only 24.6% amino acid identity. An NCBI database against all available *Pinus* genomes identified that the top hit to RLP30 and RE02 was PsRLK (GenBank accession CAC20842.1), a receptor-like kinase protein from Scots pine (*Pinus sylvestris*) (Ávila et al., 2006).

Considering the strong sequence and structural (**Figure 3.15**) similarity between Ds131885, Cm2721, Pp10632 and VmE02, it is possible that the proteins from the pine pathogens might also be recognized by *N. benthamiana* RE02 to elicit a defence response from the plant in the form of a HR (e.g., cell death). The presence of a RE02 homologue protein in *Pn. radiata* would explain the cell death observed in both non-host (*N. benthamiana*) and host (*Pn. radiata*) species following protein delivery. The fact that both RLP30 and RE02 have the ability to recognize the same protein from *S. sclerotiorum*, even though they have low sequence similarity, indicates that the same might be possible for the *N. benthamiana* and *Pn. radiata* receptors responsible for Ds131885, Cm2721 and Pp10632 recognition. The full-length amino acid identity between all three proteins RLP30, RE02 and PsRLK is 20.1%. It will be interesting to determine if Ds131885-, Cm2721- and Pp10632-triggered cell death in *N. benthamiana* requires RE02 using VIGS, for example, and also to identify and characterise the pine receptor that might be involved in recognizing these proteins. Alternatively, rather than recognition leading to defence, considering that *Ds131885* is highly up-regulated in the Mid and Late stages of *D. septosporum* infection (likely the beginning and end of the necrotrophic phase), the recognition by a pine receptor, and the cell death triggered by such recognition, might be beneficial to the pathogen.

In conclusion, it was possible to identify CE proteins shared between pine pathogens *D. septosporum*, *C. minus* and *P. pluvialis*. In this Chapter, bioinformatic methods and protein delivery into host and non-host plants helped to identify proteins that trigger plant chlorosis or cell death and might have important roles during pine infection. However, further studies are needed to confirm if the cell death is due to recognition by an R protein, and to identify possible pine targets. Tertiary structure predictions also provided more information on possible functions these proteins have and suggested how rapid diversification might have led some of these effectors to have low or no sequence similarity despite their structural similarity. The common β -trefoil

fold identified in six of the 29 predicted *D. septosporum* CEs is compatible with suggestions that the effector reservoir that pathogens have is actually finite, and they secrete structurally related proteins during host infection (Outram et al., 2022).

4 CRISPR/Cas9 gene disruption of *Dothistroma septosporum* NZE10 candidate effectors for functional characterization

4.1 Introduction

It is important to determine the functions of candidate effectors (CEs) in order to understand the molecular mechanisms underpinning plant-pathogen interactions. After identifying fungal/oomycete CE proteins that are possibly involved in pathogenicity or virulence, the next step is to characterize their functions, by either disrupting or deleting the genes that encode them and addressing what biological role they might have in the host-pathogen interaction. One way of doing this is through genome editing by generating gene loss-of-function mutants.

Genome editing is a genetic engineering technique, in which a DNA molecule can be inserted into, replaced, or deleted from a genome. There are three main genome editing techniques, zinc-finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPR). The first two methods, ZFNs and TALENs, both involve the fusion of a DNA-binding domain and a DNA-cleavage domain. While the DNA-cleavage domain is the same for both methods, the recognition sites of the DNA-binding domains differ between ZFNs and TALENs. Despite being similar methods, TALENs can be more easily manipulated for practical applications than ZFNs (reviewed in Sushmita et al. (2021)).

More recently, CRISPR systems have become a popular approach for genome engineering. The method is based on the adaptative immune system of bacteria which can recognize and eliminate invading viruses and plasmids. In bacteria and archaea, as the name suggests, the CRISPR locus is a cluster of repetitive sequences interspersed with spacers. Spacers are variable sequences that come from previous viral or plasmid invaders. Adjacent to this, there are conserved CRISPR-associated (*Cas*) genes, encoding Cas proteins. Exogenous DNA can be sampled and integrated as new spacers in the CRISPR locus. These spacers are then transcribed and processed into small interfering CRISPR RNAs (crRNAs) with the help of a trans-activating crRNA (tracrRNA). If this crRNA-tracrRNA duplex encounters a section of viral DNA that has a sequence which is

complementary to this crRNA, the Cas9 will cleave it. Upstream of these spacers is the protospacer adjacent motif (PAM) site, the target site for Cas endonucleases (Doudna & Charpentier, 2014; Wiedenheft et al., 2012).

There are three major types of CRISPR-Cas systems: Types I–III (Makarova et al., 2011). Due to its lower complexity, Type II is used for genome engineering and consists of a single Cas protein (Cas9) and an RNA duplex of a crRNA and a tracrRNA. CRISPR technology currently uses a crRNA-tracrRNA fusion, called single-guide RNA (sgRNA or gRNA) to direct the cleavage of sequences of interest by the Cas9 endonuclease, through the recognition of the PAM site ('NGG', where N is any nucleotide, followed by two guanines) (Doudna & Charpentier, 2014; Jinek et al., 2012), creating a double-strand break (DSB). The genome editing process happens during the repair of such a DSB (**Figure 4.1**).

There are two main repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (**Figure 4.1**). NHEJ is an efficient, but error-prone DNA repair pathway, in which DNA ends are joined together without a template, often creating small insertions or deletions (INDELs). In HDR, the DSB repair is based on complementarity with a homologous template, resulting in most cases in a perfect repair of the DNA. Therefore, an exogenous repair template (donor DNA (dDNA); e.g., an antibiotic selection marker) with the desired alteration can be delivered into the cell to allow HDR to occur, replacing the endogenous segment.

Most of the studies using CRISPR/Cas9 in phytopathology have been focused on the plant side for disease resistance engineering. It first started in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* and then expanded to maize, tomato, rice, wheat, and sorghum, notably, all angiosperm species (Dort et al., 2020). Engineering disease resistance in forest trees proves to be more complex than in other species (see **Section 1.6** and **Section 1.7** of **Chapter 1**), which in turn makes CRISPR/Cas9 research in forest pathology limited. An exception to this is CRISPR/Cas9 studies in the genus *Populus*. Poplar trees have an ecological and economical importance around the world and are threatened by pathogens belonging to the *Melampsora* and the *Sphaerulina* genera. Poplars are considered a model system in molecular studies of forest trees. It is important to note, however, that poplars are angiosperms, with a shorter life-cycle when

compared with those of most gymnosperm trees. Poplars also have relatively small genomes, making it easier for its complete sequencing and assembly. So far, most CRISPR/Cas9 studies in poplar species have focused on gene targeting with the aim of achieving growth improvements. There are, however, two studies targeting genes related to disease resistance. Poplar CRISPR/Cas9 mutants were generated by overexpressing two different transcription factors involved in resistance against fungi, leading to increased disease resistance (Jiang et al., 2017; Wang et al., 2017).

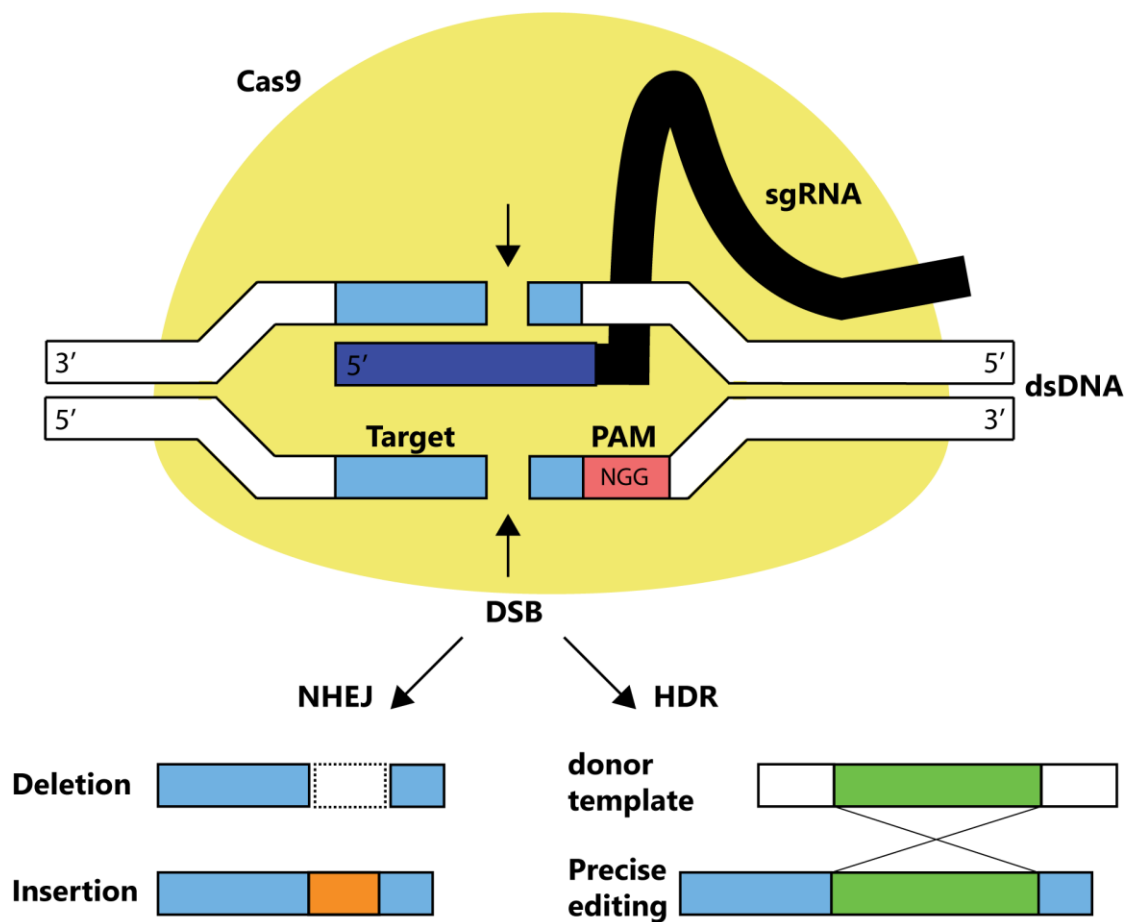


Figure 4.1. CRISPR/Cas9 genome editing system and DNA repair pathways. Single-guide RNA (sgRNA) is complementary in sequence to the target sequence, which is next to a protospacer adjacent motif (PAM) site that directs the CRISPR-associated endonuclease 9 (Cas9) to the cleavage site (Cas9 recognizes a 'NGG' PAM, where 'N' is any nucleotide, followed by two guanines), creating a double-strand break (DSB). The cell repairs the DSB in the double-strand DNA (dsDNA) by two main pathways: non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ creates small insertions or deletions, while HDR, in the presence of a donor DNA template, involves repair by homologous recombination, resulting in more precise DNA editing.

In filamentous fungi and oomycetes, most published applications of the CRISPR/Cas9 method so far have involved proof-of-concept experiments. Once these protocols have been established, it will be easier to disrupt or delete genes for functional characterization in host-pathogen interactions. Other applications include tagging proteins with fluorescent markers to determine their subcellular localization, and assessments of gene regulation, either by gene overexpression or gene silencing (Wang & Coleman, 2019). Many plant-pathogenic fungal and oomycete species have been edited using the CRISPR/Cas9 system, including *Phytophthora sojae* (Fang & Tyler, 2016), *Phytophthora capsica* (Wang et al., 2018), *Ustilago maydis* (Schuster et al., 2016), *Magnaporthe oryzae* (Arazoe et al., 2015) and Dothideomycetes like *Alternaria alternata* (Wenderoth et al., 2017), *Leptosphaeria maculans* (Idnurm et al., 2017) and *Venturia inaequalis* (Rocafort et al., 2022).

The best way to determine the function of an effector is to observe phenotypic variation of mutants regarding its virulence. For this reason, three *Dothistroma septosporum* genes were chosen to be disrupted, resulting in mutants that were used in virulence assays to try to determine their biological roles during the infection of *Pinus radiata*.

Two cross-kingdom CEs from *D. septosporum*, Ds69335 and Ds131885, with homologues in *Cyclaneusma minus* and *Phytophthora pluvialis*, were selected. As mentioned before, Ds69335 has strong up-regulation in the Early and Mid *in planta* stages that mark the start and the end of the biotrophic stage, when compared with the Late (necrotrophic) stage and *in vitro* (**Appendix Table A.8**). Furthermore, Ds69335 has a predicted 'cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins' (CAP) domain and structural homology to Pry1 proteins, from *Saccharomyces cerevisiae*, with some CAP proteins having known virulence roles in other pathogenic fungi (see **Section 3.3.3.2** of **Chapter 3**), making it a good candidate for a study of function. Cell death elicitor Ds131885 triggers cell death in both non-host and host plants, and its gene is also highly expressed and up-regulated during pine infection, especially during the necrotrophic stage (**Appendix Table A.8**). Ds131885 is a homologue of cross-kingdom pathogen-associated molecular pattern (PAMP), VmE02, from apple pathogenic fungus *Valsa mali* (Nie et al., 2019), and the predicted tertiary

structure of the protein is similar to a characterized protein involved in pathogen invasion (Parker et al., 2016). Thirdly, Ds74283, even though not present in the other two pine pathogens analysed, was also selected on the basis that it triggered cell death responses in *Nicotiana* species and *Pn. radiata*, and its gene is also highly expressed during the Mid and Late *in planta* stages of pine infection (**Appendix Table A.8**). Structural prediction of this protein indicates it has similarity to proteins with a β -trefoil fold, a fold shown to also be present in other *D. septosporum* CEs, as well as proteins from *C. minus* and *P. pluvialis* (**Chapter 3** and **Chapter 5**).

4.2 Methods

4.2.1 Designing the sgRNA and CRISPR/Cas9 plasmid

As mentioned above, one of the two main components of a CRISPR/Cas9 system is a sgRNA. The sgRNA is a 20-base pair (bp) target sequence adjacent to a PAM site which directs the Cas9 to the cleavage site, creating a DSB and thereby disrupting the gene of interest. The sgRNAs for the three CE genes were designed according to Rocafort et al. (2022). For that, the "Find CRISPR sites" tool, implemented in Geneious v9.1.8 (Kearse et al., 2012), was employed, using the nucleotide sequence of each CE gene as a target. This tool searches for sgRNA sites in the gene (or region) of interest, and also searches for off-target binding sites in the *D. septosporum* NZE10 genome. For each CRISPR site, the programme calculates an Activity Score (or on-target score), which is a measure of efficiency of sgRNA binding and Cas9 cleavage at that site, and a Specificity Score (or off-target score), that is based on the presence of off-target binding sites in other parts of the genome. It is best to choose sites in the first exon, because mutations at the start of a gene are more likely to produce a truncated or non-functional protein. Therefore, the first exon of each gene was targeted for possible CRISPR sites.

The sgRNA was generated by annealing the 5' and 3' complementary sgRNA primers. The primers (designed without the PAM site) had the addition of SapI 3 bp overhangs specific for Golden Gate cloning into the expression vector Cas9HygAMAccdB (**Appendix Figure A.25**). Primers were synthesized by Integrated DNA Technologies (IDT) and are shown in **Appendix Table A.2**. The sgRNA was generated by pre-annealing 40 ng of the 5' and 3' primers, in annealing buffer (50 mM NaCl, 1 M EDTA pH 8 and

10 mM Tris-HCl pH 8) with the following reaction conditions: 5 min at 95°C, 20 s at 95°C, decreasing 0.5°C each cycle, for 140 cycles, and 1 min at 25°C (Rocafort et al., 2022).

The expression vector Cas9HygAMAcdb is an autonomously replicating plasmid and contains the required components to enable correct genome editing (**Appendix Figure A.25**) (Rocafort et al., 2022). It contains an *Aspergillus niger cas9*, tagged with a nuclear localization signal, in order to import the Cas9 into the fungal nucleus, and also has an RNA polymerase III promoter for sgRNA expression *in vivo*.

A Golden Gate reaction was performed to ligate the pre-annealed sgRNA into the expression vector Cas9HygAMAcdb, with the SapI restriction enzyme. For this, 20 fmol of Cas9HygAMAcdb, 10 fmol of pre-annealed primers, 10% (v/v) 1 x T4 ligase buffer, 5% (v/v) T4 ligase (New England Biolabs), 1% (v/v) SapI restriction enzyme (New England Biolabs) and sterile dH₂O were combined in a total reaction volume of 10 µL. The reaction conditions were: 30 cycles of restriction-ligation at 37°C for 1 min and 16°C for 1 min, digestion at 55°C for 5 min, heat inactivation at 80°C for 5 min (Rocafort et al., 2022). The sgRNAs for the three *CE* genes, cloned into Cas9HygAMAcdb, were generated, and confirmed by sequencing.

4.2.2 Generating donor DNA plasmid for homologous recombination

Using CRISPR alone requires the fungus to repair the DSB using the NHEJ pathway, which is an efficient, but error-prone DNA repair pathway. The other natural cell repair system, HDR, occurs in the presence of a template, generating a perfect copy of the DNA. Therefore, exogenous repair templates, in this case a selectable marker flanked by sequence matching the target gene, can be delivered into the cell to allow HDR to occur such that the target gene is disrupted.

The dDNA (template for the HDR) for each transformation had two flanks which included part of the targeted *CE* gene, with the *nptII* cassette (*P_{trpC}-nptII-T_{trpC}*) in between. The gene *nptII* confers resistance to geneticin. Geneticin is a selective antibiotic produced by *Micromonospora rhodorangea*, that inhibits the elongation step of translation, blocking polypeptide synthesis in prokaryotic and eukaryotic cells. The 5' and 3' flanks both start 3 bp from each side of the DSB (which occurs 3 bp downstream from the PAM site) (Jinek et al., 2012). Both flanks were designed to avoid the coding sequence

(CDS) from genes upstream and downstream from the targeted *CE* gene. The 2,808 bp *nptII* cassette sequence was amplified from plasmid pII99 (**Appendix Figure A.26**) (Lara-Ortíz et al., 2003), and the 2,591 bp backbone vector sequence was amplified from plasmid pAN7-1 (**Appendix Figure A.27**) (Punt et al., 1990), which is a Gibson Assembly-compatible plasmid, used for dDNA assembly. Flanks were amplified from wild-type (WT) *D. septosporum* NZE10 genomic DNA (gDNA) by PCR (**Table 4.1**). All PCRs were performed with Phusion High-Fidelity DNA polymerase (New England Biolabs) (described in **Section 2.2.6.1 of Chapter 2**). Fragments were then assembled via Gibson Assembly (Gibson et al., 2009) and the correct assembly was verified by sequencing. The resulting dDNA plasmid was linearised with the *NdeI* (New England Biolabs) restriction enzyme prior to fungal transformation.

Table 4.1. *Dothistroma septosporum* sequence details for the flanks used to generate donor DNA for homologous recombination.

CE gene^a	Flank	Size (bp)^b	Chromosome^c	nt position^d
<i>Ds69335</i>	5'	1395	2	1,021,170 to 1,022,564
	3'	1000		1,020,163 to 1,021,162
<i>Ds74283</i>	5'	966	9	714,367 to 715,332
	3'	859		715,340 to 716,198
<i>Ds131885</i>	5'	1000	6	1,668,852 to 1,669,851
	3'	840		1,668,005 to 1,668,844

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (*CE*) gene of interest that was disrupted.

^b Length of the flank in base pairs (bp).

^c Chromosome location of the candidate effector gene in the *D. septosporum* NZE10 genome (de Wit et al., 2012).

^d Nucleotide (nt) position of the start and end of the flank sequence.

4.2.3 Construction of complementation plasmids

To make *D. septosporum* NZE10 *Ds69335*, *Ds74283* and *Ds131885* complementation plasmids, the targeted *CE* genes, along with 5' and 3' regions flanking each *CE* gene (**Table 4.2**), were amplified using specific primers carrying *Apal* (5') and *XbaI* (3') restriction digestion sites (**Appendix Table A.2**). Both flanks were designed to avoid the CDS from genes upstream and downstream from the targeted *CE* gene. Each PCR product was digested with the appropriate enzymes and ligated into the pBC-phleo vector (**Appendix Figure A.28**) (Silar, 1995), and the correct assembly was verified by sequencing prior to *D. septosporum* transformation.

Table 4.2. *Dothistroma septosporum* sequence details for the flanking regions used to generate complementation strains.

CE gene ^a	CDS position – chromosome ^b	CDS + flanks position ^c	Flank size (bp) ^d
<i>Ds69335</i>	1,020,671 to 1,021,564 – Chr 2	1,020,133 to 1,022,264	5' – 700 and 3' – 538
<i>Ds74283</i>	715,296 to 715,932 – Chr 9	714,596 to 716,198	5' – 700 and 3' – 266
<i>Ds131885</i>	1,668,375 to 1,668,916 – Chr 6	1,668,005 to 1,669,616	5' – 700 and 3' – 370

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b Nucleotide and chromosome (Chr) location of the coding sequence (CDS) of each targeted candidate effector gene in the *D. septosporum* NZE10 genome (de Wit et al., 2012).

^c Nucleotide position of the genomic region (CDS plus flanks) used to generate the complementation plasmids.

^d Length of the flank in base pairs (bp).

4.2.4 Transformation of *Dothistroma septosporum* NZE10

4.2.4.1 *Dothistroma septosporum* media

D. septosporum medium (DM): 5% (w/v) malt extract (Oxoid), 2.3% (w/v) nutrient broth (Oxoid). DM agar was made with the addition of 1.5% (w/v) bacteriological agar (Acumedia®).

D. septosporum Sporulating Media (DSM): 1.5% (w/v) malt extract (Oxoid), 1.5% (w/v) yeast extract (Merck). DM agar was made with the addition of 1.5% (w/v) bacteriological agar (Acumedia®).

Pine Needle Minimal Media (PMMG): 60 g of pine needles were soaked in 600 mL of distilled water (dH₂O) for 24 h, then, needles were removed, and the following was added: 0.02% (w/v) MgSO₄·7H₂O, 0.09% (w/v) KCl, 0.1% (w/v) NH₄NO₃, 0.0002% (w/v) FeSO₄, 0.0002% (w/v) ZnSO₄·7H₂O, 0.0002% (w/v) MnCl₂, 0.2% (w/v) asparagine, 0.3% glucose. PMMG agar was made with the addition of 1.5% (w/v) bacteriological agar (Acumedia®).

4.2.4.2 Preparation of protoplasts

100 µL of freshly grown *D. septosporum* NZE10 mycelium was ground in sterile dH₂O and inoculated into 25 mL DM broth in 125 mL flasks. The fungal culture was grown at 22°C with shaking at 160 rpm (Ecotron) for 7 days and mycelia were harvested by filtration using an autoclaved funnel and miracloth (Merk). Mycelia were washed 3 times

with approx. 30 mL of sterile dH₂O, and once with 10 mL OM buffer (1.4 M MgSO₄, 10 mM Na₂HPO₄, pH adjusted with 100 mM NaH₂PO₄·2H₂O to 5.8). Then, 30 mL of glucanex digestion buffer (10 mg/mL of Glucanex® 200G (Novozyme, Bagsværd, Denma) in OM buffer) was added to the mycelia and incubated at 30°C overnight with shaking at 100 rpm (Ecotron). Protoplast formation was checked under a microscope the next day. Protoplasts were harvested at room temperature (RT) by filtering through sterile miracloth then aliquoted in 5 mL volumes in 15 mL falcon tubes. Two mL of ST buffer (0.6 M sorbitol and 100 mM Tris pH 8) was carefully overlaid on top of the protoplast/glucanex solution and centrifuged at 2880 *g* for 5 min. A white layer of protoplasts formed between the glucanex solution and ST buffer. The protoplasts were transferred to a 15 mL falcon tube. Five mL of STC buffer (1 M sorbitol, 50 mM CaCl₂ and 50 mM Tris pH 8) was added to the tube which was centrifuged at 2880 *g* for 5 min. The STC washing and centrifugation step was repeated 3 times. Finally, protoplasts were resuspended in STC buffer to a concentration of 1.25 x 10⁸ protoplasts/mL.

4.2.4.3 *Dothistroma septosporum* NZE10 transformation using the PEG method

Protoplast-mediated transformation of *D. septosporum* was performed based on the method of Yelton et al. (1984). Three µg of circular CRISPR/Cas9-sgRNA plasmid and 6 µg of linearized dDNA plasmid (5 µg in the case of complementation plasmids) were mixed with 80 µL of protoplast suspension and 20 µL of 40% PEG solution (40% (w/v) PEG 4000 (BDH, Poole, UK)), 50 mM CaCl₂, 50 mM Tris pH 8.0 and 1 M sorbitol) in a microcentrifuge tube and kept on ice for 30 min. A total of 900 µL of 40% PEG solution was added to the reaction and incubated at room temperature (RT) for 30 min. Next, 100 µL of the reaction mixture was mixed with 5 mL of molten 1.5% Regeneration Media (RG) (5% (w/v) malt extract, 2.3% (w/v) nutrient broth, 27.4% (w/v) sucrose and 1.5% (w/v) bacteriological agar), cooled to 50°C and poured over RG agar plates (5% (w/v) malt extract, 2.3% (w/v) nutrient broth, 27.4% (w/v) sucrose and 1.5% (w/v) bacteriological agar). The plates were incubated overnight at 22°C. The next day, 5 mL of molten 0.8% RG overlay (same as 1.5% RG, but with 0.8% (w/v) bacteriological agar), cooled to 50°C, and with the appropriate antibiotic, was poured over the RG agar plates to make a final concentration of 70 µg/mL hygromycin B (Roche) and 100 µg/mL geneticin (Thermo Fisher Scientific) for gene disruption, or 100 µg/mL geneticin and 14 µg/mL phleomycin

(InvivoGen, California, USA) for gene complementation. The plates were incubated at 22°C for 3–4 weeks. Undiluted protoplasts were spread on appropriate antibiotic plates as a negative control.

4.2.5 Screening for positive transformants by PCR

gDNA was initially extracted using a crude colony method to facilitate screening according to Liu et al. (2000). For that, 500 µL of lysis buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl and 1% (w/v) SDS) was added to a 1.5 mL tube containing a small piece of mycelia, disrupted with a sterile toothpick. After 10 min at RT, 150 µL of potassium acetate buffer (60 mL of 5 M CH₃CO₂K, 11.5 mL of glacial acetic acid and 28.5 mL H₂O, pH 4.8) was added and vortexed briefly, followed by centrifugation at 10,000 *g* for 1 min. The supernatant was transferred to a new tube and centrifuged as before. After transferring the supernatant to a new tube, an equal volume of isopropyl alcohol was added, and samples were centrifuged at 10,000 *g* for 2 min. The supernatant was then discarded, and the pellet was washed in 300 µL of 70% ethanol and centrifuged at 8,000 *g* for 1 min. The pellet was air-dried and resuspended in 50 µL of TE buffer and 2 µL were used as template for PCRs.

Two sets of primers (**Appendix Table A.2**) were used to screen for positive CRISPR/Cas9 transformants. The first pair amplified the sequence between the start and stop codon of each *CE* gene that was disrupted, while the second pair, binding outside of the targeted gene region, amplified the 5' flank, the *nptII* cassette and 3' flank. For gene complementation screening, the same set of primers that amplified the sequence between the start and stop codon of each *CE* gene was used.

4.2.6 Screening for positive transformants by Southern hybridization

Confirmation of gene disruption was done by Southern hybridization with a digoxigenin (DIG)-labelled probe using a DIG High Prime DNA Labelling and Detection Starter Kit I (Roche). Probes binding to both the CRISPR/Cas9 transformants and WT fungus were generated with DIG-High Prime according to the random primed labelling technique, which contains Digoxigenin-11-dUTP for colour detection by enzyme immunoassay. **Table 4.3** shows the position in the *D. septosporum* genome of the probes

targeting each gene, and the primers used to amplify them are shown in **Appendix Table A.2**.

Table 4.3. *Dothistroma septosporum* sequence details for the probes used for Southern hybridization.

CE gene^a	Size (bp)^b	Chromosome^c	Nt position^d
<i>Ds69335</i>	664	2	1,020,239 to 1,020,902
<i>Ds74283</i>	636	9	715,563 to 716,198
<i>Ds131885</i>	679	6	1,668,005 to 1,668,683

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (*CE*) gene of interest that was disrupted for which the hybridization probe was designed.

^b Length of the probe in base pairs (bp).

^c Chromosome location of the candidate effector gene in the *D. septosporum* NZE10 genome (de Wit et al., 2012).

^d Nucleotide (nt) position of the start and end of the probe sequence.

gDNA from WT *D. septosporum* and transformants was extracted according to Doyle and Doyle (1987) (described in **Section 2.2.5.1** of **Chapter 2**), then 2 µg aliquots were digested with the appropriate restriction enzymes overnight. Restriction fragments were separated on a 0.8% agarose gel. The gel was incubated in 0.25 M HCl for 10 min, then transferred to denaturation buffer (0.5 M NaCl and 0.5 M NaOH) for 30 min and finally incubated for 60 min in neutralization buffer (0.5 M NaCl and 0.5 M Tris pH 7.4). DNA was then transferred to a positively charged nylon membrane (Roche). For that, a blotting stand was assembled by putting two layers of Whatman paper (Whatman, Maidstone, UK) on top of glass plate over a plastic container and filling the wells with 20 x SSC buffer (3 M NaCl and 0.3 M Na₃C₆H₅O₇). The gel was placed upside down on the Whatman paper, and the membrane, pre-soaked in 2 x SSC buffer, was put on top of it. Two more sheets of Whatman paper pre-soaked in 2 x SSC were placed on membrane, along with a pile of paper towels and a heavy weight. The assembled stand was left to blot overnight. The next day, the membrane was washed in 2 x SSC and fixed under a UV crosslinker Cex-800 (Ultra-Lum Inc, California, USA).

For hybridization, the membrane was prehybridized for 60 min at 42°C in DIG Easy Hyb solution (10 mL/100 cm² of membrane) in a hybridization tube with rotation. The DIG-labelled DNA probe was denatured by boiling for 5 min and rapidly placed on ice. The denatured probe was added to the hybridization buffer (3.5 mL of probe/hybridization mixture for each 100 cm² of membrane) and incubated with rotation

at 42°C overnight. The hybridized membrane was washed twice in 2 x SSC and 0.1% (v/v) SDS at RT for 5 min with constant agitation, and then washed twice in 0.5 x SSC and 0.1% (v/v) SDS (prewarmed to 70°C) with constant agitation.

Colour detection was performed by enzyme immunoassay using the DIG High Prime DNA Labelling and Detection Starter Kit I, according to manufacturer's instructions. Here, the membrane was incubated for 30 min in 1 x Blocking solution (10 x Blocking solution (Roche) diluted 1:10 in maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl, final pH 7.5), and then incubated for 30 min in antibody solution (Anti-DIG-AP diluted 1:5000 in 1 x Blocking solution). Next, the membrane was washed twice for 15 min in washing buffer (maleic acid buffer containing 0.3% (v/v) Tween 20), then incubated for 5 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, final pH 9.5). Finally, the membrane was incubated without shaking in colour-substrate solution (NBT/BCIP (Roche) diluted 1:50 in Detection buffer) overnight in the dark.

4.2.7 Copy number determination in complementation strains

gDNA was extracted following Doyle and Doyle (1987) (described in **Section 2.2.5.1 of Chapter 2**) and 50 µg/µL DNA was used in real time-quantitative PCR (qPCR) (described in **Section 3.2.3 of Chapter 3**). A known *D. septosporum* single-copy gene, *DsAflR* (JGI ID 75566) (Chettri et al., 2013), was used as reference, and the ratio between the target gene and the *DsAflR* gene was calculated using cycle threshold (Ct) values according to the following formula:

$$Ratio: \frac{(E_{target})^{\Delta Ct \text{ target (control-sample)}}}{(E_{ref})^{\Delta Ct \text{ ref (control-sample)}}$$

E: primer efficiency

ΔCt target (control – sample): (Ct of the targeted gene in WT) - (Ct of the targeted gene in complemented strain)

ΔCt ref (control – sample): (Ct of *DsAflR* in WT) - (Ct of *DsAflR* in complemented strain)

4.2.8 Phenotypic characterization of *Dothistroma septosporum* NZE10 transformants

4.2.8.1 Radial growth rate assay

Mycelia of actively growing colonies of *D. septosporum* transformants were cut with a 5 mm diameter sterile cork borer and plugs were placed on DM plates. Three plugs were grown on each DM plate and three replicate plates were used for each transformant and WT fungus. Plates were incubated at 22°C for 3 weeks, when radial colony growth was measured along two perpendicular axes and calculated as mm/day over this period.

4.2.8.2 Sporulation assay

Sporulation was assessed by spreading 50 µL spore suspension (1×10^5 spores/mL) on PMMG plates with three replicate plates for each transformant and WT fungus. Plates were incubated at 22°C for 7 days. Two mL of sterile dH₂O was added to each plate and incubated for 10 minutes at RT. Spores were collected using a sterile glass spreader, then the concentration was quantified using a cytometer (Weber Scientific, Middlesex, UK) and expressed as spores/mL.

4.2.9 Virulence assays of *Dothistroma septosporum* transformants on *Pinus radiata*

4.2.9.1 *Pinus radiata* seedlings

Pine seeds were provided by the Radiata Pine Breeding Company (RPBC). These seeds were from a cross between two parents with a similarly low level of *D. septosporum* resistance. Seeds were germinated at Scion (Rotorua, New Zealand) and seedlings were grown in small root trainer pots (approximately 2 x 2 cm²). These were then sent to Massey University (Palmerston North, New Zealand) and transplanted to pots of 9 x 9 cm² using standard garden potting mix. Seedlings were kept in a glasshouse with natural light and ambient temperature (heated to maintain 20°C during the winter months) until inoculation. Plants were approximately 16 months old at the time of inoculation and approximately 40 cm in height.

4.2.9.2 *Dothistroma septosporum* inoculation on *Pinus radiata* seedlings

The *D. septosporum* inoculations on *Pn. radiata* seedlings were performed according to Kabir et al. (2013). For that, *D. septosporum* spores were obtained from seven-day-old PMMG plate cultures and were sprayed onto *Pn. radiata* seedlings using a sterile universal bottle with a plastic bottle sprayer head screwed onto it. Two separate sets of seedlings were inoculated in two different weeks (Week 1 and Week 2). This was done because not all strains produced enough spores in Week 1. Each week had seedlings inoculated with the WT fungus, so transformants could be compared to the WT fungus of the respective week they were inoculated. **Table 4.7 (Section 4.3.5)** shows how many replicate pines were inoculated for each strain and the concentration and volume of spores used, as well as which strains were inoculated in Week 1 and Week 2. After the pines were sprayed, they were left to air-dry for 20 min. Each plant was then covered with a clean plastic sleeve (Petri dish bag) to maintain very high needle wetness.

Plants were placed in growth chambers (**Figure 4.2**) (one replicate seedling inoculated with each strain per chamber), that consisted of a plastic container and a plastic frame covered with clear plastic. A ventilation opening was cut at the top that allowed for it to be opened or closed. Seedlings were placed on top of tip boxes to elevate the pots above the water level in the chamber. After four days, the plastic sleeve covering each plant was removed. Inside each chamber, two custom-made water misters/foggers were then placed in to order to keep high needle wetness. Water was also sprayed through the ventilation opening at the top of the chamber to apply additional wetness once or twice a day, as necessary.

For the first two weeks (for the first set of inoculated seedlings; and one week for the second set of inoculated seedlings), the experiment was conducted in a growth room with artificial light (12 h light/12 h dark photoperiod with $180 \mu \text{mol m}^{-2} \text{s}^{-1}$) at 22°C. After that, seedlings were transferred to a 22°C growth room, with approximately 80% humidity and under natural light (15 to $750 \mu \text{mol m}^{-2} \text{s}^{-1}$) for an additional eight weeks. The experiments were conducted for a total of ten weeks for both sets of inoculated seedlings.

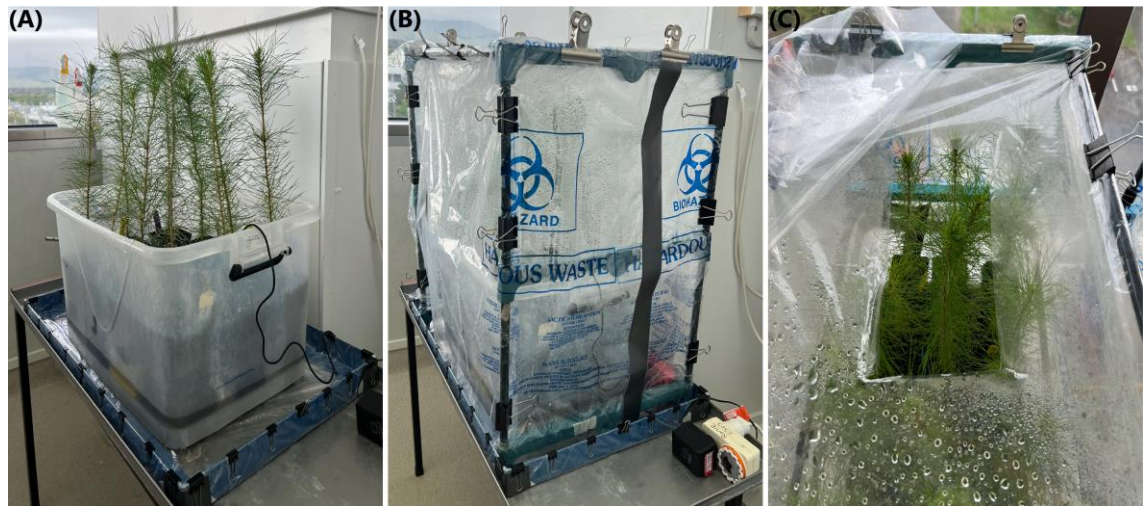


Figure 4.2. Growth chambers for *Pinus radiata* virulence assays with *Dothistroma septosporum* transformants. **(A)** After inoculation with *D. septosporum* spores, seedlings were placed in plastic containers. **(B)** Plastic containers were covered with a plastic frame covered with plastic. **(C)** Detail of the ventilation opening cut at the top of the chamber.

4.2.9.3 Lesion counts and fungal biomass determination

After ten weeks, needles were sampled from each seedling and separated into three categories: (1) alive/healthy (no visible characteristic *Dothistroma* needle blight (DNB) symptoms), (2) necrotic lesions (visible characteristic DNB symptoms; see **Section 1.2.1** of **Chapter 1**) and (3) dead/dried without DNB symptoms. Dead/dried needles were discarded. Healthy needles and needles with DNB symptoms were counted for each inoculated plant, and the proportion of needles with DNB symptoms compared with total needles (healthy plus needles with DNB symptoms) was calculated.

Next, characteristic DNB lesions were cut out of needles along with 1–2 mm of adjacent tissue and freeze-dried overnight (Dura-Dry MP, Kinetics Thermal Systems, New York, USA). The next day, the dry-weight was determined. The gDNA was first extracted using the CTAB method described in **Section 2.2.5.1** of **Chapter 2**, followed by purification with the GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Because of the low quality of gDNA obtained, remaining samples were extracted using the Plant Genomic DNA Mini Kit (Geneaid, New Taipei City, Taiwan) following the manufacturer's instructions (**Appendix Table A.12** indicates the method each sample was extracted with). gDNA

concentration was quantified using a Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific) in a Invitrogen™ Qubit™ 3 Fluorometer. gDNA was used as template in qPCR (described in **Section 3.2.3** of **Chapter 3**) analysis to estimate the amount of fungal biomass present in the pine needle lesions. This was performed in two different ways. First, relative quantification of fungal biomass was calculated by comparing the *D. septosporum* target gene polyketide synthase A (*DsPksA* – JGI ID 48345) with the *Pn. radiata* reference gene cinnamyl alcohol dehydrogenase (*CAD*) (Chettri et al., 2012). Secondly, an absolute quantification of fungal biomass was calculated based on amplification of the same *D. septosporum DsPksA* gene in comparison to a standard curve of Ct values versus gDNA concentration. In both cases, the fungal biomass was normalized by the dry-weight and by the number of lesions used for gDNA extraction in each sample. The qPCR primers are shown in **Appendix Table A.2**. Standard curves used WT *D. septosporum* NZE10 and *Pn. radiata* gDNA and were performed as described in **Section 3.2.3** of **Chapter 3**.

4.3 Results

4.3.1 Single-guide RNA selection for CRISPR/Cas9 disruption of *Dothistroma septosporum* candidate effector genes

As mentioned above, careful selection of the sgRNA used in the CRISPR/Cas9 genome editing method is an important step required for a high transformation efficiency. For each of the *D. septosporum* CE genes selected for disruption, the sgRNA sequence that had the highest on-target score in exon 1 and no other targets in the fungal genome, to prevent off-targets, was selected (**Table 4.4**).

Table 4.4. Selected single guide RNA (sgRNA) sequences used to target candidate effector genes of *Dothistroma septosporum* for CRISPR/Cas9 disruption.

sgRNA protospacer ^a	Sequence (5' – 3') ^b	Binding site ^c	Direction	Off-targets ^d	On-target score ^e
<i>Ds69335</i>	ACTCCGTCTCTGACATCGCG TGG	Exon 1	Forward	0	0.626
<i>Ds74283</i>	GTTGTTGTAGGCAGAGACGA AGG	Exon 1	Reverse	0	0.635
<i>Ds131885</i>	CCTTGCTGTTGGAGGTCGCA GGG	Exon 1	Reverse	0	0.617

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector gene of interest that was disrupted.

^b sgRNA sequence targeting each of the candidate effector genes, followed by the PAM site in red.

^c Region of the candidate effector gene where the sgRNA was designed.

^d Off-target binding sites in other parts of the *D. septosporum* genome.

^e Measure of efficiency of sgRNA binding and Cas9 cleavage at that site, based on sequence features of the sgRNA, calculated using Geneious (v9.1.8). Score was 0-1, with a score closer to 1 indicating higher activity.

Following their selection and synthesis, sgRNAs were cloned into the expression vector Cas9HygAMAcclB. Both sgRNA and dDNA were used in separate *D. septosporum* transformations, targeting *Ds69335*, *Ds74283* and *Ds131885*.

4.3.2 Targeted gene disruption of *Dothistroma septosporum* candidate effectors *Ds69335*, *Ds74283* and *Ds131885* through CRISPR/Cas9

The first co-transformation of *D. septosporum* with sgRNA and dDNA, targeted *Ds74283*, and produced 82 transformants of which 42 were stable on selective hygromycin B and geneticin media. Of these, 17 were randomly selected to be screened by PCR. All 17 transformants appeared to show the same colony morphology phenotype as the WT fungus and were screened by PCR with two sets of primers that each spanned the *Ds74283* gene (**Figure 4.3A**). Compared to the WT fungus, all 17 *D. septosporum* transformants had PCR products that were 2.8 kb larger in size, as expected for gene disruption by homologous recombination with the *nptII* cassette (**Figure 4.3B** and **C**). Five positive transformants (3, 25, 45, 52 and 56) from five independent transformation plates were randomly selected for verification by Southern hybridization (**Figure 4.3D**). As expected, none of them showed hybridization to fragment sizes corresponding to the WT fungus, suggesting successful disruption of *Ds74283*. However, only two of the transformants (3 and 56) showed hybridization to fragment sizes expected from integration of the *nptII* gene by homologous recombination at the disruption site. Moreover, all five transformants showed additional hybridizing fragments that did not

correlate with predicted sizes from partial digests or insertion of whole dDNA plasmids.

Ds74283 T3-D and *Ds74283* T56-D were selected for further analysis.

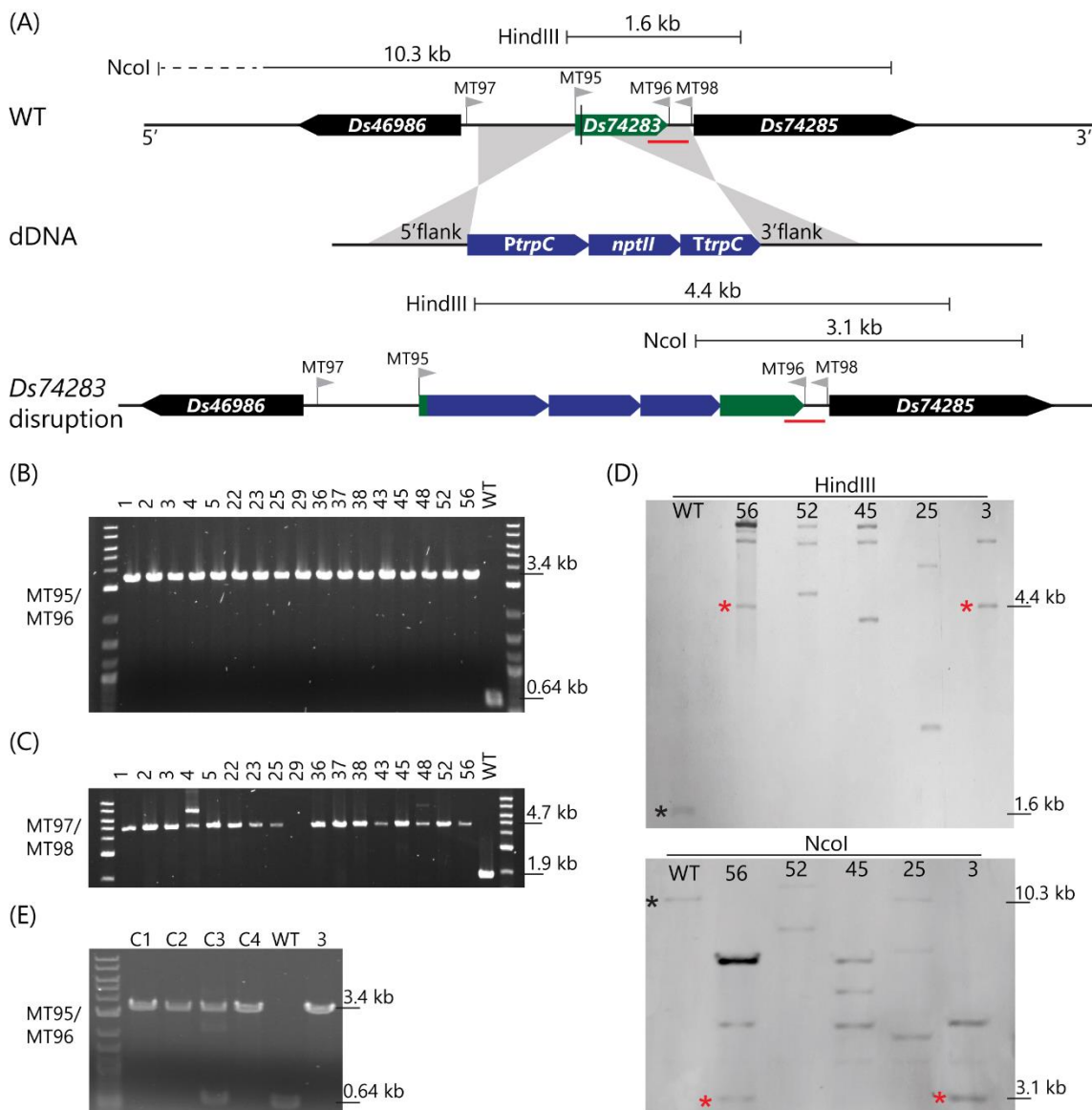


Figure 4.3. *Dothistroma septosporum* CRISPR/Cas9 *Ds74283* disruption and confirmation by PCR and Southern hybridization. **(A)** Schematic diagram showing the disruption of *Ds74283* and insertion of the *nptII* geneticin gene cassette (*PtpC*-*nptII*-*TtpC*) through homologous recombination, using donor DNA (dDNA) as template. The dDNA was constructed with two flanks (5' and 3') from *Ds74283*, starting 3 bp from the double-strand break (shown as the vertical black line crossing the gene), with the *nptII* cassette in the middle. Positions of primers are illustrated by grey flags with the primer name above. Also shown are the restriction enzyme sites and probe binding site (red line) used for Southern hybridization and the fragment sizes expected from the disruption of *Ds74283* by insertion of the *nptII* cassette. **(B)** PCR amplicons generated derived

from primers MT95 and MT96, which bind to the start and stop of the coding sequence, respectively. *Ds74283* mutants should have a product of 3.4 kb, and the wild-type (WT) fungus 0.64 kb. **(C)** PCR amplicons generated with primers MT97 and MT98, which bind either side of the target genomic region. *Ds74283* mutants should have a product of 4.7 kb, and the WT fungus 1.9 kb. Relevant size labels are shown on the right of each gel. **(D)** Southern hybridization of HindIII and NcoI-digested gDNA from *D. septosporum* WT fungus and five candidate *Ds74283* mutants, 3, 25, 45, 52 and 56, using a digoxigenin (DIG)-11-dUTP-labeled probe binding to the 3' flank region of *Ds74283* that was present in the dDNA. Expected fragment sizes are marked with a red asterisk for *Ds74283* mutants and black for WT fungus. Each of the five *Ds74283* mutants were sampled from independent transformation plates. **(E)** PCR screening of putative *Ds74283*-T3 complementation strains with primers MT95 and MT96, which bind to the start and stop of the coding sequence, respectively. Complementation strains should have a product of 0.64 kb, the same as the WT fungus, and a 3.4 kb from the *nptII* cassette insertion.

The next co-transformation with sgRNA and dDNA plasmids targeted the *Ds131885* gene and resulted in 84 transformants. From those, 67 were stable on selective hygromycin B and geneticin media, and 10 were randomly selected, with all 10 visually having the same colony morphology phenotype as the WT fungus. From these 10 transformants, nine had the 2.8 kb insertion expected for gene disruption and *nptII* cassette insertion (**Figure 4.4B** and **C**). Five PCR positive transformants (2, 9, 13, 25 and 47) and one PCR negative transformant (46) from six independent transformation plates were selected for verification by Southern hybridization (**Figure 4.4D**). As expected, none of the five PCR positive transformants showed hybridization to fragment sizes corresponding to the WT fungus, suggesting a successful disruption of *Ds131885*, while transformant 46 showed a fragment of the same size as the one expected for the WT fungus. The five PCR positive transformants (2, 9, 13, 25 and 47) showed hybridization to fragment sizes expected from the integration of the *nptII* gene by homologous recombination at the disruption size. However, just as observed in the previous hybridisation from the other transformation mentioned above, all transformants showed additional hybridization fragments that did not match with the predicted sizes from partial digestion of the gDNA, or insertions of the whole dDNA plasmid. Transformants *Ds131885* T9-D and *Ds131885* T47-D were selected for further analysis.

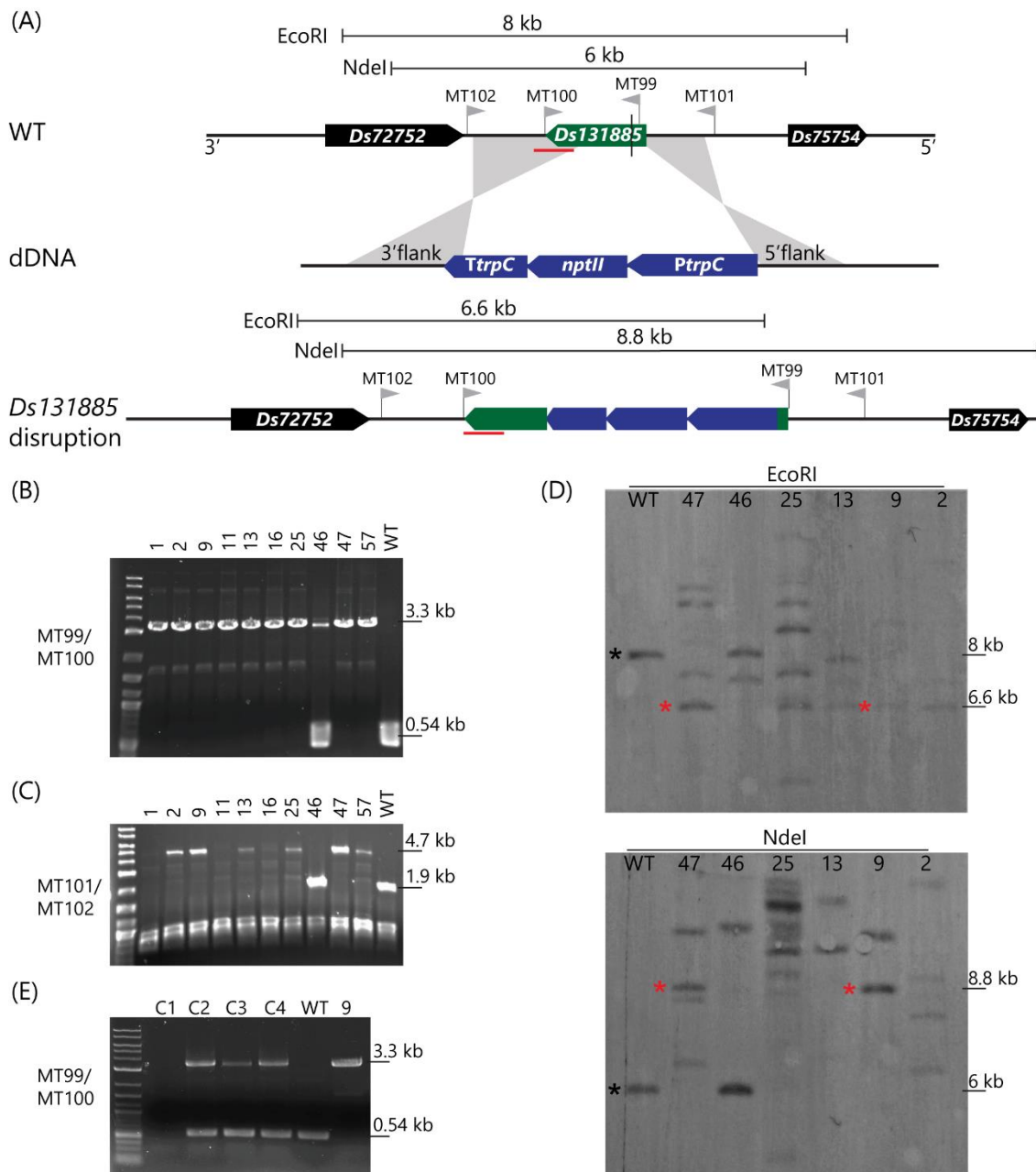


Figure 4.4. *Dothistroma septosporum* CRISPR/Cas9 *Ds131885* disruption and confirmation by PCR and Southern hybridization. **(A)** Schematic diagram showing the disruption of *Ds131885* and insertion of the *nptII* geneticin gene cassette (*PtrpC-nptII-TtrpC*) through homologous recombination, using donor DNA (dDNA) as template. The dDNA was constructed with two flanks (5' and 3') from *Ds131885*, starting 3 bp from the double-strand break (shown as the vertical black line crossing the gene), with the *nptII* cassette in the middle. Positions of primers are illustrated by grey flags with the primer name above. Also shown are the restriction enzyme sites and probe binding site (red line) used for Southern hybridization and the fragment sizes expected from the disruption of *Ds131885* by insertion of the *nptII* cassette. **(B)** PCR amplicons generated with primers MT99 and MT100, which bind to the start and stop of the coding sequence, respectively.

Ds131885 mutants should have a product of 3.3 kb, and the wild-type (WT) fungus 0.54 kb. **(C)** PCR amplicons generated with primers MT101 and MT102, which bind either side of the target genomic region. *Ds74283* mutants should have a product of 4.7 kb, and the WT fungus 1.9 kb. Relevant size labels are shown on the right of each gel. **(D)** Southern hybridization of EcoRI and NdeI-digested gDNA from *D. septosporum* WT fungus and six candidate *Ds131885* mutants, 2, 9, 13, 25, 46 and 47, using a digoxigenin (DIG)-11-dUTP-labeled probe binding to the 3' flank region of *Ds131885* that was present in the dDNA. Expected fragment sizes are marked with a red asterisk for *Ds131885* mutants and black for WT fungus. Each of the six *Ds131885* mutants were sampled from independent transformation plates. **(E)** PCR screening of putative *Ds131885*-T9 complementation strains with primers MT99 and MT100, which bind to the start and stop of the coding sequence, respectively. Complementation strains should have a product of 0.54 kb, the same as the WT fungus, and a 3.3 kb from the *nptII* cassette insertion.

The last co-transformation of *D. septosporum* with sgRNA and dDNA targeted *Ds69335*, and produced 87 transformants, of which 48 were subcultured only to selective geneticin media. This was done to try to avoid any possible off-target effects due to the maintenance of the autonomously replicating Cas9HygAMAcclB plasmid (once hygromycin B selection is removed, the plasmid can be lost). From these 48 transformants, 14 were randomly selected. All 14 transformants showed the same colony morphology phenotype as the WT fungus and were screened with two sets of primers that each spanned the *Ds69335* gene (**Figure 4.5A**). Compared to the WT fungus, eight transformants (1, 2, 4, 7, 9, 19, 36 and 40) seemed to have PCR products that were 2.8 kb larger in size, as expected for gene disruption by homologous recombination with the insertion of the *nptII* cassette (**Figure 4.5B and C**). Transformants 3, 5 and 10 had products that matched in size with the WT and were considered negative or ectopic transformants. Six positive transformants (1, 2, 4, 7, 36 and 40) from six independent transformation plates were selected for verification by Southern hybridization (**Figure 4.5D**). As expected, none of them showed hybridization to fragment sizes corresponding to the WT fungus, suggesting a successful disruption of *Ds69335*. Five of them (1, 2, 7, 36 and 40) showed hybridization to fragment sizes expected from the integration of the *nptII* gene by homologous recombination at the disruption size. However, all transformants, except transformant 2, showed additional hybridization fragments that did not match with the predicted sizes from partial digestion of the gDNA, or insertions

of the whole dDNA plasmid. For this reason, *Ds69335* T2-D and *Ds69335* T40-D were selected as the two independent transformants to be phenotypically characterized and tested for their effect in *Pn. radiata* virulence assays.

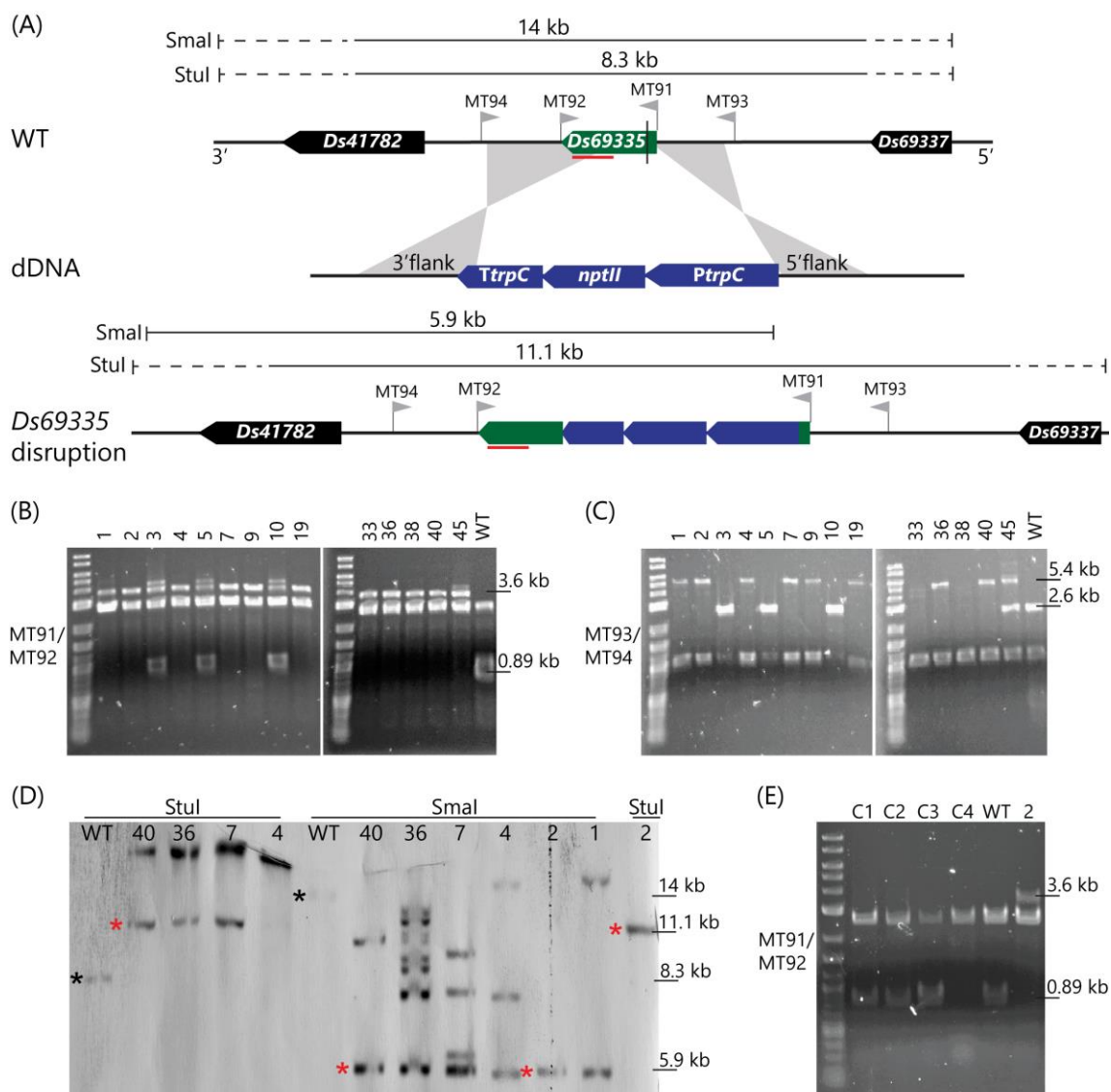


Figure 4.5. *Dothistroma septosporum* CRISPR/Cas9 *Ds69335* disruption and confirmation by PCR and Southern hybridization. **(A)** Schematic diagram showing the disruption of *Ds69335* and insertion of the *nptII* geneticin gene cassette (*PtrpC-nptII-PtrpC*) through homologous recombination, using donor DNA (dDNA) as template. The dDNA was constructed with two flanks (5' and 3') from *Ds69335*, starting 3 bp from the double-strand break (shown as the vertical black line crossing the gene), with the *nptII* cassette in the middle. Positions of primers are illustrated by grey flags with the primer name above. Also shown are the restriction enzyme sites and probe binding site (red line) used for Southern hybridization and the fragment sizes expected from the disruption of *Ds69335* by insertion of the *nptII* cassette. **(B)** PCR amplicons generated with primers

MT91 and MT92, which bind to the start and stop of the coding sequence, respectively. *Ds69335* mutants should have a product of 3.6 kb, while the wild-type (WT) should have one of 0.89 kb. **(C)** PCR amplicons generated with primers MT93 and MT94, which bind either side of the target genomic region. *Ds69335* mutants should have a product of 5.4 kb, and the WT fungus 2.6 kb. Relevant size labels are shown on the right of each gel. **(D)** Southern hybridization of *Stu*I and *Sma*I-digested gDNA from *D. septosporum* WT fungus and six candidate *Ds69335* mutants (1, 2, 4, 7, 36, 40) with *Sma*I and five with *Stu*I (2, 4, 7, 36, 40) using a digoxigenin (DIG)-11-dUTP-labeled probe binding to the 3' flank region of *Ds69335* that was present in the dDNA. Expected fragment sizes are marked with a red asterisk for *Ds69335* mutants and black for WT fungus. Each of the five *Ds69335* mutants were sampled from independent transformation plates. **(E)** PCR screening of putative *Ds69335*-T2 complementation strains with primers MT91 and MT92, which bind to the start and stop of the coding sequence, respectively. Complementation strains should have a product of 0.89 kb, the same as the WT fungus, and a 3.6 kb from the *nptII* cassette insertion.

4.3.3 Complementation of *Dothistroma septosporum* CRISPR/Cas9 gene disruption mutants

To confirm that any phenotypes of the mutants were due to the correct gene disruption and not to a non-specific effect of the transformation process, one mutant for each of the targeted *CE* genes (*Ds69335* T2-D, *Ds74283* T3-D and *Ds131885* T9-D) was selected for complementation by protoplast-mediated transformation. The complementation transformants were screened by PCR using the primer pair that amplified the sequence between the start and stop codon from each of the three genes (pairs MT95/MT96 for *Ds74283*, MT99/MT100 for *Ds131885* and MT91/MT92 for *Ds69335*) (**Figure 4.3**, **Figure 4.4** and **Figure 4.5**, respectively). For each gene, these primers were expected to amplify two PCR products if complementation was successful: one of the same size as the WT due to insertion of the complementing gene and one larger sized product from the gene disruption through insertion of the *nptII* cassette. Four transformants from each targeted gene were screened by PCR. For *Ds69335* T2-D, three showed the expected PCR product sizes including those matching that of the WT fungus (0.89 kb) (**Figure 4.5E**). For *Ds74283* T3-D, only one transformant showed the expected PCR product sizes, including 0.64 kb from the insertion of the complementing *Ds74283* gene (**Figure 4.3E**). Finally, *Ds131885* T9-D had three transformants with the

expected PCR products size, including 0.54 kb from the insertion of the complementing *Ds131885* gene (**Figure 4.4E**).

4.3.3.1 Copy number determination in *Dothistroma septosporum* complementation strains

Next, qPCR was used to determine gene copy numbers from each of the selected complementation strains; one each for *Ds69335* (C1), *Ds74283* (C3) and *Ds131885* (C2). All three complementation strains from each of the targeted *CE* genes showed an estimated copy number of two (**Table 4.5**). This was expected, since the primers selected for the qPCR also amplified the disrupted region of each gene that is still present in the fungal genome after CRISPR/Cas9 editing, and the sequence of each newly introduced gene. So, a copy number of two means there was only one copy of the complementation gene introduced into each of the gene-disruption mutants.

Table 4.5. Copy number determination of *Dothistroma septosporum* NZE10 complementation strains.

<i>CE</i> gene ^a	Strain name ^b	Ct reference	Δ Ct reference ^c	Ct target	Δ Ct target ^c	Ratio ^d	Copy number
<i>Ds69335</i>	WT	19.2	-	19.7	-	1.0	1
	<i>Ds69335</i> T2-Co	19.8	-0.58	19.3	0.49	1.98	2
<i>Ds74283</i>	WT	19.3	-	19.4	-	1.0	1
	<i>Ds74283</i> T3-Co	19.6	-0.36	18.3	1.1	2.44	2
<i>Ds131885</i>	WT	19.3	-	19.9	-	1.0	1
	<i>Ds131885</i> T9-Co	19.5	-0.22	18.7	1.24	2.49	2

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (*CE*) gene of interest that was disrupted.

^b *D. septosporum* strains with a targeted candidate effector gene disruption used for gene complementation (Co). WT: wild-type.

^c Δ Ct reference: difference in the cycle threshold (Ct) between the complementation strain and the WT fungus from amplification of each of the target and reference genes. A single copy *DsAflR* gene was used as reference (Chettri et al., 2013).

^d The ratio was calculated using an adapted $2^{-\Delta\Delta CT}$ method, considering the primer efficiency (see **Section 4.2.7**). *DsAflR* had a primer efficiency of 1.93.

In summary, mutants 2 and 40, in which *Ds69335* was disrupted, and *Ds69335* complementation strain C1 (from T2-D) were selected to be further single-spored purified, phenotypically characterised and used in *Pn. radiata* virulence assays. For *CE* *Ds74283*, mutants 3 and 56, and *Ds74283* complementation strain C3 (from T3-D) were selected and, for *Ds131885*, mutants 9 and 56, and *Ds131885* complementation strain C2 (from T9-D) were selected to be further characterised.

4.3.4 Phenotypic characterization of *Dothistroma septosporum* transformants

All *D. septosporum* candidate effector gene disruption and complementation strains, *Ds69335* T2-D, T40-D and C1 (*Ds69335* T2-Co), *Ds74283* T3-D and T56-D and C3 (*Ds74283* T3-Co), *Ds131885* T9-D, T40-D and C2 (*Ds131885* T9-Co), showed no significant difference in growth rate when compared with the WT fungus (**Table 4.6**). These results suggests that the genes *Ds69335*, *Ds74283* and *Ds131885* might not be involved in *D. septosporum* growth. Also, no significant difference in sporulation was found between the WT fungus and all the transformants after 7 days of growth, suggesting these genes do not affect sporulation (**Table 4.6**).

Table 4.6. Growth rate and sporulation assays involving candidate effector gene disruption and complementation strains of *Dothistroma septosporum*.

CE gene ^a	Strain name ^b	Growth rate (mm/day) ^c	P (T-test) ^d	Sporulation (spores/mL) ^e	P (T-test) ^d
	WT	0.63±0.03	-	3.79E+06±1.32E+06	-
<i>Ds69335</i>	<i>Ds69335</i> T2-D	0.61±0.06	0.50	2.65E+06±2.95E+04	0.29
	<i>Ds69335</i> T40-D	0.60±0.03	0.08	3.00E+06±4.87E+05	0.47
	<i>Ds69335</i> T2-Co	0.59±0.05	0.09	3.33E+06±5.64E+05	0.67
<i>Ds74283</i>	<i>Ds74283</i> T3-D	0.61±0.03	0.32	2.96E+06±6.05E+05	0.46
	<i>Ds74283</i> T56-D	0.61±0.04	0.28	7.73E+06±2.62E+06	0.13
	<i>Ds74283</i> T3-Co	0.61±0.06	0.56	1.67E+06±6.42E+05	0.11
<i>Ds131885</i>	<i>Ds131885</i> T9-D	0.58±0.08	0.16	4.00E+06±2.22E+05	0.84
	<i>Ds131885</i> T47-D	0.59±0.04	0.10	2.83E+06±8.48E+05	0.44
	<i>Ds131885</i> T9-Co	0.60±0.04	0.11	2.83E+06±1.79E+05	0.37

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* strains with a targeted candidate effector gene disruption are indicated by "D"; complementation strains are indicated by "Co". WT: wild-type.

^c Mean (± standard deviation) radial growth measured from three replicate plates per strain with three agar plugs each, after 3 weeks growth, shown as millimetres per day (mm).

^d $P > 0.05$, using Student's *t*-test, indicates no significant difference between WT and candidate effector gene disruption or complementation strains.

^e Mean (± standard deviation) sporulation measured from three replicate plates per strain inoculated with 1×10^5 spores/mL, incubated for 7 days, shown as spores per millilitre (mL).

4.3.5 Virulence assays of *Dothistroma septosporum* transformants in *Pinus radiata*

Candidate effector gene disruption and complementation strains of *D. septosporum*, as well as the WT fungus, were inoculated onto *Pn. radiata* seedlings according to **Table 4.7**. Two sets of experiments were performed in two separate weeks,

and each week had its own WT control. All analyses were done by comparing the *D. septosporum* disruption strain either to the WT fungus in the same week they were inoculated, or with its corresponding complementation strain, regarding the number of DNB lesions and fungal biomass estimations.

Table 4.7. Details of the *Dothistroma septosporum* candidate effector gene disruption and complementation strain virulence assay experiment in *Pinus radiata*.

CE gene ^a	Strain name ^b	Concentration (spores/mL) ^c	Volume (mL) ^d	Pine seedlings replicates
Week 1				
	WT	1.6 x 10 ⁶	65	3
<i>Ds69335</i>	<i>Ds69335</i> T2-D	1.6 x 10 ⁶	65	4
	<i>Ds69335</i> T40-D	1.6 x 10 ⁶	62	4
	<i>Ds69335</i> T2-Co	1.6 x 10 ⁶	55	4
<i>Ds74283</i>	<i>Ds74283</i> T3-D	1.6 x 10 ⁶	60	4
	<i>Ds74283</i> T56-D	1.6 x 10 ⁶	50	4
	<i>Ds74283</i> T3-Co	1.6 x 10 ⁶	65	4
<i>Ds131885</i>	<i>Ds131885</i> T9-Co	1.6 x 10 ⁶	65	4
Week 2				
	WT	6 x 10 ⁵	65	3
<i>Ds131885</i>	<i>Ds131885</i> T9-D (L)	7.5 x 10 ⁵	35	3
	<i>Ds131885</i> T9-D (M)	1 x 10 ⁶	50	3
	<i>Ds131885</i> T47-D	1 x 10 ⁶	50	3

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (Ds) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* strains with a targeted candidate effector gene disruption are indicated by "D"; complementation strains are indicated by "Co". WT: wild-type. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings.

^c Spore concentration in spores per millilitre (mL).

^d Total volume of spore solution sprayed on all replicates for each strain in mL. Note that these values are approximate, as only a proportion of the sprayed spore solution will have coated the fine needles.

After ten weeks, all needles were harvested and counted, and the percentage of needles with DNB lesions was calculated. The number of lesions caused by each disruption and complementation strain was compared to those caused by the corresponding WT fungus, and disruptants were also compared to their respective complement (**Table 4.8** and **Figure 4.6**). The results suggested that gene disruption had no effect on the number of lesions when compared with the WT, except for *Ds131885* T47-D, which caused fewer lesions than the WT fungus, even though this strain was inoculated at a higher spore concentration than the WT (**Table 4.7**). Also, disruption strains did not show any differences when compared to their complement. There was

high variability between replicate seedlings inoculated with the same strain, especially with *Ds69335* T2-Co, *Ds74283* T56-D and *Ds131885* T9-D (M).

Table 4.8. Percentage of needles with *Dothistroma* needle blight (DNB) lesion symptoms in response to infection with candidate effector gene disruption and complementation strains of *Dothistroma septosporum*.

CE gene ^a	Strain name ^b	Percentage (%) of needles with DNB lesions ^c	P (T-test) - WT ^d	P (T-test) - Co ^e
Week 1				
	WT	13.8±3.22	-	-
<i>Ds69335</i>	<i>Ds69335</i> T2-D	12.4±3.29	0.65	0.33
	<i>Ds69335</i> T40-D	16.2±7.76	0.68	0.67
	<i>Ds69335</i> T2-Co	19.9±12.02	0.50	-
<i>Ds74283</i>	<i>Ds74283</i> T3-D	22.8±5.25	0.08	0.81
	<i>Ds74283</i> T56-D	23.9±9.92	0.21	0.75
	<i>Ds74283</i> T3-Co	21.4±7.93	0.24	-
<i>Ds131885</i>	<i>Ds131885</i> T9-Co	13.3±3.83	0.89	-
Week 2				
	WT	24.9±3.53	-	-
<i>Ds131885</i>	<i>Ds131885</i> T9-D (L)	19.3±6.37	0.34	0.24
	<i>Ds131885</i> T9-D (M)	26.6±10.21	0.83	0.10
	<i>Ds131885</i> T47-D	7.9±7.9	0.003*	0.10

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* strains with a targeted candidate effector gene disruption are indicated by "D"; complementation strains are indicated by "Co". WT: wild-type. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings according to **Table 4.7**.

^c Mean (± standard deviation) of needles with DNB lesions compared with total number of needles in each inoculated seedling, shown as percentage (%). Full data shown in **Appendix Table A.11**.

^d $P > 0.05$, using Student's *t*-test, indicates no significant difference between WT and each transformant. The asterisk * indicates significant difference.

^e $P > 0.05$, using Student's *t*-test, indicates no significant difference between the disruption strain and its respective complement.

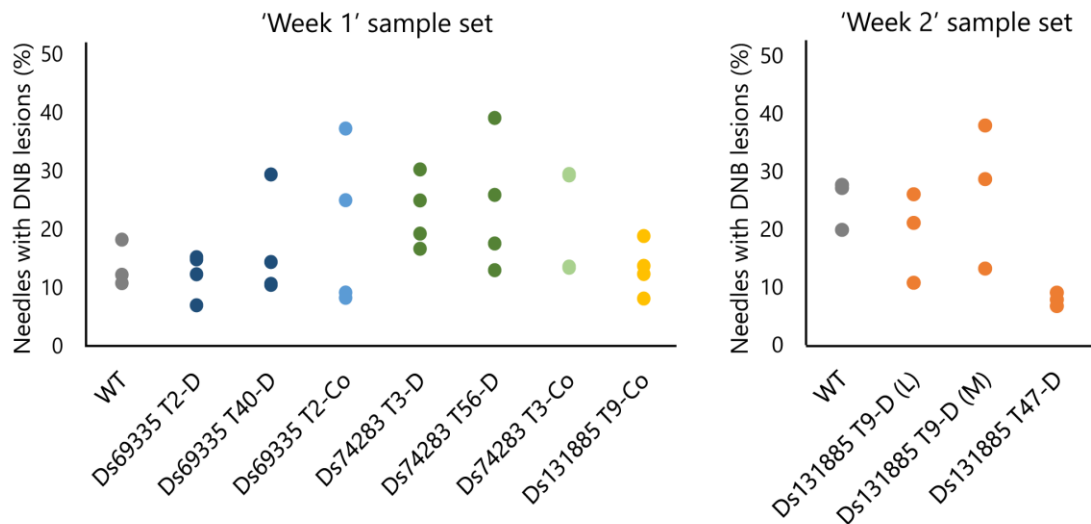


Figure 4.6. The percentage (%) of *Pinus radiata* needles with *Dothistroma* needle blight (DNB) symptoms does not differ across wild-type (WT) and candidate effector (CE) gene disruption and complementation strains of *Dothistroma septosporum*. Percentage of needles with characteristic DNB symptoms compared to the total number of needles in each seedling inoculated with each CE gene disruption and complementation strain of *D. septosporum* transformant and WT fungus. 'Week 1' and 'Week 2' refers to the experiment set up according to **Table 4.7**. D: disrupted *D. septosporum* strain; Co: complemented *D. septosporum* strain; L: low spore concentration; M: moderate spore concentration compared to other strains. Full data shown in **Appendix Table A.11**.

To determine if the gene disruption had any effects on pathogen growth on *Pn. radiata*, fungal biomass in characteristic DNB lesions was estimated using a qPCR assay comparing the *DsPksA* gene from *D. septosporum* and the *CAD* gene from *Pn. radiata*. The results (**Table 4.9**) showed that in Week 1 all disruption strains differed in the amount of biomass normalized by dry-weight when compared with the WT fungus, however, when compared to their respective complement, none of the disruption strains showed any differences in biomass. The amount of fungal biomass was also normalized by the number of lesions. In this case, no significant differences in the amount of fungus biomass were observed between any of the samples. In Week 2, *Ds131885* T9-D (L) and *Ds131885* T47-D did not show any differences when compared to the WT fungus in ng/mg dry-weight, although *Ds131885* T9-D (M) appeared to have a lower amount of fungal biomass than the WT. None of the disrupted transformants were different from their complements regarding the amount of biomass per dry-weight or number of lesions. **Figure 4.7** shows a graphical representation of the values obtained for each

inoculated seedling for all transformants (both disruption and complementation strains) and WT, in relative fungal biomass per dry-weight, and per number of lesions, and highlighting the variability between replicates for most of the samples.

Table 4.9. Relative quantification of *Dothistroma septosporum* candidate effector gene disruption and complementation strain biomass in *Pinus radiata*.

CE gene ^a	Strain name ^b	Fungal DNA (rel quant) (ng/mg DW) ^c	P (T-test) WT/Co ^d	Fungal DNA (rel quant) (ng/lesion) ^e	P (T-test) WT/Co ^f
Week 1					
	WT	7.1±0.6	-	2.3±0.4	-
<i>Ds69335</i>	<i>Ds69335</i> T2-D	3.6±1.6	0.025*/0.23	3.2±1.4	0.45/0.31
	<i>Ds69335</i> T40-D	3.0±2.7	0.078*/0.58	2.8±2.7	0.80/0.64
	<i>Ds69335</i> T2-Co	1.9±1.5	0.006*/ -	1.9±1.4	0.72/ -
<i>Ds74283</i>	<i>Ds74283</i> T3-D	2.4±1.5	0.007*/0.51	1.2±0.8	0.14/0.99
	<i>Ds74283</i> T56-D	2.7±1.0	0.003*/0.24	2.0±0.9	0.72/0.34
	<i>Ds74283</i> T3-Co	1.6±1.0	0.001*/ -	1.2±0.9	0.18/ -
<i>Ds131885</i>	<i>Ds131885</i> T9-Co	1.3±1.9	0.008*/ -	1.0±1.4	0.27/ -
Week 2					
	WT	2.0±0.5	-	0.6±0.2	-
<i>Ds131885</i>	<i>Ds131885</i> T9-D (L)	3.5±1.6	0.30/0.24	1.7±1	0.18/0.54
	<i>Ds131885</i> T9-D (M)	0.7±0.2	0.03*/0.65	0.4±0.2	0.44/0.59
	<i>Ds131885</i> T47-D	3.3±1.0	0.18/0.22	2.2±0.6	0.02*/0.28

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* strains with a targeted gene disruption are indicated by "D"; complementation strains are indicated by "Co". WT: wild-type. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings according to **Table 4.7**.

^c Fungal biomass (ng) relative to pine and normalised to dry-weight (DW) of infected needle lesions (mean ± SD), determined by the cycle threshold (Ct) amplification values from qPCR of *DsPksA* (fungal target) and *CAD* (pine reference), based on regression equations: $y = -3.7597x + 26.877$ (target) and $y = -3.365x + 27.84$ (reference). Full data shown in **Appendix Table A.12**.

^d *P*-value, using Student's *t*-test, between WT and each transformant (first value) and between the disruption strain and its respective complement (second value). The asterisk * indicates significant difference ($P < 0.05$).

^e Fungal biomass (ng) relative to pine and normalised per disease lesion (mean ± SD), determined by the Ct amplification values from qPCR of *DsPksA* (fungal target) and *CAD* (pine reference), based on regression equations. Full data shown in **Appendix Table A.12**.

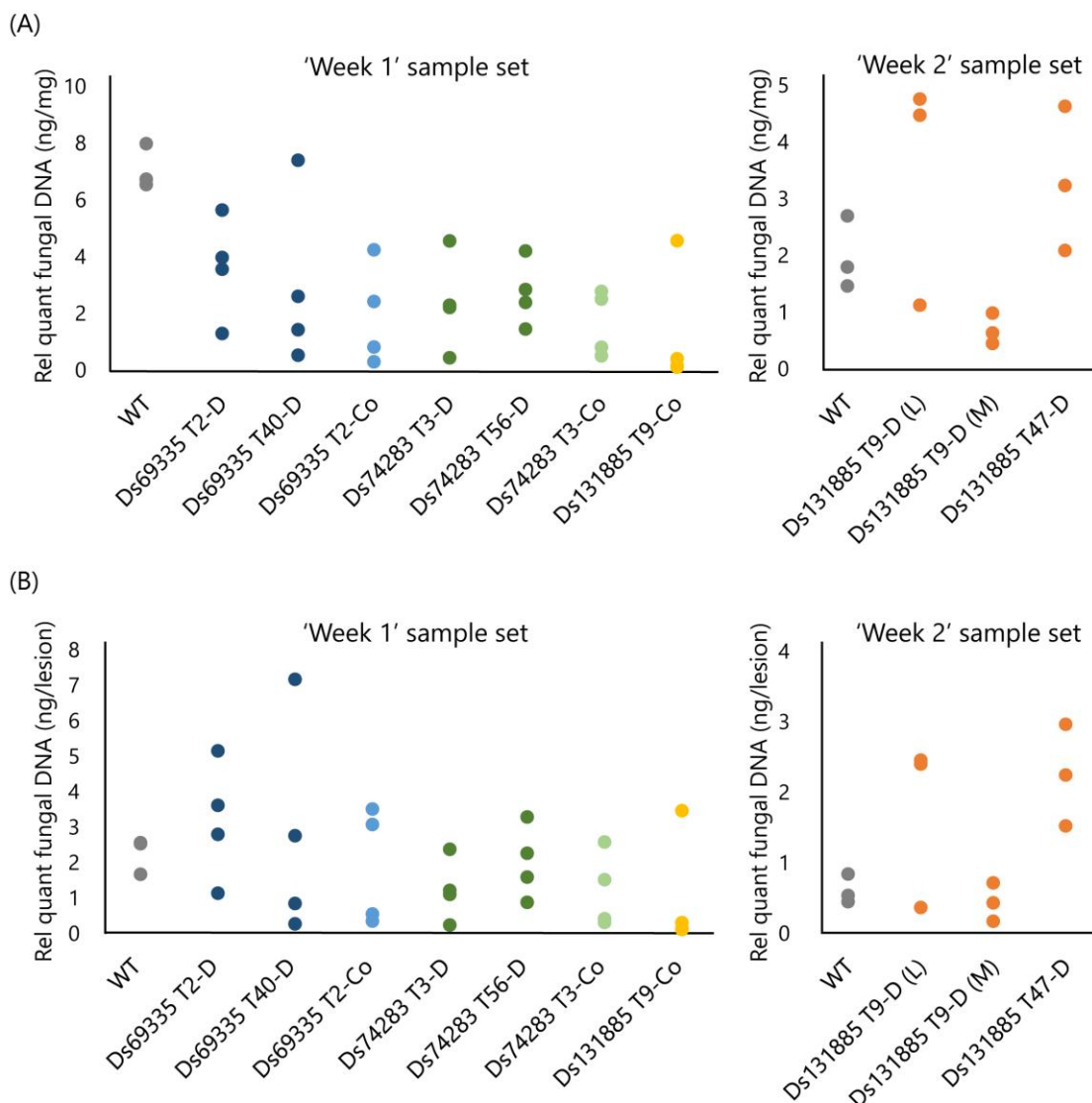


Figure 4.7. Relative quantification of *Dothistroma septosporum* candidate effector gene disruption and complementation strain biomass following *Pinus radiata* infection. Fungal biomass (ng) in characteristic *Dothistroma* needle blight lesions was estimated using a qPCR assay comparing the *DsPksA* gene from *D. septosporum* and the *CAD* gene from *Pn. radiata* for each *D. septosporum* disruption or complementation strain and wild-type (WT) fungus. Values were normalized by dry-weight (mg) **(A)** and number of lesions **(B)**. 'Week 1' and 'Week 2' refers to the experiment set up according to **Table 4.7**. D: disrupted *D. septosporum* strain; Co: complemented *D. septosporum* strain; L: low spore concentration; M: moderate spore concentration compared to other strains. Full data shown **Appendix Table A.12**.

Next, absolute quantification to estimate fungal biomass in characteristic DNB lesions was also determined. This was done because the Ct values for the *CAD* gene from *Pn. radiata* determined in the qPCR assay had considerable variation with many high

values (**Appendix Table A.12**) and suggested that the *Pn. radiata* DNA extracted from some lesion samples might have been degraded and therefore affecting the relative quantification results shown above. Based on the absolute quantification fungal biomass estimations, all Week 1 disruption and complementation strains had significantly less biomass per dry-weight when compared with the WT fungus (**Table 4.10**), the same as seen in the relative quantification analysis (**Table 4.9**). However, the absolute quantification estimations also showed significantly less fungal biomass in disruption and complementation strains compared to WT when normalized by number of lesions (**Table 4.10**), whilst these values were previously not significant in the relative analysis (**Table 4.9**). The one exception was *Ds69335* T40-D, which showed no significant difference to WT when normalised by lesion number, although there was one clear outlier among the replicates for this sample (**Appendix Table A.13** and **Figure 4.8B**). For Week 2, no differences were observed between disrupted strains and WT fungus (**Table 4.10**). **Figure 4.8** shows a graphical representation of the values obtained for each inoculated seedling for all disruption and complementation strains and WT tested, in absolute fungal biomass per dry-weight and per number of lesions. In Week 1 a clear trend of lower biomass in both mutants and complements, compared to the WT, is shown - i.e., for *Ds69335* and *Ds74283*. In Week 2, the WT biomass was lower than in Week 1 (due possibly to the lower spore inoculum), but no clear trend was seen for *Ds131885* compared to the WT.

No differences were observed when the disruptants were compared to their respective complementation strains. So, despite trends suggesting reduced virulence of *Ds69335* and *Ds74283* when assessed by absolute quantification of fungal biomass, the lack of complementation of the mutant phenotype meant that a conclusion could not be made regarding the possible role in virulence for these genes during *D. septosporum* infection of *Pn. radiata*.

Table 4.10. Absolute quantification of *Dothistroma septosporum* candidate effector gene disruption and complementation strain biomass in *Pinus radiata*.

CE gene ^a	Strain name ^b	Fungal DNA (abs quant) (ng/mg DW) ^c	P (T-test) WT/Co ^d	Fungal DNA (abs quant) (ng/lesion) ^e	P (T-test) WT/Co ^d
Week 1					
	WT	85.0±13.7	-	26.9±5.2	-
<i>Ds69335</i>	<i>Ds69335</i> T2-D	5.8±4.1	0.0002*/0.84	4.9±3.2	0.002*/0.85
	<i>Ds69335</i> T40-D	14.2±15.0	0.0029*/0.35	12.9±15.1	0.25/0.38
	<i>Ds69335</i> T2-Co	5.2±3.8	0.0002*/ -	4.5±2.6	0.001*/ -
<i>Ds74283</i>	<i>Ds74283</i> T3-D	8.5±5.7	0.0004*/0.71	4.4±3.0	0.002*/1.0
	<i>Ds74283</i> T56-D	8.6±4.2	0.0003*/0.67	6.4±3.5	0.003*/0.58
	<i>Ds74283</i> T3-Co	6.5±6.9	0.0004*/ -	4.4±4.6	0.004*/ -
<i>Ds131885</i>	<i>Ds131885</i> T9-Co	5.0±4.4	0.0002*/ -	5.0±4.7	0.005*/ -
Week 2					
	WT	5.3±2.3	-	1.6±0.7	-
<i>Ds131885</i>	<i>Ds131885</i> T9-D (L)	10.0±3.6	0.19/0.23	4.9±2.4	0.13/0.99
	<i>Ds131885</i> T9-D (M)	11.8±2.5	0.05/0.05	7.1±2.8	0.05/0.59
	<i>Ds131885</i> T47-D	1.6±1.8	0.15/0.34	1.1±1.2	0.64/0.29

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* strains with a targeted gene disruption are indicated by "D"; complementation strains are indicated by "Co". WT: wild-type. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings according to **Table 4.7**.

^c Absolute quantification of fungal DNA (ng) per mg dry-weight (DW) of infected needle lesions (mean ± SD), determined by the cycle threshold (Ct) amplification values from qPCR of *DsPksA* (fungal target), based on regression equation: $y = -3.7597x + 26.877$ (target). Full data shown in **Appendix Table A.13**.

^d *P*-value, using Student's *t*-test, between WT and each disruption or complementation strain (first value) and between the disruption strain and its respective complement (second value). The asterisk * indicates significant difference ($P < 0.05$).

^e Absolute quantification of fungal DNA (ng) per lesion (mean ± SD), determined by the Ct amplification values from RT-qPCR of *DsPksA* (fungal target), based on regression equation: $y = -3.7597x + 26.877$ (target). Full data shown in **Appendix Table A.13**.

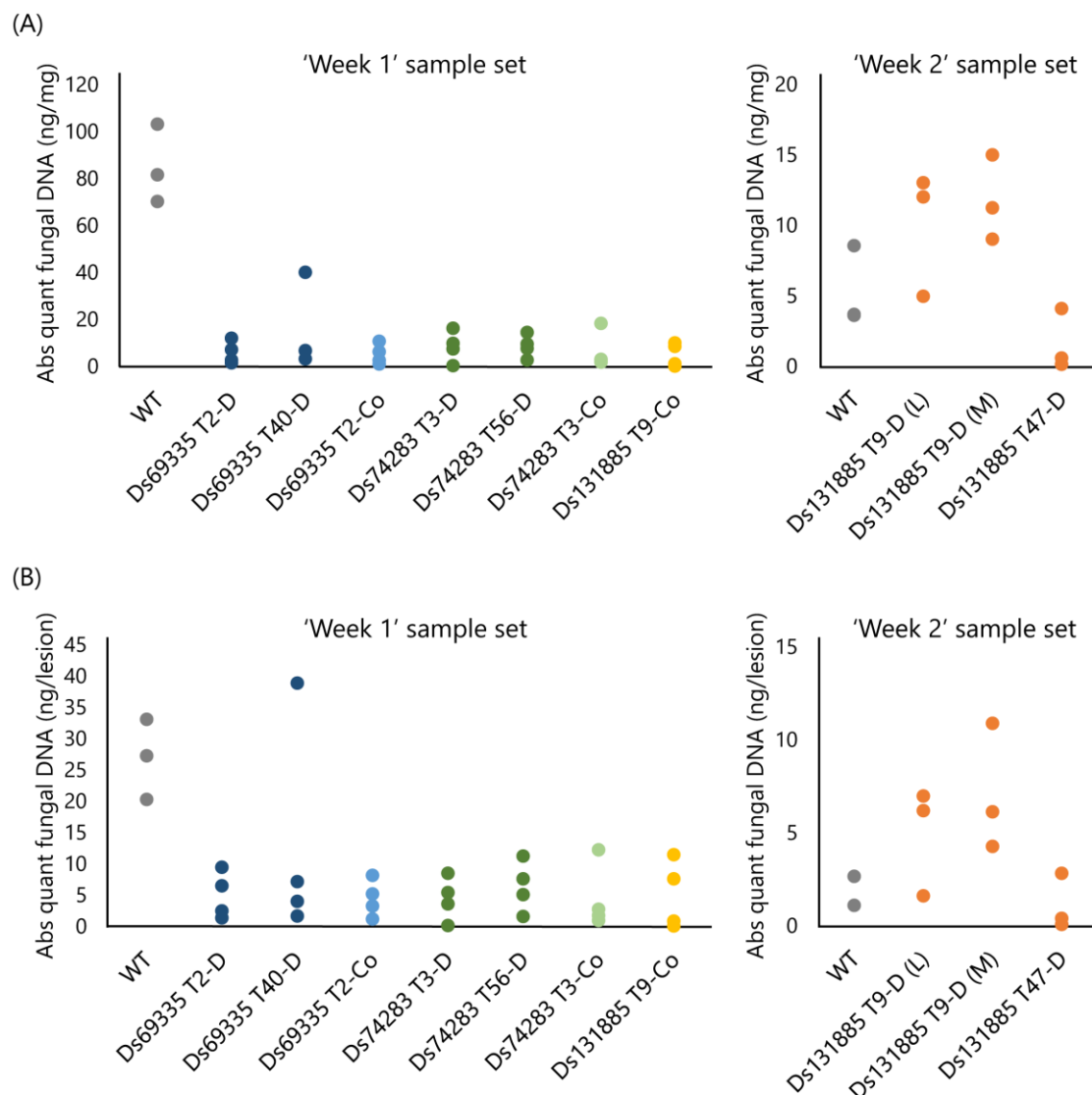


Figure 4.8. Absolute quantification of *Dothistroma septosporum* candidate effector gene disruption and complementation strain biomass following *Pinus radiata* infection. Fungal biomass (ng) in characteristic *Dothistroma* needle blight lesions was estimated using the cycle threshold values of the *DsPksA* gene from *D. septosporum* from a qPCR assay for each *D. septosporum* transformant and wild-type (WT) fungus. Values were normalized by dry-weight (mg) **(A)** and number of lesions **(B)**. 'Week 1' and 'Week 2' refers to the experiment set up according to **Table 4.7**. D: disrupted *D. septosporum* strain; Co: complemented *D. septosporum* strain; L: low spore concentration; M: moderate spore concentration compared to other strains. Full data shown in **Appendix Table A.13**.

4.4 Discussion

To understand the role *CE* genes from *D. septosporum* have during infection of *Pn. radiata*, *Ds69335*, *Ds74283* and *Ds131885* were each disrupted using CRISPR/Cas9 in

separate transformations targeting each of the three genes. Based on the findings in **Chapter 3**, all three genes were suggested to be potentially important virulence factors of *D. septosporum*. To identify the functions of these CEs, mutants for each gene were generated to observe any phenotypic effects on virulence.

The CRISPR/Cas9 genome editing method was used for gene disruption, along with the use of a dDNA to enable homologous recombination. For genes whose phenotype cannot be predicted, such as the three CEs, the use of dDNA can facilitate faster and more efficient screening for transformants. In this Chapter, for *Ds69335*, *Ds74283* and *Ds131885* disruption, *D. septosporum* was co-transformed with an autonomously replicating plasmid containing the Cas9 endonuclease, along with the specific sgRNA, and a dDNA plasmid harbouring a selective resistance marker, with flanking arms of approximately 1 kb. This same strategy has been successfully used in other fungal and oomycete species. In *P. sojae*, homologous recombination was stimulated using the *nptII* gene in between 1 kb flanking sequences from the candidate gene (Fang & Tyler, 2016), as used here. The combination of a Cas9 protein and a dDNA-sgRNA expression plasmid resulted in almost 90% of *Colletotrichum sansevieriae* transformants with the candidate gene disrupted, with flanking sequences (1–1.5 kb) resulting in efficient dDNA recombination (Nakamura et al., 2019). However, shorter flanking arms can also work efficiently; 100% efficiency of homologous integration of the dDNA in CRISPR/Cas9 transformation of *Beauveria bassiana* was obtained when 250 bp flanking sequences were used (Chen et al., 2017).

Here, all three candidate genes were successfully disrupted but with some differences in efficiency between the transformations that targeted each of them. Based on PCR screening, *Ds74283* was disrupted in all stable transformants tested, while *Ds69335* was disrupted in 57.15% of the transformants screened, and *Ds131885* in 90%. The sgRNA targeting *Ds74283* had the highest on-target score, which could explain why the disruption was more efficient than in the other two genes. However, the sgRNA targeting *Ds131885* was lower than the one for *Ds69335*, which indicates that the differences observed might have been for other reasons. For example, different expression levels of Cas9 in each transformation (Schuster & Kahmann, 2019) and target

gene accessibility through chromatin dynamics and DNA packing (Daer et al., 2017), might explain differences in gene disruption rates.

Nevertheless, *Ds69335*, *Ds74283* and *Ds131885* were successfully disrupted, and, through Southern blot analysis, it was possible to detect the expected size of disruption fragment in at least two independent transformants for each of these genes. Next, two disruption strains and one complementation strain from each transformation were selected for further characterization. Phenotypic characterization suggested that none of the candidate genes directly affected growth or sporulation, since no difference was observed between the deletion and complementation strains and the WT fungus.

The virulence assay showed that none of the disrupted genes were required for pathogenicity in *D. septosporum*, since all transformants were still able to cause characteristic DNB lesions. As for virulence, all 'Week 1' strains (with disruption of *Ds69335* or *Ds74283*) showed a decrease in fungal biomass in the infected needle tissue, especially when observing the results for absolute biomass quantification (**Figure 4.8**). However, when comparing the amount of fungal biomass between all the complementation strains and WT, no clear conclusion could be drawn, as the complementation strains also had significantly less fungal biomass. This was observed despite all inoculations in Week 1 having the same starting concentration and similar volumes of spores, and the seedlings being maintained in the same experimental conditions. These results suggest that the complementation strains were not restored in virulence. It is important to note that non-clonal *Pn. radiata* seedlings were used in this experiment, which could have contributed to variability between replicates, as differences in host genotype could lead to different responses to *D. septosporum* infection.

Complementation is carried out to ensure that the phenotype of a transformant with a targeted gene mutation is due only to that specific mutation and not ectopic effects. It is possible that the *Ds69335*- and *Ds74283*-disrupted strains (Week 1) actually did have reduced fungal biomass and therefore reduced virulence compared to the WT, but that factors associated with the complementation process prevented restoration of virulence. When transforming the mutant to generate a complementation strain, the functional copy of the gene is often inserted at a random ectopic place in the genome. The position of the *CE* genes in the genome could be important for their expression and

some might be under chromatin regulation, which compromised their expression. Future experiments should include a larger number of complementation strains for testing; here only one from each gene was used due to space limitations in the virulence assay.

Another possibility is that off-target effects occurred in the complementation strains due to Cas9 expression. Constitutive expression of Cas9 is known to increase the number of off-target effects and DNA damage (Cho et al., 2014; Fu et al., 2013; Pattanayak et al., 2013). The *Cas9* gene may have integrated into the *D. septosporum* genome during the transformation process, enabling the endonuclease to target and cleave the inserted functional copy of the target gene in the three complementation strains. Also, mismatches in the complementarity between the sgRNA and target gene sequences are tolerated and might have led the Cas9 to cleave other regions of the genome (Pattanayak et al., 2013), also generating off-target events. All transformants had additional hybridising fragments in the Southern analysis that did not match with sizes expected for additional gene copies or partial digestion. This suggests that parts of the Cas9HygAMAcdb plasmid or pAN-7 might have integrated into the genome, concomitant with the *CE* genes being disrupted. In the case of *Ds74283* and *Ds131885*, disrupted transformants were kept on selective hygromycin medium after protoplast regeneration. The expression vector Cas9HygAMAcdb is only lost after the removal of the hygromycin selection, which means the Cas9 was retained for longer, increasing the chance of integration into the genome. This was shown to be case for some *V. inaequalis* CRISPR/Cas9 transformants, in which the Cas9HygAMAcdb plasmid had integrated into their genomes (Rocafort et al., 2022).

Southern blot analysis in which probes are designed to specifically hybridize with the Cas9HygAMAcdb and pAN-7 plasmids should be performed, as well as sequencing of the PCR products from the amplification of the targeted gene in the complementation strains to determine any mutations that might be inactivating the inserted *CE* gene. Otherwise, performing whole-genome sequencing of the transformants would help to determine if and what parts of these plasmids have integrated, and where. For the complementation strains, this would reveal mutations that might be inactivating the inserted gene as well as any potential off-target effects due to ectopic integration of the complementing gene. Other forms of Cas9 delivery could be exploited, such as transient

delivery of Cas9, in the form of a ribonucleoprotein which has a limited half-life, directly into the cell, in order to limit off-target effects and possible integration into the genome of the transformant (Yip, 2020).

The integration of the *Cas9* gene, if accompanied with integration of the specific sgRNA in the genomes of the disruption strains, would explain why all complementation strains have similar low fungal biomass amount when compared to the disruption strains, as the additional gene copy in each of these strains might also have been mutated.

Aside from problems with the complementation strains, there were also limitations in the virulence assay due to variation in inoculum concentrations and the availability of growth rooms. Comparing the WT fungus inoculated in Weeks 1 and 2, there was a substantial difference in the amounts of fungal biomass. This could be due to differences in spore concentrations used to inoculate the seedlings (Week 1 spore concentration was approx. 2.5 times higher than that used in Week 2; **Table 4.7**). Also, seedlings in Week 1 were kept in a growth room with artificial lights for two weeks before being moved to a growth room with natural light, while seedlings in Week 2 were kept for only one week under artificial light. This time difference could have affected *D. septosporum* at the beginning of the infection process.

Another limitation concerned the sampling time of the experiment. Here, the experiment was conducted for ten weeks after inoculation. After ten weeks, DNB lesion symptoms were advanced with abundant sporulating lesions and some needle death, which corresponds to the Late (necrotrophic) infection stage of *D. septosporum* according to previous studies (Bradshaw et al., 2016; Kabir et al., 2015). By focusing on the single 10 weeks post-inoculation time point it was not possible to determine if the rate of infection differed between the strains. Instead, the single late sampling point might have masked any real differences in the virulence capacity of the disrupted strains that may have been evident at earlier stages. Sampling from earlier time points and analysing differences in the infection rate could be informative in future experiments, as well as early-stage phenotypic characterization of spore germination rates of transformant strains *in planta*.

While the quantification of fungal biomass showed a lower trend for both disruption and complementation transformants when compared to the WT, the percentages of infected needles were not significantly different between any of these transformants and the WT fungus, or between the gene-disrupted strains and their respective complementation strain. The percentage of needle infection, however, does not always correlate with changes in fungal biomass. For example, deletion of *DsEcp2-1* did not have any impact on number of lesions but had an impact on lesion size and increased fungal biomass (Guo et al., 2020). Measuring the lesion sizes produced by the transformants in this study might also provide additional information rather than just counting the total number of lesions produced.

Similar virulence assay experiments to those performed in this study showed that the deletion of *D. septosporum* CE genes *DsAvr4*, *DsHdp1* and *DsEcp6* did not seem to affect the pathogen virulence on *Pn. radiata* (Guo et al., 2020). As mentioned above, deletion of another CE gene, *DsEp2-1*, on the other hand, showed an increase in fungal biomass, while complements restored the WT phenotype, suggesting this CE might be recognized by a pine immune receptor. In all of the experiments mentioned above, however, clonal *Pn. radiata* seedlings were used and deletion of candidate genes was performed by a targeted replacement strategy based on homologous recombination. Nevertheless, these results indicate that functional redundancy might occur; for example, *D. septosporum* has three other hydrophobin genes, which suggests that the virulence function of *DsHdp1* was masked by the presence of the other proteins in the deleted strain.

Homologues of the CE genes tested in the study described in this thesis were deleted in other pathosystems. When deleted, *Va. mali* *VmE02*, the orthologue of *Ds131885*, generated mutants that had the same filamentous growth as the WT fungus (Nie et al., 2019). Interestingly, however, these mutants had reduced numbers of pycnidia when compared to the WT, suggesting this protein might have a role in *Va. mali* conidiation (Nie et al., 2019). This does not seem to be case for *Ds131885* in *D. septosporum*, as no differences in sporulation levels were observed between transformants and WT fungus. However, just as deletion of *VmE02* did not affect the

virulence of *Va. mali* in apple leaves, the results here suggest that *D. septosporum* Ds131885 is also not required for virulence in pine.

As mentioned before, the other disrupted gene, *Ds69335*, encodes a protein that is predicted to belong to the CAP superfamily, which have members that are known to suppress host defence responses. The lower fungal biomass observed in the disrupted strains could be due to the activation of the pine immune system in the absence of the CAP protein, or possibly due to *Ds69335* interacting with and inhibiting plant targets, such as pathogenesis-related (PR) proteins. However, because no differences were observed between disrupted and complementation strains, the role that *Ds69335* has during pine infection is not clear.

CAP proteins have been deleted from other fungal pathogens. Fpr1, a CAP protein from *Fusarium oxysporum*, is required for virulence in mice but, interestingly, not on tomato plants (Prados-Rosales et al., 2012). The fungal pathogen *Candida albicans* also secretes two CAP proteins, RBE1 and RBT4, and both are important for virulence in mice (Röhm et al., 2013). Single deletion of either *RBE1* or *RBT4* resulted in moderate attenuation of disease, while the virulence of the double mutant was dramatically reduced (Röhm et al., 2013). *D. septosporum* is predicted to secrete two other CAP proteins (see **Section 3.3.3.4** of **Chapter 3**), based solely on amino acid searches (structural prediction might uncover a higher number). It would be interesting to determine the expression levels of these genes in the *Ds69335*-disrupted strains following pine infection. This could be more rapidly tested by growing the disrupted strain in media supplemented with pine needles, for example, and then assessing their expression by RT-qPCR. Alternatively, strains in which all three predicted CAP-encoding genes are disrupted might reveal the real mode of action of these proteins, similar to what was observed in *C. albicans*.

Additional CAP proteins could mask any virulence effect of disrupting *Ds69335* in *D. septosporum*, or *Ds69335* may have other functions that are not involved in virulence. As discussed in **Chapter 3**, CAP proteins have the ability to bind to small hydrophobic molecules. Also, *Ds69335* has strong structural similarity with Pry1 proteins from *S. cerevisiae* that, in turn, are similar to PR-1 from plants. PR-1 proteins from plants have antimicrobial activity. The fact that *Ds69335* is strongly up-regulated at the beginning of

pine infection, suggests that an alternative role for Ds69335 might be in defence against other microbes. For example, PR-1 proteins isolated from tomato and *Nicotiana tabacum* were shown to inhibit *Phytophthora* species growth (Breen et al., 2017).

Ds74283 is predicted to adopt a β -trefoil fold and has structural similarities with cytolysins (see **Section 3.3.2.2** of **Chapter 3**), proteins that have a known toxic effect to cells through plasma membrane disruption (Alouf, 2003). Ds74283 might therefore be a necrotrophic effector, facilitating disease lesion expansion by destruction of plant cells. This role would correlate with the up-regulation of Ds74283 at the Late stage of infection and Ds74283 mutants would also be expected to have lower virulence than the WT. However, because *D. septosporum* is predicted to secrete several proteins that also adopt a β -trefoil fold (see **Section 3.3.3.3** of **Chapter 3**) there may be functional redundancy if any of these proteins perform the same function as Ds74283 during pine infection; if this was the case then little or no effect on virulence would be seen in Ds74283 disruption mutants.

Gene-disrupted strains showed a decrease in fungal biomass *in planta* when compared to the WT fungus. But because complementation strains also showed a decrease in biomass, there was no clear evidence that these CE genes are involved in *D. septosporum* virulence on *Pn. radiata*. Or, if they are involved, the proteins encoded by these genes may have only small contributions to virulence, and the experiment lacked the resolution to detect these small changes in virulence by analysing the fungal biomass. Whilst future work is needed, it is important to note that pine virulence assays take a long time; once the trees are grown and ready for use (1–2 years depending on if seedlings or clones are used), it takes at least four months from obtaining sufficient inoculum through to completing fungal biomass analysis of the infected seedlings. Simpler and more rapid types of assays to study the function of these CE genes should be pursued. CE genes could be overexpressed and transformant strains inoculated onto clonal pine shoots (as used in protein infiltrations in **Chapter 2** and **Chapter 3**), although these are very expensive and not without their own limitations.

Overall, although two of the CE gene disruptions presented in this chapter provided only tentative evidence for an effect on virulence, the CRISPR/Cas9 gene editing method was successfully applied in *D. septosporum* for the first time. This will enable the

functional characterization of other *D. septosporum* CE genes identified in this thesis and in future studies, with the possibility of application to other forest pathogens, such as *C. minus* and *P. pluvialis*.

5 Identification and characterization of candidate effector proteins from different morphotypes of *Cyclaneusma minus*

5.1 Introduction

Cyclaneusma needle cast (CNC) is a disease of *Pinus* species in many parts of the world (Bednářová et al., 2013). CNC affects more than 30 pine species, including *Pinus radiata* in New Zealand and Australia. Scots pine (*Pinus sylvestris*) is also prone to the disease in forests of Europe and North America (Merrill & Wenner, 1996; Müller & Kurkela, 2012). The disease results in premature needle loss, and therefore losses in production. For New Zealand's plantation forestry, the losses caused by CNC were estimated to be around \$38 million per year in 2009 (Bulman, 2009), however the impact has likely been reduced since then by the planting of less susceptible genotypes. Regardless, the disease persists in some locations, and there are no alternative control measures (Bulman, 1993).

CNC is caused by the Leotiomycete fungus *Cyclaneusma minus*. At first, *C. minus* was reported as *Naemacyclus niveus* (Gilmour, 1959) and later separated into two species, *N. niveus* and *Naemacyclus minor* (Von Butin, 1973). This separation was based on morphological characters on needles, the formation of the sexual and asexual stages in culture and the relationship with different host species. *N. niveus* and *N. minor* were later reassigned to the genus *Cyclaneusma* as *Cyclaneusma niveum* and *Cyclaneusma minor*, respectively (Dicosmo et al., 1983). Further investigations found that *C. niveum* isolates had bacilliform pycnidiospores and much shorter apothecia, consistent with the species *C. minus*, rather than those recorded for *C. minor* (Dick et al., 2001). It was subsequently proposed that these isolates were actually two morphotypes of one species: *C. minus*, which was tagged as *C. minus* 'simile' for those isolates tending towards *C. niveum*, and *C. minus* 'verum' for those isolates tending towards *C. minus* (Dick et al., 2001). A relationship was shown between apothecium dimensions from needles and these morphotypes. However, there was considerable variation within each morphotype, and modern molecular techniques were required to determine whether further morphotypes exist.

Hunter et al. (2016) developed molecular tools for differentiating the verum and simile morphotypes and looked at their distribution throughout New Zealand. The study found that 'simile' was the most common morphotype in New Zealand, and that the molecular classification developed was much more accurate than morphological assessments.

Amongst the isolates studied, one (NZFS3325) showed DNA sequence variability in the ITS region of its genome, when compared to the corresponding region in both the simile and verum molecular taxonomic units (MOTUs), which indicated that additional morphotypes might exist (Hunter et al., 2016). In order to characterize these morphotypes, genome sequencing was carried out by researchers at Scion for eight *C. minus* isolates: two isolates belonging to morphotype simile (NZFS759 and 809), four belonging to morphotype verum (NZFS110, 725, 1800 and 3276) and two isolates (NZFS3305 and 3325) that could not be differentiated into verum or simile by previous molecular tools.

In addition to providing information on taxonomic classification, another benefit of these genomic resources is that they can provide further insights into the molecular interactions of different *C. minus* morphotypes with *Pn. radiata*. In **Chapter 3**, the genome sequence of *C. minus* NZFS809 (simile) was screened for the presence of genes encoding orthologues of candidate effector (CE) proteins from *Dothistroma septosporum* that elicited cell death in the model non-host plants *Nicotiana benthamiana* and *Nicotiana tabacum*. It was found that *C. minus* NZFS809 had several orthologous proteins that also triggered such cell death responses. The availability of the seven other *C. minus* genome sequences provided the opportunity to compare the level of sequence variability in these CEs and to determine whether the cell death-eliciting activity exhibited by the representatives from *C. minus* NZFS809 was conserved amongst the different morphotypes. In this Chapter, the genome sequences of the different *C. minus* morphotypes were examined for genes encoding CE proteins, with a specific focus on homologues of the cell death-eliciting Ecp32 protein family from *D. septosporum* and *Fulvia fulva* (discussed in **Chapter 2**). In addition to performing comparisons of their sequences and predicted tertiary structures, the aim of this chapter was to screen these homologs for their ability to trigger cell death in *N. benthamiana* and *N. tabacum* using

Agrobacterium tumefaciens-mediated transient transformation assays (ATTAs). In doing so, it was hoped that this analysis would provide novel insights into whether the Ecp32 family of *C. minus* plays an important role during host infection.

5.2 Methods

The work described in **Sections 5.2.1, 5.2.2 and 5.2.3** (culturing through to preliminary genome analysis) was carried out at Scion by Rebecca L. McDougal, Tammy Walters, Kiryn Dobbie and Luciano Nunes Leite.

5.2.1 Microorganisms

Cyclaneusma minus isolates NZFS110, 725, 759, 809, 1800, 3305, 3276 and 3325 (**Table 5.1**) were used in this study, and were grown on 2% (w/v) Difco™ Malt Extract Agar (BD&Co) and incubated at 22°C. Once the mycelium filled half of the plate, it was scraped into a sterile 15 mL falcon tube and stored at -80°C in preparation for freeze-drying and genomic DNA (gDNA) extraction.

5.2.2 Colony morphology molecular analysis

After freeze-drying, gDNA was extracted from the eight *C. minus* isolates using the REDExtract-N-Amp Plant PCR Kit (Sigma-Aldrich), following the manufacturer's instructions, and qPCR was performed to identify the morphotype of each isolate. Primers are shown in **Appendix Table A.2**. Each 10 µL reaction contained PerfeCTa qPCR ToughMix 2X (Qiagen), 300 nM forward and reverse primers, and 100 nM probe, sterile dH₂O and 2 µL of template DNA. Cycling conditions were 95°C 5 min with 45 cycles of 95° for 20 secs, 63°C for 20 secs, using a 48-well plate Eco™ Real-Time PCR System (Illumina, California, USA) (Hunter et al., 2016).

5.2.3 Genome sequencing, assembly and annotation

After the mycelia of *C. minus* isolates were freeze-dried, total gDNA was extracted for sequencing using a DNeasy Plant Maxi Kit (Qiagen), according to the manufacturer's instructions. For each isolate, 200 mg of tissue was added to Lysing Matrix C tubes containing 1.0 mm silica spheres (MP Biomedical, California, USA) and homogenised to a fine powder using a FastPrep-24™ (MP Biomedical) with two cycles of 20 s at 5 m/sec.

The extracted gDNA was concentrated by ethanol precipitation and resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8). gDNA concentration was quantified by spectrophotometry using both the Denovix DS-11 FX (DeNovix, Delaware, USA) and the DeNovix dsDNA Broad Range Assay (Denovix).

gDNA from eight isolates of *C. minus* (**Table 5.1**) was sequenced. Paired-end libraries having insert sizes of 327–353 bp were sequenced on a NovaSeq 6000 system (Illumina). Data filtering to remove adapters, contamination and low-quality reads resulted in at least 41 million reads (150 bp) for each isolate (**Appendix Table A.14**).

De novo genome assemblies of filtered reads were generated using SPAdes v3.14.0 (Nurk et al., 2013) with default settings. Contigs were assembled into scaffolds with redundans v0.13c (Pryszcz & Gabaldón, 2016). Genome assembly quality was measured in QUAST (Gurevich et al., 2013). Assembly completeness was estimated with BUSCO comparisons to the fungal dataset (Simão et al., 2015).

Annotations were predicted in each masked genome assembly using the Funannotate v.1.8.9 pipeline (Palmer & Stajich, 2020). Gene prediction was carried out using predicted protein sequences of *Elytroderma deformans* CBS183.68 (available at: <https://mycocosm.jgi.doe.gov/Elyde1/Elyde1.home.html>) as a guide. At first, protein sequences of *E. deformans* were mapped to the *C. minus* assembly file and the orthologs from *Cyclaneusma* were extracted. These orthologous sequences were first used to train Augustus, followed by three rounds of iterative gene prediction. Genes were predicted by Augustus v3.3.3 (Stanke et al., 2004), GeneMark ES v4.68 (Ter-Hovhannisyan et al., 2008), snap 2006-07-28 (Korf, 2004) and GlimmerHMM v3.0.4 (Majoros et al., 2004) and transfer RNAs predicted by tRNAscan-SE v2.0.9 (Chan et al., 2021). EvidenceModeler v1.1.1 (Haas et al., 2008) was used to generate consensus gene models. Putative gene products were determined by searches to MEROPS v12.0 (Rawlings et al., 2018), UniProt DB 2021_03 (The UniProt, 2021), dbCAN v10.0 (Yin et al., 2012), PFAM v34.0 (Mistry et al., 2021) and BUSCO v2.0 (Simão et al., 2015) databases.

5.2.4 Effector prediction

Following genome assembly and annotation of the eight *C. minus* isolates, the pipeline described in **Section 3.2.1** of **Chapter 3** was used to identify CE proteins from

all genomes, based on their repertoire of predicted proteins. Briefly, signal peptides were predicted using SignalP v3.0 (Bendtsen et al., 2004). Then, to exclude membrane-bound proteins, the following programmes were used: TMHMM v2.0 (Krogh et al., 2001) and BIG-PI (Eisenhaber et al., 2004). Proteins that were predicted to be ≤ 300 amino acids in length with ≥ 4 cysteine residues were selected and classified as small, secreted and cysteine-rich proteins (SSCPs). EffectorP v3.0 (Sperschneider & Dodds, 2021) was also used to select proteins classified as apoplastic or cytoplasmic effectors.

The Cms835 protein sequence from *C. minus* isolate NZFS809, which is an orthologue of DsEcp32-1 (Ds75860), was used to identify other members of the *C. minus* Ecp32 family from the eight isolates, through reciprocal BLASTp and tBLASTn searches and using an E-value cut-off of $<10^{-5}$.

Protein sequence alignments, construction of phylogenetic trees and protein tertiary structure predictions were performed as described in **Section 2.2.1 of Chapter 2**, and the prediction of intrinsically disordered regions (IDR) was carried out as described in **Section 3.2.1 of Chapter 3**.

5.2.5 *Agrobacterium tumefaciens*-mediated transient expression assays (ATTAs)

The methods used to generate ATTA expression vectors and for ATTAs in non-host plants *N. benthamiana* and *N. tabacum* are described in **Sections 2.2.6 and 2.2.7 of Chapter 2**. All genes coding for proteins from *C. minus* isolates other than NZFS809 used in ATTAs were synthesized directly into the pICH86988 expression vector by Twist Biosciences (**Appendix Figure A.1**).

5.3 Results

The results described in **Sections 5.3.1 and 5.3.2** (culturing through to preliminary genome analysis) were obtained at Scion by Rebecca L. McDougal, Tammy Walters, Kiryn Dobbie and Luciano Nunes Leite.

5.3.1 Molecular differentiation of *Cyclaneusma minus* isolates

Eight *C. minus* isolates were collected from different parts of New Zealand and were classified into two different morphotypes, verum (Cmv) and simile (Cms), according

to molecular analysis (Hunter et al., 2016). However, two of these isolates, NZFS3305 and 3325 (the same one previously identified in Hunter et al. (2016)), could not be differentiated based on those molecular tools and were, therefore, classified as a new *C. minus* morphotype, novus (Cmn). This classification was supported by morphological analyses carried out at Scion (McDougal et al, unpublished).

Table 5.1. Isolates of *Cyclaneusma minus* used in this study.

NZFS Number ^a	New Zealand Location ^b	Host	<i>C. minus</i> 'simile' qPCR	<i>C. minus</i> 'verum' qPCR	Morphotype ^c
110	South Canterbury	<i>Pinus radiata</i>	-	+	verum
725	Coromandel	<i>Pinus radiata</i>	-	+	verum
1800	Dunedin	<i>Pinus radiata</i>	-	+	verum
3276	Bay of Plenty	<i>Pinus radiata</i>	-	+	verum
759	Northland	<i>Pinus radiata</i>	+	-	simile
809	Southland	<i>Pinus radiata</i>	+	-	simile
3305	Bay of Plenty	<i>Pinus radiata</i>	-	-	novus*
3325	Bay of Plenty	<i>Pinus radiata</i>	-	-	novus*

^a Scion Forest Health Reference Laboratory Culture Collection number (NZFS).

^b Location in New Zealand where the isolate was collected, according to (Crosby et al., 1998).

^c Morphotype based on molecular analysis according to Hunter et al. (2016). * New morphotype name proposed for this study.

5.3.2 Genome sequencing, assembly and annotation

The final genome assemblies produced by Scion for the eight *C. minus* isolates ranged in length from 32 to 43 Mb (**Table 5.2** and **Appendix Table A.14**). The final set of gene models for each genome showed a completeness of >96% with the retrieval of at least 728 complete BUSCO hits from a total of 758 fungal BUSCOs (**Table 5.2** and **Appendix Table A.14**).

Table 5.2. *Cyclaneusma minus* genomes statistics.

NZFS ^a	Morpho- type ^b	NCBI Accession	Scaffolds ^c	Gene models	N50 ^d (bp)	Assembly length (bp)	Complete BUSCOs (%)	GC (%)
110	verum	JAKZHM000000000	2616	10.445	570.2	42,876,071	96.6	45.6
725	verum	JAKZHN000000000	1804	10.409	1,103.7	41,971,199	96.9	45.8
1800	verum	JAKZHQ000000000	1644	10.455	438.3	42,298,080	97.1	45.6
3276	verum	JAKZHT000000000	2004	10.397	528.9	42,485,107	97.3	45.7
759	simile	JAKZHO000000000	2588	9.659	833.0	36,285,465	96.3	47.9
809	simile	JAKZHP000000000	1548	9.684	666.1	35,864,553	97.3	48.0
3305	novus	JAKZHR000000000	610	9.266	126.2	31,933,912	97.6	45.8
3325	novus	JAKZHS000000000	732	9.269	119.02	32,988,501	97.7	50.2

^a Scion Forest Health Reference Laboratory Culture Collection number (NZFS).

^b Morphotype according to Hunter et al. (2016). Novus: new morphotype proposed for this study.

^c Number of scaffolds assembled using Redundans v0.13c (Pryszcz & Gabaldón, 2016).

^d The minimum contig length among contigs required to cover 50% of the assembled genome sequence.

5.3.3 Prediction and functional characterization of candidate effector proteins from different *Cyclaneusma minus* morphotypes

Following genome sequencing and assembly of the eight *C. minus* isolates from the three different morphotypes, their predicted proteins were used for effector prediction. Genomes from the same morphotype have similar numbers of predicted proteins and effectors (**Table 5.3**). **Appendix Table A.15** shows all amino acid sequences of the CEs predicted with EffectorP for all isolates. From the 56 apoplastic CEs of isolate NZFS809, three (including one from the Ecp32 family) were known to trigger cell death responses in *N. benthamiana* and *N. tabacum* (see **Section 3.3.3** of **Chapter 3**). For this reason, these three were chosen to be further analysed in the seven other *C. minus* isolates, along with cross-kingdom CE Cms8840 (the orthologue of Ds69335 from *D. septosporum* and Pp7927 from *Phytophthora pluvialis*).

Table 5.3. Effector prediction in eight *Cyclaneusma minus* isolates.

NZFS ^a	Morpho- type ^b	Predicted proteins ^c	Secreted proteins ^d	No membrane affiliation ^e	SSCP ^f	Effectors ^g	Cyto effectors	Apo effectors
110	verum	10,445	729	582	117	92	16	76
725	verum	10,409	714	568	109	90	17	73
1800	verum	10,455	721	578	120	100	18	82
3276	verum	10,397	715	568	111	94	17	77
759	simile	9,659	642	504	95	72	17	55
809	simile	9,684	640	497	91	69	13	56
3305	novus	9,266	639	509	91	70	17	53
3325	novus	9,269	629	499	87	68	15	53

^a Scion Forest Health Reference Laboratory Culture Collection number (NZFS).

^b Morphotype according to Hunter et al. (2016). Novus: new morphotype proposed for this study.

^c Number of proteins predicted from each of the assembled *Cyclaneusma* genomes.

^d Number of secreted proteins after being queried using SignalP 3.0 (Bendtsen et al., 2004).

^e Number of secreted proteins with no membrane affiliation after being queried using the programmes TMHMM v2.0 (Krogh et al., 2001) and BIG-PI (Eisenhaber et al., 2004).

^f Small, secreted cysteine-rich proteins, of ≤ 300 amino acids with ≥ 4 cysteine residues.

^g Number of effector proteins predicted using EffectorP v3.0 (<http://effectorp.csiro.au/>). Effector proteins were classified as cytoplasmic (cyto) or apoplastic (apo).

5.3.4 Candidate effectors from different morphotypes of *Cyclaneusma minus* trigger cell death in non-host species

Based on their similarity to cell death elicitors from *D. septosporum*, the CE proteins Cms835, Cms2492, Cms2721 from *C. minus* isolate NZFS809, which is of morphotype simile (Cms), were previously tested for their ability to trigger cell death in non-host *Nicotiana* species (see **Section 3.3.3 of Chapter 3**). Cms8840 was also included in the analysis as orthologues of this protein are shared between all three pine pathogens. Out of these proteins, all but Cms8840 triggered cell death in both *N. benthamiana* and *N. tabacum* (**Figure 3.7** and **Figure 3.8 of Chapter 3**). Next, to build on these results, all four of the CEs described above from *C. minus* NZFS809 were assessed for their presence in other sequenced isolates of the Cms morphotype, as well as in all sequenced isolates of the morphotypes verum and novus. Amino acid sequences for orthologues of Cms835, Cms2492, Cms2721 and Cms8840 in other *C. minus* isolates and morphotypes are shown in **Appendix Figure A.29**.

Orthologues of Cms2492 are present in all of the *C. minus* isolates and morphotypes assessed. A phylogenetic tree constructed based on an alignment of Cms2492 with its orthologues from all other sequenced *C. minus* isolates and

morphotypes (**Figure 5.1**) shows that proteins from morphotypes classified as Cmn are identical in sequence, while the two Cms proteins are also identical to each other (**Figure 5.2A**). Proteins from Cmv isolates NZFS110, 725 and 1800 are identical and have only one amino acid difference (S60G) compared with the protein sequence from NZFS3276 (**Figure 5.1** and **Figure 5.2A**). Between morphotypes, the pairwise amino acid identity varies between 70.3% (between Cms and Cmn) and 75.2% (between Cms and Cmv).

NZFS809 Cms2492	1	MKSAVFASIF	AFAASSALAQYDT	QSPSFYLQL	ISDNST	INGSYLT	SCHSGA	51
NZFS759 Cms163	1	MKSAVFASIF	AFAASSALAQYDT	QSPSFYLQL	ISDNST	INGSYLT	SCHSGA	51
NZFS110 Cmv8527	1	MKSAVFASIL	AFAASSALAQYDAQSP	PFYLQLL	SDNSTL	NGSYLAS	CHTGA	51
NZFS725 Cmv5485	1	MKSAVFASIL	AFAASSALAQYDAQSP	PFYLQLL	SDNSTL	NGSYLAS	CHTGA	51
NZFS1800 Cmv2944	1	MKSAVFASIL	AFAASSALAQYDAQSP	PFYLQLL	SDNSTL	NGSYLAS	CHTGA	51
NZFS3276 Cmv4855	1	MKSAVFASIL	AFAASSALAQYDAQSP	PFYLQLL	SDNSTL	NGSYLAS	CHTGA	51
NZFS3305 Cmn4577	1	MKSAVCASVL	AFAASSALAQYDVQSP	PFYLQVL	SDNST	INGTYLAS	CHTGA	51
NZFS3325 Cmn4351	1	MKSAVCASVL	AFAASSALAQYDVQSP	PFYLQVL	SDNST	INGTYLAS	CHTGA	51
NZFS809 Cms2492	52	AIESLCLYGPEA	ADPDMAETFK	LNNTTSPYVANATGL	ITWVL	QGSNFNESE	I	102
NZFS759 Cms163	52	AIESLCLYGPEA	ADPDMAETFK	LNNTTSPYVANATGL	ITWVL	QGSNFNESE	I	102
NZFS110 Cmv8527	52	AIESLCLYSSTD	- - DSDHETYNLNTTDPYVANATGYL	TWVL	HGGNFNESEP			100
NZFS725 Cmv5485	52	AIESLCLYSSTD	- - DSDHETYNLNTTDPYVANATGYL	TWVL	HGGNFNESEP			100
NZFS1800 Cmv2944	52	AIESLCLYSSTD	- - DSDHETYNLNTTDPYVANATGYL	TWVL	HGGNFNESEP			100
NZFS3276 Cmv4855	52	AIESLCLYGSTD	- - DSDHETYNLNTTDPYVANATGYL	TWVL	HGGNFNESEP			100
NZFS3305 Cmn4577	52	AIESLCLLDEDV	- - PSNYETYNLNTTTPYVANGTGIL	TWVL	PASNFNESEP			100
NZFS3325 Cmn4351	52	AIESLCLLDEDV	- - PSNYETYNLNTTTPYVANGTGIL	TWVL	PASNFNESEP			100
NZFS809 Cms2492	103	LNFFYYNPTSN	IAMPLFEPSETGQA	IAFDCEDNAY	ISAYL	NYSTSPPSYYGE		153
NZFS759 Cms163	103	LNFFYYNPTSN	IAMPLFEPSETGQA	IAFDCEDNAY	ISAYL	NYSTSPPSYYGE		153
NZFS110 Cmv8527	101	LTFDYDATSNV	AMPVFEPSETGQGIAFDCEDNAY	ISSYL	NYSTSPPSYYED			151
NZFS725 Cmv5485	101	LTFDYDATSNV	AMPVFEPSETGQGIAFDCEDNAY	ISSYL	NYSTSPPSYYED			151
NZFS1800 Cmv2944	101	LTFDYDATSNV	AMPVFEPSETGQGIAFDCEDNAY	ISSYL	NYSTSPPSYYED			151
NZFS3276 Cmv4855	101	LTFDYDATSNV	AMPVFEPSETGQGIAFDCEDNAY	ISSYL	NYSTSPPSYYED			151
NZFS3305 Cmn4577	101	LVFYDPTTN	IAMPLFAPSDEGQAVAFDCEDNAY	INGYL	DYSTSPPTYLED			151
NZFS3325 Cmn4351	101	LVFYDPTTN	IAMPLFAPSDEGQAVAFDCEDNAY	INGYL	DYSTSPPTYLED			151
NZFS809 Cms2492	154	DGASVQAFYNWYL	CRTNDEGYTYETVWV	TAGEPVT	PD	CSAVDLH	RVFV*	203
NZFS759 Cms163	154	DGASVQAFYNWYL	CRTNDEGYTYETVWV	TAGEPVT	PD	CSAVDLH	RVFV*	203
NZFS110 Cmv8527	152	AGDNGQQFYNWVF	CHTNFAGTYETL	VWSTSGNPV	IPDC	SAVSLH	REFV*	201
NZFS725 Cmv5485	152	AGDNGQQFYNWVF	CHTNFAGTYETL	VWSTSGNPV	IPDC	SAVSLH	REFV*	201
NZFS1800 Cmv2944	152	AGDNGQQFYNWVF	CHTNFAGTYETL	VWSTSGNPV	IPDC	SAVSLH	REFV*	201
NZFS3276 Cmv4855	152	AGDNGQQFYNWVF	CHTNFAGTYETL	VWSTSGNPV	IPDC	SAVSLH	REFV*	201
NZFS3305 Cmn4577	152	G - - PKEFY	NWYVCQTNQEGYEYTTV	VWATAGEPV	IPDC	SAISLH	REFV*	198
NZFS3325 Cmn4351	152	G - - PKEFY	NWYVCQTNQEGYEYTTV	VWATAGEPV	IPDC	SAISLH	REFV*	198

Figure 5.1. Protein sequence alignment of Cms2492 with its orthologues from *Cyclaneusma minus* morphotypes simile, verum and novus. The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined. The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus.

Together with Cms2492, proteins representing each morphotype were tested for their ability to elicit cell death in *N. benthamiana* and *N. tabacum*. These proteins were:

Cmv8527 (NZFS110) and Cmn4577 (NZFS3305). All three proteins consistently triggered cell death in *N. benthamiana*, while Cms2492 and Cmn4577 only triggered weak cell death in *N. tabacum* and Cmv8527 did not trigger any visible plant response (Figure 5.2B).

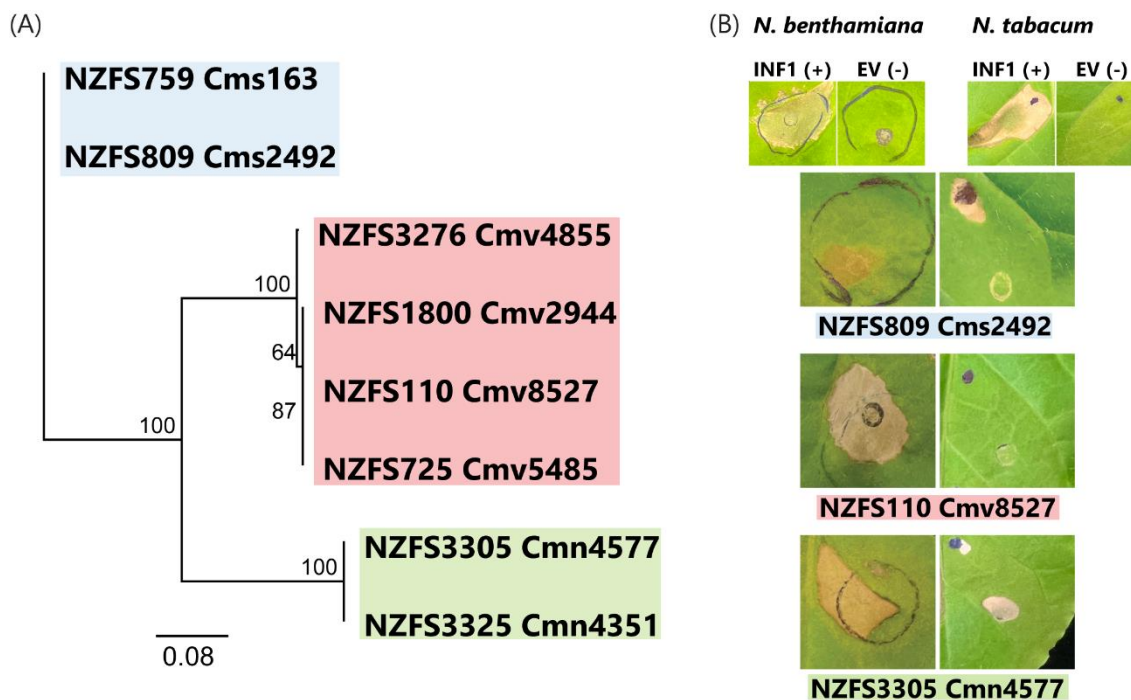


Figure 5.2. Orthologues of *Cyclaneusma minus* candidate effector Cms2492 from three different morphotypes trigger cell death in *Nicotiana* species. **(A)** Protein phylogeny of Cms2492 and its respective orthologues from other *C. minus* isolates. The tree was constructed with the neighbour-joining method using Geneious Software v9.1.8. Bootstrap values are shown at the nodes as percentages. The scale bar represents 0.08 substitutions per site. The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes. Morphotypes are colour coded: simile – blue; verum – red; novus – green. **(B)** Cms2492, Cmv8527 and Cmn4577 were expressed in *Nicotiana benthamiana* and *Nicotiana tabacum* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12 infiltration zones) from three independent experiments. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. Photos were taken 7 days after infiltration.

The same analysis was carried out for cell death elicitor Cms2721. Orthologues of this protein are also present in all of the *C. minus* isolates and morphotypes assessed. In

a phylogenetic tree, all *C. minus* isolates grouped together, with identical proteins identified within each of the three morphotypes (**Figure 5.3** and **Figure 5.4A**). Between morphotypes, the full-length amino acid identity varied from 85.8% (between Cmv and Cmn, and between Cms and Cmn) to 91.2% (between Cmv and Cms). Together with Cms2721, proteins representing each morphotype were tested for their ability to elicit cell death in *N. benthamiana* and *N. tabacum*. These proteins were: Cmv scf53 (NZFS110) and Cmn7980 (NZFS3305). All three proteins consistently triggered cell death in *N. benthamiana* and *N. tabacum* (**Figure 5.4B**).

NZFS809 Cms2721	1	MASLLKSL	SLLLAATLV	SNVDS	SSNTKGK	CPGTL	KCSASASSSA	IQAA	50					
NZFS759 Cms394	1	MASLLKSL	SLLLAATLV	SNVDS	SSNTKGK	CPGTL	KCSASASSSA	IQAA	50					
NZFS110 scf53	1	MPSLLKSL	SLLLAATLV	SNIDACS	SSNTKGK	CPGTL	NCSASASSSA	IQAA	50					
NZFS725 scf36	1	MPSLLKSL	SLLLAATLV	SNIDACS	SSNTKGK	CPGTL	NCSASASSSA	IQAA	50					
NZFS1800 scf13	1	MPSLLKSL	SLLLAATLV	SNIDACS	SSNTKGK	CPGTL	NCSASASSSA	IQAA	50					
NZFS3276 Cmv1489*	1	MPSLLKSL	SLLLAATLV	SNIDACS	SSNTKGK	CPGTL	NCSASASSSA	IQAA	50					
NZFS3305 Cmn7980*	1	MPSLLKNL	TLLLAATLV	SATADSC	VSNKGK	CPGTL	NCSASASSSA	IQAA	50					
NZFS3325 Cmn7756*	1	MPSLLKNL	TLLLAATLV	SATADSC	VSNKGK	CPGTL	NCSASASSSA	IQAA	50					
NZFS809 Cms2721	51	EC	SHNTRT	SETQT	FAVFV	TDHEYD	SSHGAPYGT	CEAYTCT	PTEDEM	TDV	100			
NZFS759 Cms394	51	EC	SHNTRT	SETQT	FAVFV	TDHEYD	SSHGAPYGT	CEAYTCT	PTEDEM	TDV	100			
NZFS110 scf53	51	EC	SHNTRT	SETQT	FAVFV	TDHEYD	SVYGAPYGT	CEAYTCT	APTEDEM	TDV	100			
NZFS725 scf36	51	EC	SHNTRT	SETQT	FAVFV	TDHEYD	SVYGAPYGT	CEAYTCT	APTEDEM	TDV	100			
NZFS1800 scf13	51	EC	SHNTRT	SETQT	FAVFV	TDHEYD	SVYGAPYGT	CEAYTCT	APTEDEM	TDV	100			
NZFS3276 Cmv1489*	51	EC	SHNTRT	SETQT	FAVFV	TDHEYD	SVYGAPYGT	CEAYTCT	APTEDEM	TDV	100			
NZFS3305 Cmn7980*	51	EC	SHNTRT	SVTQT	FAVFMT	TDHQYD	DSHGAPYGS	CKAYTCT	APTSD	EMTDV	100			
NZFS3325 Cmn7756*	51	EC	SHNTRT	SVTQT	FAVFMT	TDHQYD	DSHGAPYGS	CKAYTCT	APTSD	EMTDV	100			
NZFS809 Cms2721	101	DD	DC	WTF	FWSG	EGEE	SGVGTNC	IEDPT	TGEC	CCEDSS	SGTFI	AGSDSCT	*	149
NZFS759 Cms394	101	DD	DC	WTF	FWSG	EGEE	SGVGTNC	IEDPT	TGEC	CCEDSS	SGTFI	AGSDSCT	*	149
NZFS110 scf53	101	DD	DC	WTF	FWSG	EGEE	EGLGTNC	IEDPST	TGEC	CCEDSD	GTFFV	AGSDSCT	*	149
NZFS725 scf36	101	DD	DC	WTF	FWSG	EGEE	EGLGTNC	IEDPST	TGEC	CCEDSD	GTFFV	AGSDSCT	*	149
NZFS1800 scf13	101	DD	DC	WTF	FWSG	EGEE	EGLGTNC	IEDPST	TGEC	CCEDSD	GTFFV	AGSDSCT	*	149
NZFS3276 Cmv1489*	101	DD	DC	WTF	FWSG	EGEE	EGLGTNC	IEDPST	TGEC	CCEDSD	GTFFV	AGSDSCT	*	149
NZFS3305 Cmn7980*	101	DD	DC	WTF	FWSG	EGEE	SGVGTNC	IEDPGT	TGEC	CCEDSD	GTFFI	DGSSSCT	*	149
NZFS3325 Cmn7756*	101	DD	DC	WTF	FWSG	EGEE	SGVGTNC	IEDPGT	TGEC	CCEDSD	GTFFI	DGSSSCT	*	149

Figure 5.3. Protein sequence alignment of Cms2721 with its orthologues from *Cyclaneusma minus* morphotypes simile, verum and novus. The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined. The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes. The asterisk * for isolates NZFS3276, 3305 and 3325 indicates that the protein was incorrectly predicted, and the corrected protein sequence is shown in this alignment. Proteins from isolates NZFS110, 725 and 1800 were not originally predicted, so Cmv scf refers to the scaffold on which the predicted protein sequence can be found in the genome. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus.

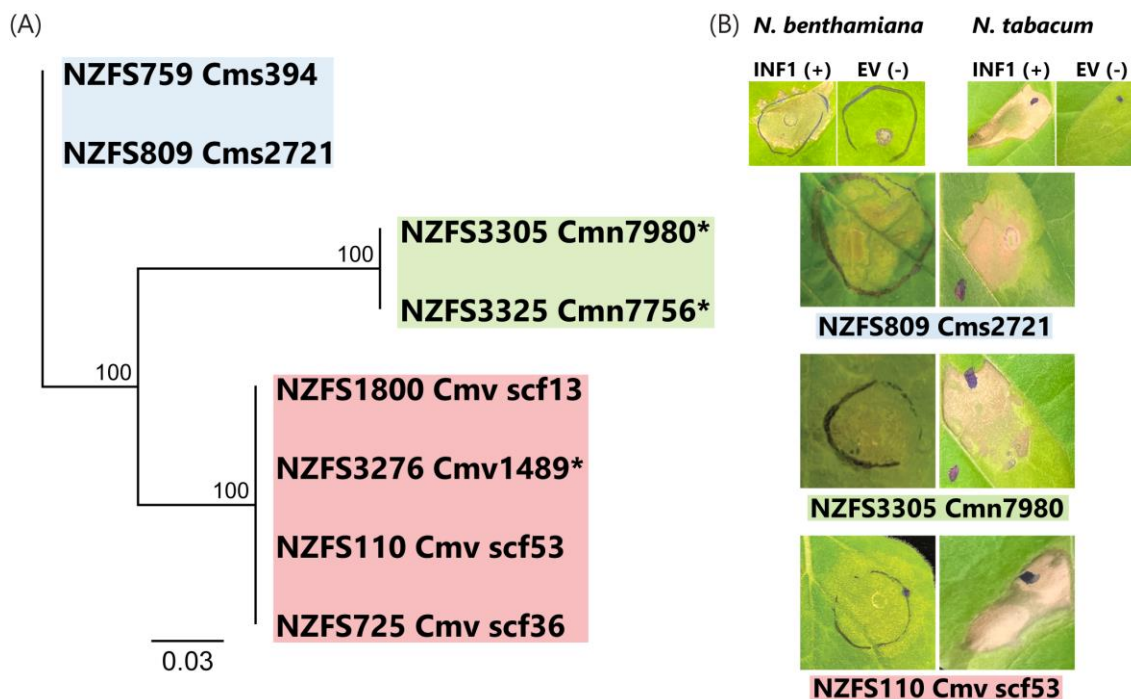


Figure 5.4. Orthologues of Cms2721 from *Cyclaneusma minus* proteins from three different morphotypes trigger cell death in *Nicotiana* species. **(A)** Protein phylogeny of Cms2721 and its respective orthologues from other *C. minus* isolates. The tree was constructed with the neighbour-joining method using Geneious Software v9.1.8. Bootstrap values are shown at the nodes as percentages. The scale bar represents 0.03 substitutions per site. The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes. The asterisk * for isolates NZFS3276, 3305 and 3325 indicates that the protein was incorrectly predicted, and the corrected protein sequence was used. Proteins from isolates NZFS110, 725 and 1800 were not originally predicted, so scf refers to the scaffold on which the predicted protein sequence can be found in the genome. Morphotypes are colour coded: simile – blue; verum – red; novus – green. **(B)** Cms2721, Cmv scf53 and Cmn7980 were expressed in *Nicotiana benthamiana* and *Nicotiana tabacum* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12 infiltration zones) from three independent experiments. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. Photos were taken 7 days after infiltration.

The third CE studied in the *C. minus* isolates and morphotypes was Cms8840. Even though Cms8840 did not trigger cell death in either of the non-host plants, it was still of interest as homologues of this protein were found in *D. septosporum* (Ds69335) and *P. pluvialis* (Pp7927), all of which have sequence and structural homology with

proteins belonging to the 'cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins' (CAP) superfamily (see **Section 3.3.3.2 of Chapter 3**). The gene coding for Cms8840 is present in all the other *Cyclaneusma* isolates. All of these proteins are predicted to have a C-terminal CAP domain and a long intrinsically disordered region (IDR) at their N-terminus (**Figure 5.5**). Unlike those from other fungi and oomycetes (see **Section 3.3.3.4 of Chapter 3**), the Cms8840 orthologues from *C. minus* are not part of a large family, with all isolates having just one copy of the gene that encodes them. Consistent with their respective morphotypes, proteins from isolates NZFS759 and NZFS809 (Cms) have no sequence differences, and NZFS3305 and NZFS3325 (Cmn) are also identical to each other. NZFS110, 725, 1800 and 3276 (Cmv) have protein sequences with 94.1% full-length amino acid identity, including a predicted deletion of 18 amino acids in the IDR of NZFS3276 when compared to the other proteins from morphotype Cmv (**Figure 5.5**).

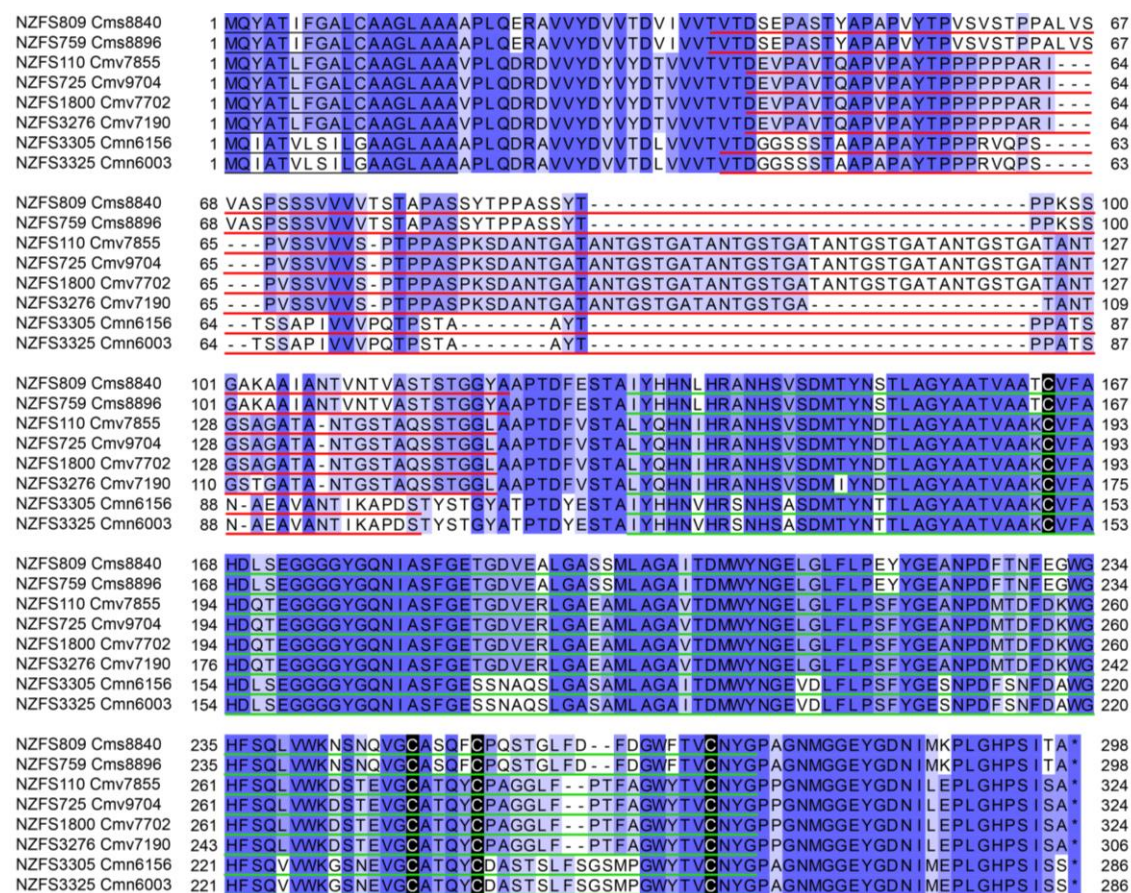


Figure 5.5. Protein sequence alignment of Cms8840 with its orthologues from *Cyclaneusma minus* morphotypes simile, verum and novus. The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined. The predicted intrinsically disordered regions (IDRs) are underlined in red and the predicted 'cysteine-rich secretory protein, antigen 5, and pathogenesis-related 1 protein' (CAP) domain is underlined in green. IDRs were predicted with Predictors of Natural Disordered Regions (PONDR®) and CAP domains were predicted through the Conserved Domain Database (CDD) from the National Centre for Biotechnology Information (NCBI). The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus.

In conclusion, it was possible to identify a set of CEs from eight isolates of *C. minus* belonging to three different morphotypes. Based on the findings that proteins from isolate NZFS809 were able to trigger non-host cell death responses (Cms2492 and Cms2721), it was also possible to determine that cell death activity is conserved amongst other isolates and morphotypes, suggesting these proteins might be important avirulence factors of *C. minus* when infecting pine, and therefore, are good candidates

to be further characterized. Cms8840 is also a good candidate for future studies since it is also present in the other two pine pathogens and because specific CAP domain proteins have been shown to be involved in suppressing host defences (see **Section 3.4** of **Chapter 3**).

5.3.4.1 Characterization of the Ecp32 cell death elicitor family from different *Cyclaneusma minus* morphotypes

As mentioned previously, the Cms835 protein from isolate NZFS809 is a homologue of DsEcp32-1 (Ds75860), which forms part of a four-member Ecp32 family in *D. septosporum*. With the Cms835 (NZFS809) amino acid sequence as reference, other Ecp32 family members in this and other isolates/morphotypes were identified from their respective proteomes and genomes using BLASTp and tBLASTn, respectively. Based on this analysis, it was determined that all isolates and morphotypes had members of the Ecp32 family of proteins (**Appendix Figure A.30**).

Even though all isolates and morphotypes appear to have proteins belonging to the Ecp32 family, the number of family members between each morphotype varies. A first BLASTp search indicated that both NZFS759 and 809 (Cms) had eight proteins as part of the Ecp32 family. However, a further look into the genes coding for these proteins indicated that three genes from each of these two isolates were pseudogenes. The predicted Cms proteins Cms834 (NZFS809) and Cms3366 (NZFS759) are identical truncated proteins that have a premature stop codon at amino acid position 54, while the predicted Cms proteins in another pair, Cms4060 (NZFS809) and Cms4411 (NZFS759), have a premature stop codon at amino acid position 149. Cms245 (NZFS809) and Cms2573 (NZFS759) are also identical predicted proteins and, in this case, a cytosine insertion at position 213 of the CDS caused a frameshift mutation that led to a premature stop codon. Interestingly, four of these pseudogenes are each clustered together with one of more functional genes in the respective genome. Specifically, genes encoding Cms835 and Cms834 (truncated) are clustered together in the NZFS809 genome. Likewise, those of Cms4058, Cms4059 and Cms4060 (truncated) are also clustered. Genes encoding the respective orthologues of these proteins from the NZFS759 isolate are also

clustered, with those for Cms3365 and Cms3366 (truncated) clustered together, as well as Cms4411 (truncated), Cms4412 and Cms4413 clustered together.

All isolates from Cmv (NZFS110, 725, 1800 and 3276) each have six paralogues in their Ecp32 family, while Cmn isolates (NZFS3305 and 3325) each have only three. **Figure 5.6** shows a protein sequence alignment of all full-length members from the *C. minus* Ecp32 family (CmEcp32); family members that had identical amino acid sequences were excluded from this alignment, as well as pseudogenes. **Figure 5.7** shows the phylogenetic tree constructed based on these alignments, including all family members identified, even those with identical amino acid sequences. These results show that proteins from the CmEcp32 family cluster into seven separate groups (all colour-coded differently in the phylogenetic tree). The clustered groups coloured in blue, yellow and green are made up of eight protein members, with one paralogue belonging to each of the eight genomes analysed. The clustered group colour-coded in red has six members, with paralogues only belonging to Cms and Cmv, and no members belonging to Cmn, while the grey group is only made up of proteins from Cmv. It is interesting to note that proteins from the same isolate (NZFS) in the red, grey, and orange groups are clustered together in their respective genomes, with the proteins from Cmv more closely related to the ones from Cms. Proteins clustered in the group coloured in pink have longer amino acid sequences than the rest of the proteins in this family; normally they range from 197 to 226 amino acids in length, however these four proteins are made up of 268 amino acids. Proteins from this cluster all belong to Cmv and have a predicted IDR at their N-terminus, immediately following their putative signal peptide (**Figure 5.6**).

Together, these results suggest that several gene duplication events may have occurred in *C. minus* or an ancestral species. Between Cms and Cmv, the pairwise amino acid identity varies between 25.1% and 71.4% across all CmEcp32 protein family members. Between Cms and Cmn, the pairwise amino acid identity varies between 32.1% and 68.9%, while between Cmv and Cmn, it varies between 28.8% and 68.6%. Between different isolates of the same morphotype, the amino acid sequence identity among the Ecp32 proteins ranges from 25.3% to 50.6% (Cmv), 30.9% to 42.3% (Cms) and 34.4% to 46.8% (Cmn).

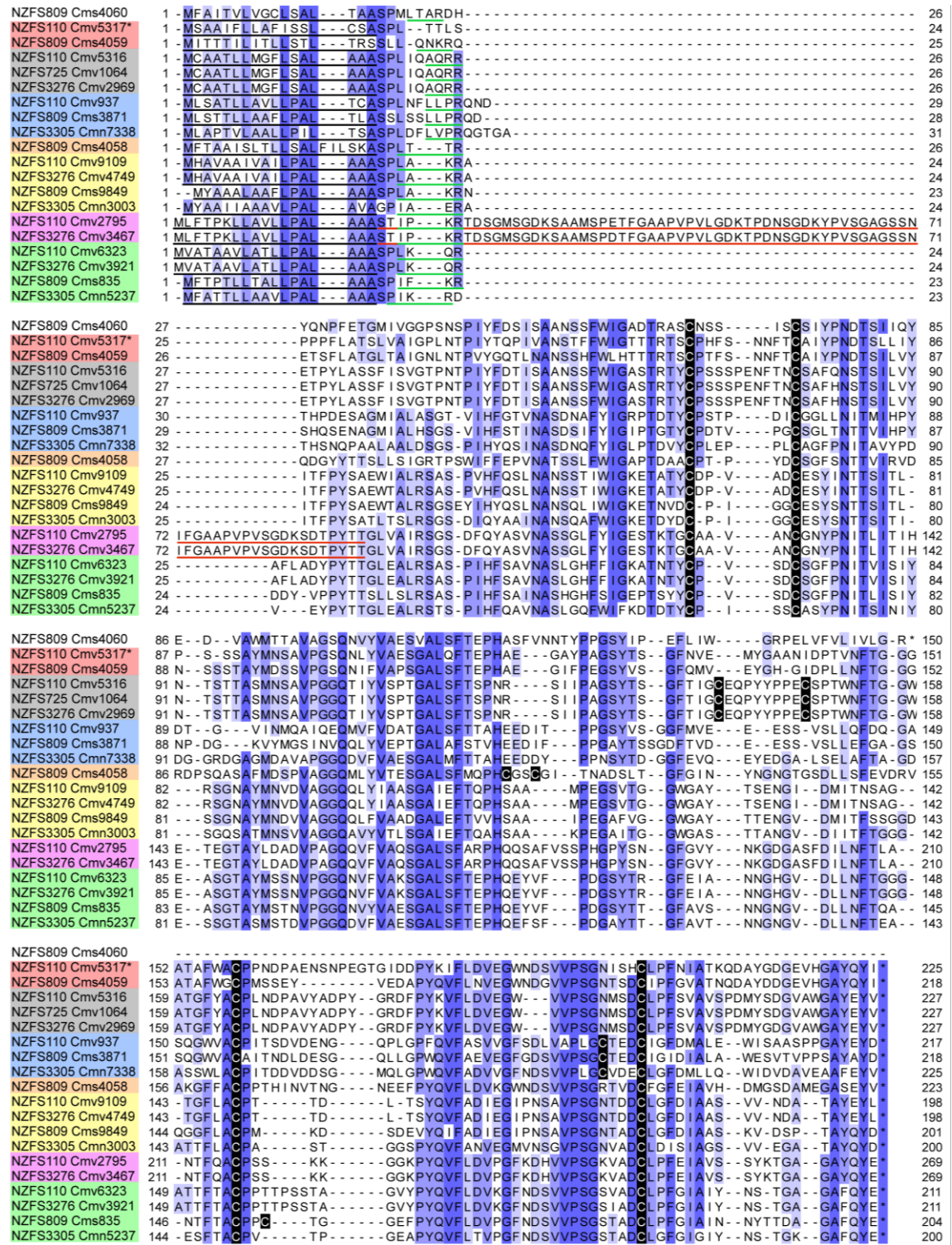


Figure 5.6. Protein sequence alignment of Ecp32 family members across eight isolates and three morphotypes of *Cyclaneusma minus*. The alignment was generated using Clustal Ω (Sievers et al., 2011), with blue shading indicating the degree of amino acid sequence conservation. Conserved cysteine residues are highlighted in black and predicted signal peptides are underlined. Intrinsically disordered regions (IDRs) predicted with Predictor of Natural Disordered Regions (PONDR®) are underlined in red. The cleavage motifs of Kex2 protease are underlined in green.

The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes; the asterisk * is to identify that the protein was incorrectly predicted, and the corrected protein sequence was used. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. Proteins were colour-coded according to their clustering in the phylogeny in **Figure 5.7**.

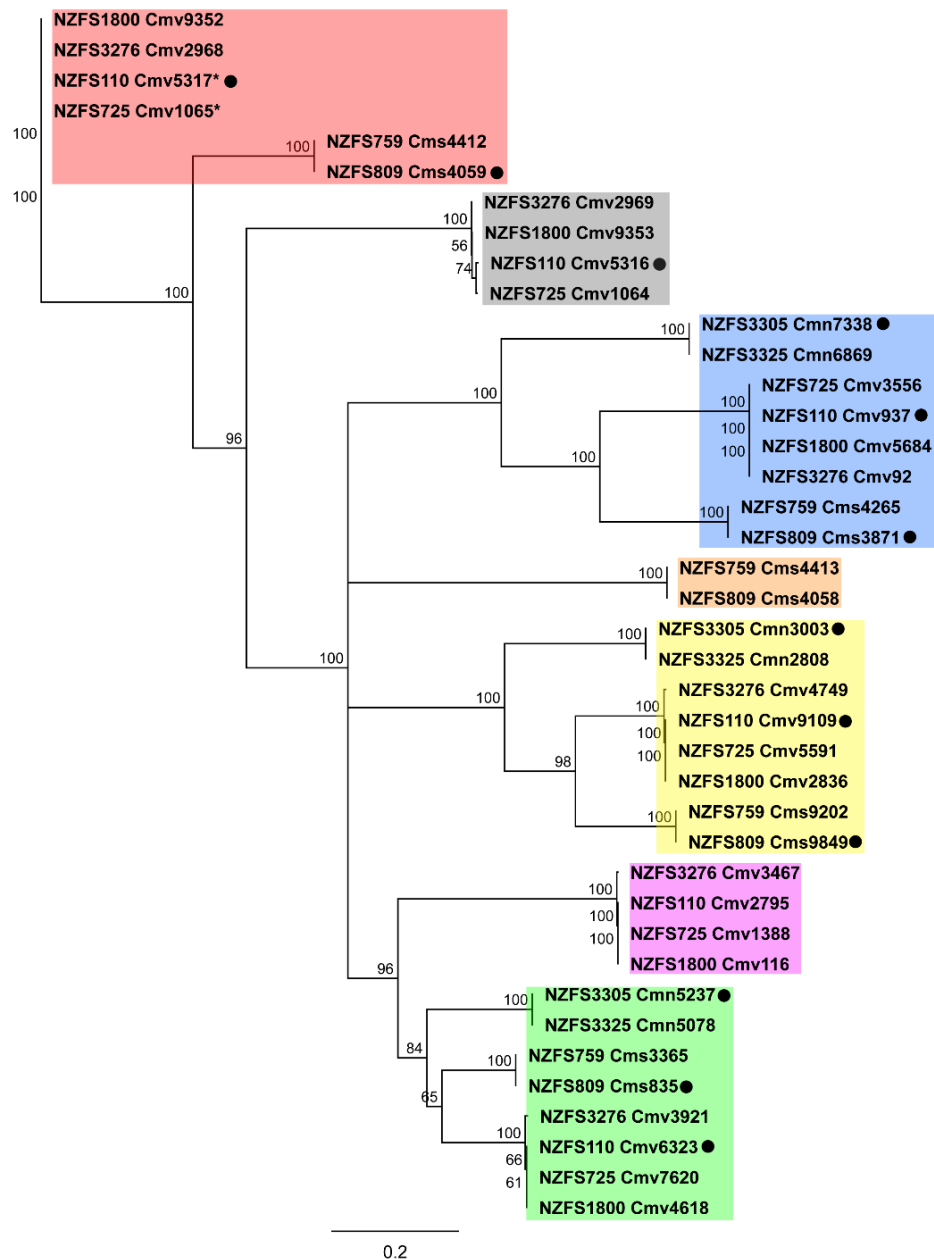


Figure 5.7. Phylogenetic tree of the *Cyclaneusma minus* Ecp32 family members across eight isolates and three morphotypes of *Cyclaneusma minus*. Neighbour-joining tree obtained from the amino acid alignment of Ecp32 proteins using Geneious Software v9.1.8. Bootstrap values are shown at the nodes as percentages. The scale bar represents 0.2 substitutions per site. The number following each morphotype ID (NZFS) refers to the protein number from the annotated genomes; the asterisk * is to identify that the protein was incorrectly predicted, and the corrected protein sequence was used. The black dot refers to the proteins that were expressed in *Nicotiana benthamiana* and *Nicotiana tabacum* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. Proteins were colour-coded according to their clustering in the phylogeny.

Unlike what was observed for orthologues between *D. septosporum* and *F. fulva*, proteins from the Ecp32 family from each *C. minus* isolate did not group with the previously identified members of the Ds/FfEcp32 family. **Figure 5.8** shows a phylogenetic tree of protein members from the Ds/FfEcp32 family and all members of the CmEcp32 family from isolate NZFS110 (Cmv).

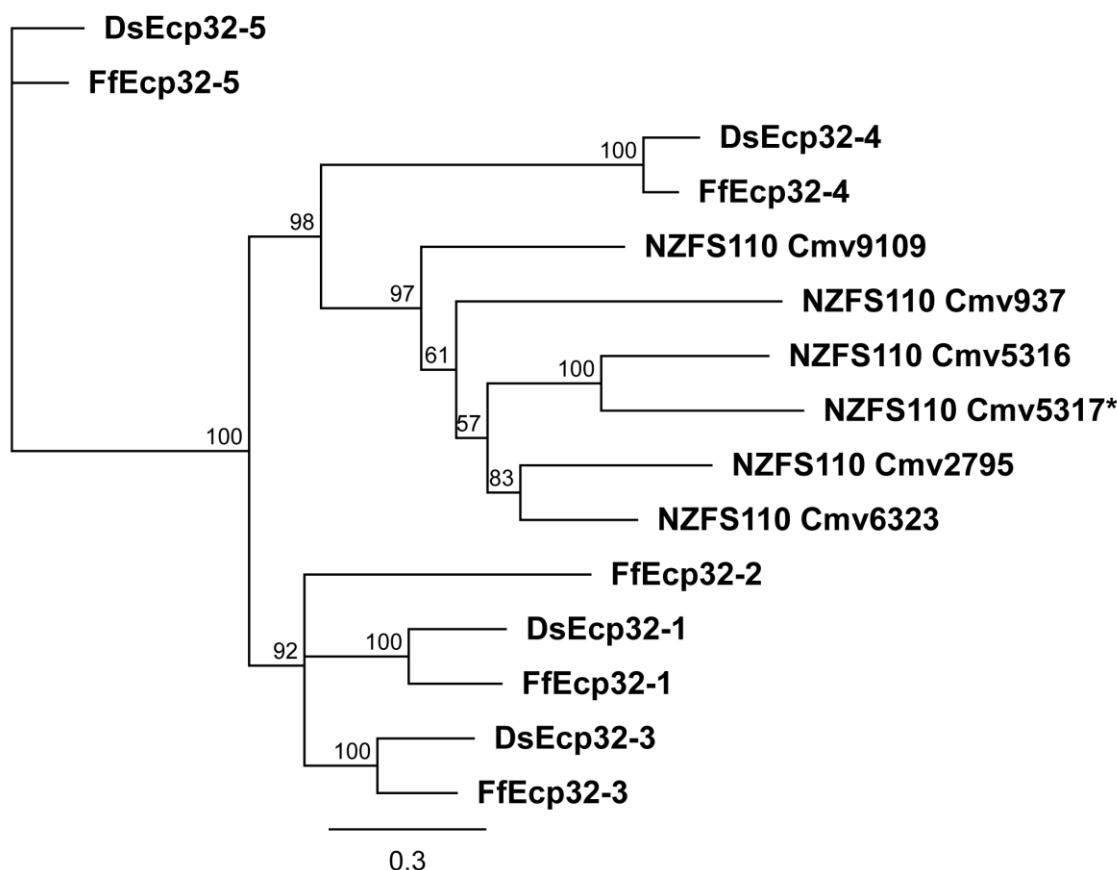


Figure 5.8. Protein phylogeny of the *Dothistroma septosporum*, *Fulvia fulva* and *Cyclaneusma minus* NZFS110 Ecp32 families. The trees were constructed with the neighbour-joining method using Geneious Software v9.1.8. Bootstrap values are shown at the nodes as percentages. The scale bar represents 0.3 substitutions per site. Ds: *D. septosporum*; Ff: *F. fulva*; Cmv: *C. minus* morphotype verum.

5.3.4.1.1 Proteins of the Ecp32 family from different *Cyclaneusma minus* morphotypes trigger cell death in *Nicotiana* species

Because Cms835 was previously shown to trigger non-host cell death, the next question was if other proteins from the CmEcp32 family in the same and in different morphotypes could also trigger cell death in *Nicotiana* species. The selected proteins

tested by ATTAs are indicated with a black dot in **Figure 5.7**. The goal was to select one protein from each morphotype in the clusters that had a higher number of members. As a result, in *N. benthamiana*, all proteins tested triggered some degree of cell death, except for Cmv937 (the Cmv representative from the blue cluster in **Figure 5.7**) (**Figure 5.9**). Proteins from the green cluster in **Figure 5.7**, Cmv6323, Cms835 and Cmn5237, consistently triggered cell death responses, the same with clustered proteins Cmv9109, Cms9849 and Cmn3003 (yellow cluster in **Figure 5.7**), with Cms9849 triggering a strong cell death response in all infiltration zones. The truncated protein Cms4060 (NZFS809) was also tested and triggered a weak cell death response. Although this protein was not included in the phylogenetic tree shown in **Figure 5.7**, it showed greatest similarity with the proteins colour-coded in grey, and an alignment between these proteins (**Figure 5.6**) indicated conservation of the N-terminus (post signal peptide), which could suggest that this region of the protein might be responsible for the cell death, since Cmv5316 also triggered a cell death response (**Figure 5.9**).

In *N. tabacum*, yellow cluster proteins Cmv9109, Cms9849 and Cmn3003 (**Figure 5.7**) consistently triggered strong cell death responses, while all other proteins inconsistently triggered either weak cell death or chlorosis (**Figure 5.10**). Interestingly, the truncated protein Cms4060 (NZFS809), as well as its paralogue Cmv5316, did not trigger cell death (**Figure 5.10B**).

Interestingly, all CmEcp32 proteins, with the exception of those from isolates NZFS110, 725, 1800 and 3276 in the red group cluster (**Figure 5.7**), have a pro-domain motif of four amino acids at the N-terminus (**Figure 5.6**). First identified in *Saccharomyces cerevisiae*, this motif is recognized by the calcium-dependent subtilisin-like serine protease Kex2 and is involved in the correct processing and maturation of proteins (Outram et al., 2021; Wickner, 1974). Other fungi have Kex2-like homologues also involved in effector processing (Outram et al., 2021). The motif is composed of a leucine or any aliphatic residue in the first position, followed by x (any residue; this residue is not involved in the recognition), followed preferably by a lysine (even though other residues are functional (Bevan et al., 1998)) and followed by an arginine in the last position (the cleavage occurs at the end of the carboxyl end of the arginine). The presence of this pro-domain motif might explain the differences observed in the cell death activity by the

CmEcp32 proteins. In some cases, like for yellow cluster proteins Cmv9109, Cms9849 and Cmn3003 (**Figure 5.7**), this processing does not seem to interfere with the proteins' ability to trigger cell death, but it might in the cases of proteins that did not trigger cell death or gave inconsistent responses. This could be tested by removing the motif when cloning the genes that encode each of these proteins in the expression vector for ATTAs. Western blots to try to detect all of these proteins should also be performed.

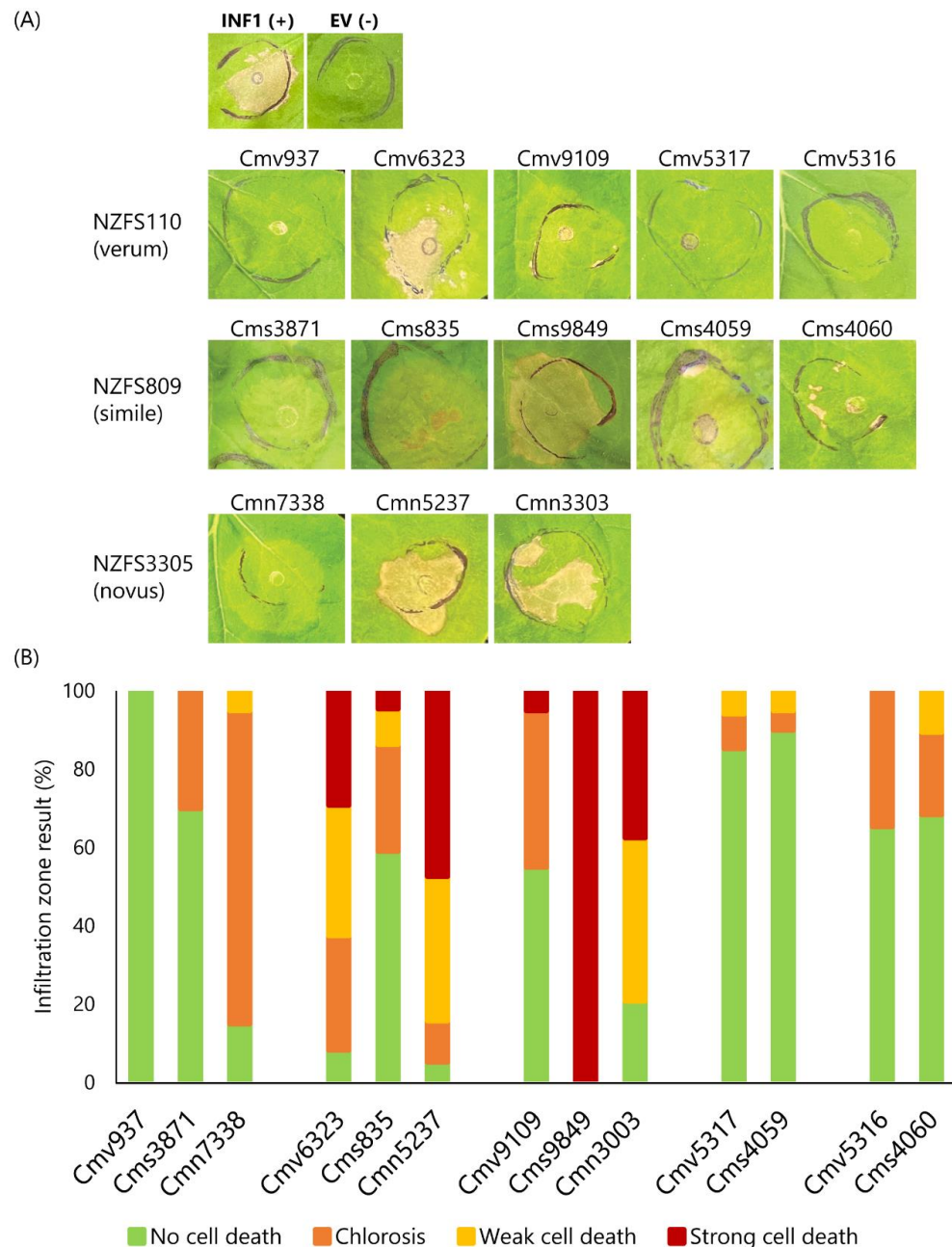


Figure 5.9. Ecp32 proteins from different morphotypes of *Cyclaneusma minus* trigger cell death in *Nicotiana benthamiana*. **(A)** Proteins were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12-30 infiltration zones), from at least three independent experiments. Cms4060 is a truncated protein. INF1, *Phytophthora infestans* elicitin positive cell death control; EV, empty vector negative no-cell death control; Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. Photos were taken 7 days after infiltration. **(B)** Graphs display the percentages of infiltration zones that showed cell death in response to each different protein, divided into four categories: strong cell death, weak cell death, chlorosis, and no cell death.

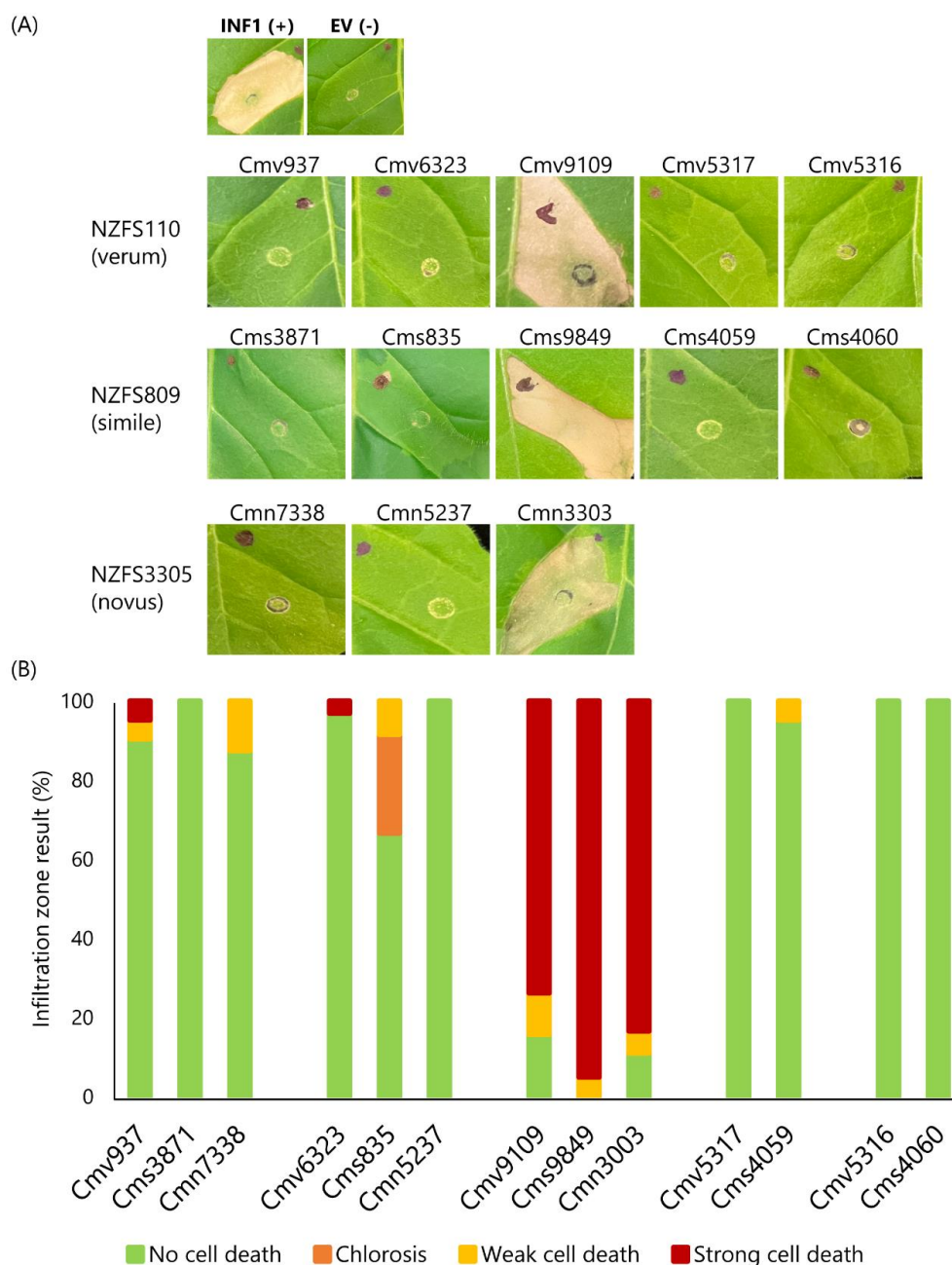


Figure 5.10. Ecp32 proteins from different morphotypes of *Cyclaneusma minus* trigger cell death in *Nicotiana tabacum*. **(A)** Proteins were expressed in *N. tabacum* using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit cell death. Representative images are shown (n = 12–30 infiltration zones), from at least three independent experiments. Cms4060 is a truncated protein. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no-cell death control; Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. Photos were taken 7 days after infiltration. **(B)** Graphs display the percentages of infiltration zones that showed cell death in response to each different protein, divided into four categories: strong cell death, weak cell death, chlorosis, and no cell death.

5.3.4.1.2 The *Cyclaneusma minus* Ecp32 family has structural similarity with β -trefoil proteins

The tertiary structures of one Ecp32 family member from each of the clusters shown in **Figure 5.7** were predicted using AlphaFold2 (**Figure 5.11**). The amino acid sequences of proteins from NZFS110 (Cmv) were used for these structural predictions since one Ecp32 family member from this isolate was present in all clusters, except for the orange one (**Figure 5.7**). The predicted structures had a pLDDT score and a TM-score of 86.4 and 0.848 for Cmv937, 89.0 and 0.874 for Cmv6323, 95.5 and 0.908 for Cmv9109, 88.2 and 0.867 for Cmv5317, 84.9 and 0.86 for Cmv5316, and 75.1 and 0.7 for Cmv2795, respectively.

As expected for proteins belonging to the Ecp32 family (see **Section 2.3.2** of **Chapter 2**), all proteins examined were predicted to be structurally similar to β -trefoil proteins, even though they had some differences in topology. The structures of Cmv937, Cmv6323, Cmv9109, Cmv5317 and Cmv2795 have two predicted disulphide bonds, while Cmv5316 has three. The two predicted disulphide bonds shared across all predicted structures are conserved with the two predicted disulphide bonds present in all members of the Ecp32 family from *D. septosporum* and *F. fulva* (except Ds/FfEcp32-5, which does not have any cysteine residues) (**Figure 5.11** and **Appendix Figure A.7**). Notably, of the two conserved disulphide bonds, one of them, S-S2, is also predicted to be present in the predicted structures of the other β -trefoil proteins discussed in **Chapter 3** (i.e., Ds69113 and Ds74283, present only in *D. septosporum*, and the orthologous pair Ds71487 and Cm2492 from *D. septosporum* and *C. minus*, respectively) (**Figure 3.16** of **Chapter 3** and **Figure 5.11**). Even though Cm2492 is also predicted to have a β -trefoil fold, it has no sequence similarity with any proteins from the CmEcp32 family, as observed previously.

The structure of the truncated Ecp32 candidate Cms4060 was also predicted since the protein triggered weak cell death in the same manner as Cmv5316 (**Figure 5.9**). AlphaFold2 predicted a tertiary structure with a pLDDT score of 73.9 and a predicted TM-score of 0.716. The alignment between the predicted structures of Cms4060 and Cmv5316 (**Figure 5.11I**) highlights the N-terminal conservation previously observed in

the sequence alignment (**Figure 5.6**); the predicted structure of Cms4060 (coloured green in the structural alignment) aligns with the region of Cmv5316 coloured in black (the grey colouring represents the C-terminal region of Cmv5316 that is not present in Cms4060), including one conserved disulphide bond (S-S1).

In conclusion, just like that observed for *D. septosporum* and *F. fulva*, the Ecp32 family is also present in *C. minus*, with different numbers of paralogues between isolates. Amino acid sequence searches (see **Section 2.3.3** of **Chapter 2**) revealed that this protein family is widespread across other fungal species, and therefore, might be an important core effector of plant-pathogenic fungi. Similarities in the structures of truncated protein Cms4060 and full-length Cmv5316, with conservation of cell death activity, might provide insights into the regions of the proteins from the CmEcp32 family that are responsible for plant receptor or target recognition.

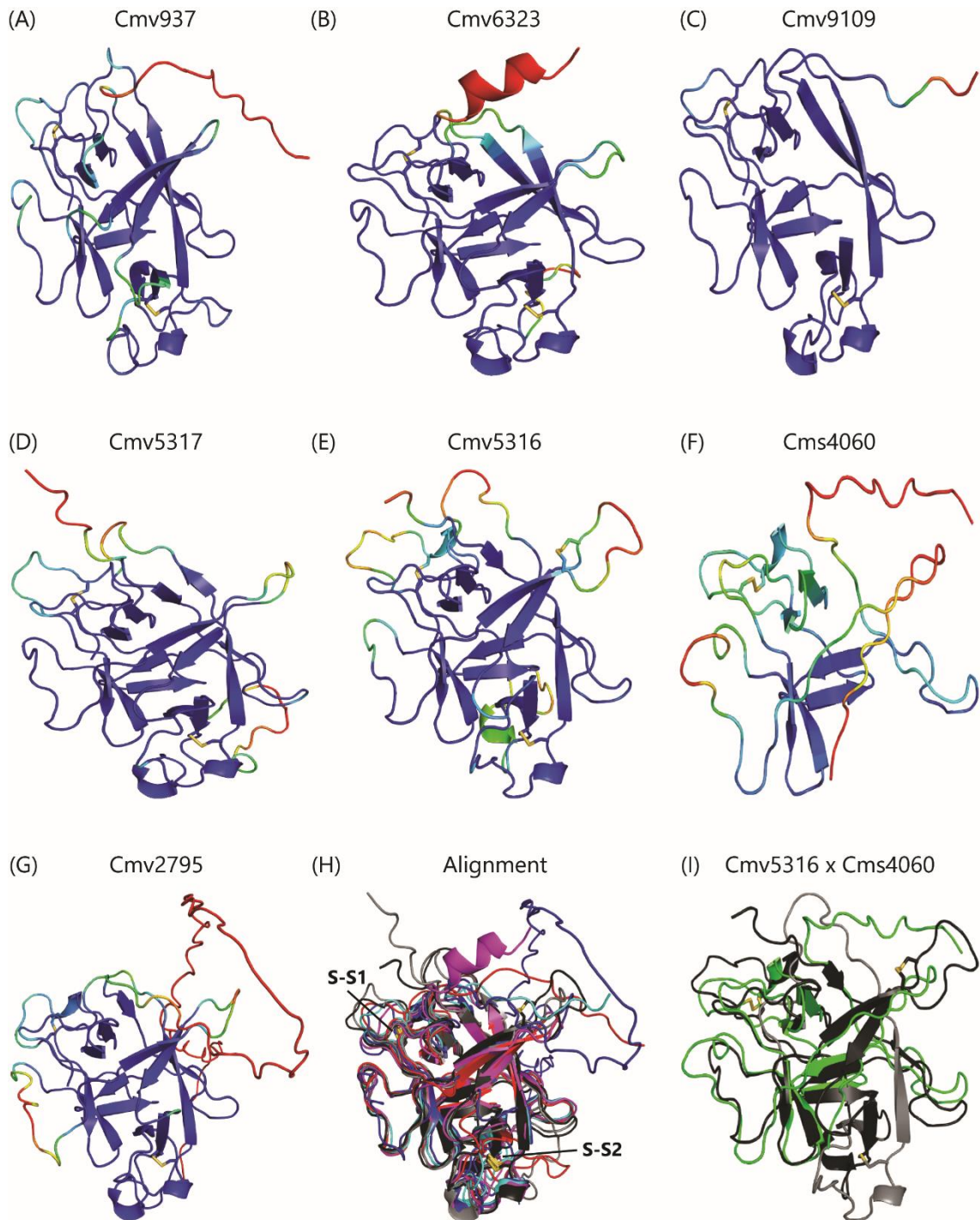


Figure 5.11. Predicted protein tertiary structures of Ecp32 family members from different isolates of *Cyclaneusma minus*. **(A–G)** Predicted structures of **(A)** Cmv937, **(B)** Cmv6323, **(C)** Cmv9109, **(D)** Cmv5317, **(E)** Cmv5316, **(F)** Cms4060 and **(G)** Cmv2795. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. **(H)** Alignment of the predicted Cmv937 (red), Cmv6323 (magenta), Cmv9109

(cyan), Cmv5317 (grey) and Cmv5316 (black) structures. S-S: disulphide bonds. **(I)** Alignment of the predicted Cmv5316 (black and grey) with the truncated Cms4060 (green). Black represents the part of the Cmv5316 structure that aligns with Cms460. Protein structures were aligned using the CEalign tool in PyMOL. Disulphide bonds are shown as yellow sticks. Cmv: *C. minus* morphotype verum; Cms: *C. minus* morphotype simile.

5.4 Discussion

C. minus is an important pathogen of pines worldwide. However, very little is known about the molecular interactions underpinning this pathosystem. At first, differences in ascospore length and characteristics of the fungus grown on agar plates indicated that there were two morphological types of *C. minus* in New Zealand, termed *C. minus* simile and *C. minus* verum (Bulman & Gadgil, 2001). However, with the development of molecular tools to differentiate these morphotypes, a new morphotype was also identified in New Zealand, being classified as novus. Isolates from these three morphotypes of *C. minus* had their genomes sequenced, which enabled the prediction and comparison of CEs.

The effector prediction for *C. minus* isolates was performed using a similar pipeline to that described in **Section 3.2.1** of **Chapter 3** and generated a first list of possible effector proteins for this pathogen. Because effectors are often described as small and cysteine-rich, this was used to first identify *C. minus* CEs. Then, these SSCPs were classified as apoplastic or cytoplasmic effectors according to EffectorP, resulting in a set of CE proteins from these eight *C. minus* isolates. However, other proteins that were not classified as SSCPs are also potential effectors that should not be discarded for future analysis. Nevertheless, this initial classification is a good starting point to functionally characterize *C. minus* effectors, and it would be interesting to assess which of those trigger cell death responses, using the non-host ATTA approach described previously.

In this Chapter, the main focus was on proteins that were selected based on their similarity with *D. septosporum* proteins and were also identified as cell death elicitors (see **Section 3.3.3** of **Chapter 3**). In particular, two proteins from isolate NZFS809 (simile), Cms2492 and Cms2721, shown to trigger cell death responses in non-host *Nicotiana* species, were investigated across the *C. minus* isolates. Orthologues of these proteins

were found in the genomes of the other seven isolates and were shown to trigger cell death responses in non-host species (**Figure 5.9** and **Figure 5.10**). The results observed for orthologues of Cms2492 and of Cms2721 suggests conservation of cell death-eliciting activity between isolates (and morphotypes) of *C. minus*. Whether the identified sequence variation contributes to virulence functions of these proteins outside of cell death elicitation remains to be determined. Also, other experiments, such as reactive oxygen species measurements and analysis of the expression of *Nicotiana* defence gene markers, should be performed to determine if these cell death responses are actually due to activation of the plant immune system.

Some pathogens are known to secrete effectors that form part of families (discussed in **Chapter 1** and **Chapter 2**). Following the observed similarity between Ds75860 (part of the Ecp32 family from *D. septosporum*) and Cms835 (from isolate NZFS809), it was assessed to what extent the Ecp32 protein family was also present in this and the other seven isolates of *C. minus*. However, different morphotypes seemed to have different numbers of paralogues, with the morphotype novus (NZFS3305 and 3325) having only three paralogues in each isolate, while morphotype simile (NZFS759 and 809) has five and verum (NZFS110, 725, 1800 and 3276) has six. All of these proteins are conserved at the primary sequence and tertiary structure levels, suggesting that these proteins have important and/or conserved roles in fungal virulence. The differences observed in the number of paralogues in each morphotype might also suggest duplication events from an ancestral gene or from other family members in some of the morphotypes.

Proteins from the CmEcp32 family were found to have members that differed in their ability to trigger cell death in the non-host species *N. benthamiana* and *N. tabacum*. This difference was also observed in members of the Ecp32 family from *D. septosporum* and *F. fulva*. Also consistent with the predicted structures of Ecp32 proteins from *D. septosporum* and *F. fulva*, predicted structures generated for representative members of the CmEcp32 family were shown to adopt a β -trefoil fold (discussed in **Chapter 2** and **Chapter 3**). The differences in cell death-triggering activity observed between different family members might be due to structural differences on the surface of the proteins, for example as a result of insertions, deletions and/or substitutions of residues, which occur

more often in loops than in α -helices and β -sheets (Fiser et al., 2000). In some cases, loops in tertiary structures of proteins are known to be involved in protein-protein interactions, recognition sites, signalling cascades and ligand binding (Fetrow, 1995), all of which could explain why some proteins from the CmEcp32 family trigger more consistent cell death responses than others. Also, sequence and structural diversification observed for the proteins in this family could have a role in evasion of host immunity, another possible explanation for why this family is expanded in *C. minus*.

Alternatively, the pro-domain identified at the N-terminus of the Ecp32 proteins might also play an important part in the correct processing of these proteins and might influence the different cell death responses observed. Removing this pro-domain prior to cloning would help determine if this is the case. Western blots would be useful in determining if the proteins that did not elicit a response, or triggered inconsistent responses, are actually being expressed in *Nicotiana* species.

The finding that Cms4060 (truncated) and Cmv5316 (full-length) triggered the same degree of cell death in both *Nicotiana* species suggests that the N-terminal part of the protein (that is also conserved between Cms4060 and the other CmEcp32 proteins) might contain the region, or conserved epitope, that is either recognized by a plant extracellular immune receptor or is interacting with the plasma membrane, triggering cell death. Previous studies have demonstrated the possibility of swapping loop regions from non-functional proteins for other loops from functional proteins (Pardon et al., 1995; Wolfson et al., 1991). Considering the consistent cell death triggered by Cmv9109, Cms9849 and Cmn3003, it would be informative to try to determine differences in the loop regions of these proteins that might be responsible for cell death, in comparison with other proteins that trigger inconsistent or no cell death responses.

As discussed in **Section 2.3.7** of **Chapter 2**, FfEcp32-3-triggered cell death, but not DsEcp32-3-triggered cell death, was compromised in *N. benthamiana* leaves expressing the TRV:BAK1, TRV:SOBIR1 or the T26 gene silencing construct (which targets two different leucine rich repeat-type receptors in *N. benthamiana*). It would be interesting to determine if these targets are also required for cell death activity for members of the CmEcp32 family (specifically proteins from the yellow cluster in **Figure 5.7**, which triggered consistent cell death in non-host plants). Or, alternatively,

given their predicted structural similarity to β -trefoil proteins such as lectins and cytolytins, cell membrane perturbation could also be involved in the cell death elicitation by these proteins, outside of immune receptor recognition.

As mentioned previously, Cms2492 is also predicted to adopt a β -trefoil fold (see **Section 3.3.3.2 of Chapter 3**), even though it has no sequence similarity with CmEcp32 family proteins. Just as observed for *D. septosporum*, whilst proteins from the CmEcp32 family have strong primary sequence and structure similarity between them, Cms2492 (and orthologues encoded by genes present in the other genomes) have lost primary sequence similarity with proteins from the Ecp32 family and only structural similarity is retained between these β -trefoil proteins. *C. minus* proteins that are predicted to adopt a β -trefoil fold might have originated from one protein and, during evolution, duplication events occurred leading to sequence dissimilarity. It remains to be determined if these, and possibly other β -trefoil proteins from *C. minus*, share the same virulence functions when infecting the host. It would also be interesting to determine the structures of other CEs from *C. minus* and identify if this fold is present in the structures of any of those proteins. For example, the Ecp20 family present in both *D. septosporum* and *F. fulva* could not be detected in *C. minus* through primary sequence similarity searches, but searches in the predicted structures of all *C. minus* CEs might identify proteins that adopt the characteristic Alt a 1 fold of Ecp20 family proteins, thereby expanding the repertoire of common effector types between these foliar fungal pathogens.

6 Conclusions and future work

The aim of this thesis was to better understand the molecular interactions between *Pinus radiata* and three of its foliar pathogens: two fungal and one oomycete. The fungal pathogens *Dothistroma septosporum* and *Cyclaneusma minus*, and the oomycete pathogen *Phytophthora pluvialis*, each have serious impacts on New Zealand *Pn. radiata* plantations, causing Dothistroma needle blight, Cyclaneusma needle cast and red needle cast, respectively. The generation of information about these pathosystems will ultimately contribute to the improvement of control measures for each of these diseases.

All three of these pathogens are thought to colonize the apoplastic environment between cells where they secrete effector proteins to promote disease (virulence factors). Notably, however, if these effectors are recognized by corresponding resistance (R) proteins from the host, they activate the plant immune system to stop pathogen growth (avirulence factors), normally due to a hypersensitive response (HR) from the host. This is only efficient against biotrophic pathogens, as this HR can be exploited for host colonization by necrotrophs. Due to these critical roles, the primary aim of this thesis was to identify and characterize effector proteins that are common between the three pine pathogens, or at least between the two fungi. The latter was included because fungi and oomycetes are distantly related and might therefore interact with their hosts in different ways. Finding common virulence or avirulence factors secreted by these pathogens can provide important insights into how they interact with their host *Pn. radiata* to cause disease and, perhaps, how the plant recognizes and defends itself from them.

Using a combination of genomic and transcriptomic information, as well as bioinformatic methods, it was possible to predict a set of candidate effector (CE) genes from these pathogens and express them in the non-host model plants *Nicotiana benthamiana* and *Nicotiana tabacum* for functional characterization. Another aim of this thesis was to further develop a method to screen such CEs of conifer pathogens directly in pine. Several CE proteins from all three pathogens were identified and shown to trigger cell death responses in both *Nicotiana* species and *Pn. radiata*. The predicted tertiary structures of these CE proteins also provided useful information about their potential biological roles. Ultimately, three CE genes from *D. septosporum* (two of which have

orthologues in *C. minus* and *P. pluvialis*) were disrupted through CRISPR/Cas9 technology to study their biological function during *Pn. radiata* infection.

6.1 Pine needle pathogen candidate effector proteins trigger cell death responses in host and non-host plants

Candidate effectors were identified from *D. septosporum* using an effector prediction pipeline based on typical amino acid sequence features. Some of these effectors had already been identified in a previous study (Hunziker, 2018), while additional ones were identified in this thesis. From these 29 identified proteins, two different CE protein families, Ecp20 and Ecp32, which each have members that are highly conserved at the primary sequence and tertiary structure levels, were firstly characterised in *D. septosporum* NZE10 and *Fulvia fulva* (**Chapter 2**). It was later shown that the Ecp32 family is also present in different isolates of *C. minus* (**Chapter 5**), but not in *P. pluvialis* (at least not at the primary sequence level) (**Table 6.1**). Both protein families have members that were able to trigger plant cell death responses.

Further experiments should be performed to determine the localization of these CEs in the plant during pathogen infection. More specifically, localization experiments could be performed by generating an N- or C-terminal fusion between the CE protein and a fluorophore like mCherry or yellow fluorescent protein, under the control of a strong promoter. After transforming it into the pathogen, fluorescence (and thus location of the CE) could be monitored during infection using confocal microscopy.

Likewise, experiments that aim to investigate if the cell death response observed is due to their direct interaction with the host plasma membrane, or recognition by a plant immune receptor (R protein), might offer insights on the nature of the cell death response. Those would involve measurements of reactive oxygen species (ROS) in both host and non-host plants and analysis of expression for genes that are known to be involved in defence responses or defence response pathways. Another possibility for assessing whether a cell death response is due to recognition by an R protein would be to express the CE proteins in *BAK1/SOBIR1*-silenced or knock-out plants; these extracellular co-receptors are required to transduce defence signals following apoplastic effector recognition. This was done for the cell death-eliciting Ecp20 and Ecp32 family

members and it was found that DsEcp20-3-triggered cell death was compromised in *BAK1/SOBIR1*-silenced plants, as well as in *N. benthamiana* leaves expressing a virus (tobacco rattle virus)-induced gene silencing (TRV VIGS) construct that targets two different plant genes (one encoding a receptor-like protein (RLP) and the other encoding an extracellular leucine-rich repeat (LRR) domain protein). In the case of direct interactions, co-immunoprecipitation assays can be performed to investigate possible interactions between DsEcp20-3 (and perhaps DsEcp20-4, which does not trigger cell death responses) and the identified *N. benthamiana* receptors. Trying to identify these receptors, either by sequence or structural similarity, in the *Pn. radiata* genome (not yet publicly available) might also provide useful information.

Some fungal proteins with an Alt a 1-like fold (a fold predicted to be adopted by all DsEcp20 family members) are known to interact with and inhibit the enzymatic activity of pathogenesis-related family 5 (PR5) proteins from different plants (Gómez-Casado et al., 2014; Kumar & Mukherjee, 2020; Zhang et al., 2019). If this is the case for the Ecp20 proteins secreted by *D. septosporum* in pine, DsEcp20-3 could be directly recognized by an R protein, resulting in the observed cell death response, or indirectly, by inhibiting a particular pine PR5 protein. The modulation of this PR5 protein could then be recognized by an R protein to give the observed cell death response. On the other hand, DsEcp20-4 might be a virulence factor that inhibits pine PR5 proteins to suppresses host defences but is not directly recognised due to lack of recognition or absence of a corresponding pine R protein. DsEcp20-4 might also not be indirectly recognised by an R protein due to an undetectable modulation of the targeted PR5 protein, or because the PR5 protein is not guarded by an R protein. It would be interesting to determine and compare the possible virulence targets of DsEcp20-3 and DsEcp20-4 (and other Ecp20 family members) in pine.

Gene disruption of individual *Ecp20* family members could be achieved using CRISPR/Cas9 technology to determine the contribution that each protein makes to pathogen virulence during pine infection. Following on from this, all family members could be sequentially disrupted, which would enable the virulence contribution of the entire Ecp20 family to be assessed. The latter is quite an attractive option, as it may be that some family members contribute redundantly to virulence and therefore do not provide a quantifiable change in virulence when individually disrupted.

Apart from the DsEcp20 and DsEcp32 families, three other CE proteins were identified in *D. septosporum* as triggering host and non-host cell death, some of which were also identified in *C. minus* and *P. pluvialis* (**Chapter 3**) (**Table 6.1**). Among these, Ds74283 is only present in *D. septosporum*, whilst Ds71487 is an orthologue of Cm2492 from *C. minus* and all of them trigger cell death responses in *Nicotiana* species. In addition to these, Ds131885 is an orthologue of Cm2721 from *C. minus* and Pp10632 from *P. pluvialis*, with all these orthologous proteins also triggering cell death. Ds74283 and Ds131885 were transiently expressed in *N. benthamiana*, and the expression of plant defence genes indicated that both proteins activate genes involved in pathogen-associated molecular pattern-triggered immunity (PTI) and the hypersensitive response (HR). For this reason, further experiments mentioned above for Ecp20 family proteins should be performed to further characterize the nature of the cell death triggered by these proteins.

Table 6.1. Summary of key candidate effector proteins identified in *Dothistroma septosporum* and whether they are present as homologues in *Cyclaneusma minus* and *Phytophthora pluvialis*.

Ds protein ^a	AlphaFold prediction ^b	Present in Cm? ^c	Present in Pp? ^d	Cell-death eliciting activity ^e
DsEcp20 family	Alt a 1-like fold	Not at the primary sequence level	Not at the primary sequence level	Yes
DsEcp32 family	β -trefoil-like fold	CmEcp32 family	Not at the primary sequence level – but β -trefoil-like fold was identified in some <i>P. pluvialis</i> predicted proteins	Yes
Ds69113		Not at the primary sequence level		No
Ds71487		Cm2492		Yes
Ds74283*		Not at the primary sequence level		Yes
Ds69335*	CAP/Pry-1-like	Cm8840	Pp7927	No
Ds131885*	AMA4-like fold	Cm2721	Pp10632	Yes

^a *Dothistroma septosporum* NZE10 (Ds) JGI protein ID. Protein IDs of the DsEcp20 and DsEcp32 family are shown in **Chapter 2**.

^b Ds candidate protein structural predictions were made using AlphaFold2 (Jumper et al., 2021) and the predicted structures were queried against those in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using the protein structure comparison server, Dali (Holm, 2020).

^c Ds Basic Local Alignment Search Tool for protein (BLASTp) searches against the predicted proteins of *Cyclaneusma minus* NZFS809 (Cm) (E-value < 10⁻⁵).

^d Ds BLASTp searches against the predicted proteins of *Phytophthora pluvialis* NZFS3000 (Pp) (E-value < 10⁻⁵).

^e Ds proteins that were expressed in *Nicotiana benthamiana* and *Nicotiana tabacum* using *Agrobacterium tumefaciens*-mediated transient expression to assess their ability to elicit cell death (see **Chapter 3**).

* Indicates that the gene coding for the respective protein was disrupted through CRISPR/Cas9 technology and Ds transformants were tested for virulence in *Pinus radiata* (see **Chapter 4**).

It is important to note that the CE prediction was initially carried out using the genomic/transcriptomic information of *D. septosporum*. However, focusing firstly on the other two pine pathogens instead might have also provided additional insights into new CEs not identified previously. Generating transcriptomic information for *C. minus* and *P. pluvialis* strains infecting *Pn. radiata* should also be performed to determine at which stage of the infection process the shared CE genes identified are being expressed in these pathogens.

Not all CEs transiently expressed in *N. benthamiana* or *N. tabacum* elicited cell death responses. However, they are still valuable virulence factor candidates. They could be suppressing plant defence responses, which could be tested by treating *Nicotiana* plants with the CE and determining whether they enhance susceptibility to pathogens of these non-host plants. These CEs could also be overexpressed or disrupted in the pine pathogens to assess whether their virulence on *Pn. radiata* is affected, which could be quantified by lesion size and number, and fungal biomass. Another reason that no cell death response was observed might be because these CEs are not recognized by any *Nicotiana* immune receptors, either directly, or indirectly via another host target, due to the evolutionary difference between angiosperms (*Nicotiana* species) and gymnosperms (pine). However, *N. benthamiana* is an alternative host to many *Phytophthora* species, so determining if this is also the case for *P. pluvialis* might help further understand molecular interactions between *P. pluvialis* and an (alternative) host. There are many approaches to studying interactions that would be more easily performed in an angiosperm host than in a gymnosperm like pine, including the generation of additional *in planta* transcriptomic data, virulence assays, TRV VIGS and protein interactions (e.g., yeast two-hybrid experiments to determine host targets). Common CEs between these three pine pathogens should be prioritized in such studies.

In conclusion, it was possible to identify a set of CEs from the three pine pathogens, with some triggering host and non-host cell death. Notably, the host and non-host screening of the CE proteins was done in isolation (i.e., in the absence of the rest of the pathogen's effector repertoire), which could also have implications on the cell death (or

lack of) observed here. Nevertheless, homologues of the identified CEs in the three pine pathogens are also present in other fungal and oomycete species, highlighting that some plant pathogens might employ similar molecular strategies to initiate infection, all of which contributes to further understanding forest tree-pathogen interactions.

6.2 β -trefoil and other structurally similar proteins from *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

At first, comparisons between proteins from *D. septosporum*, *C. minus* and *P. pluvialis* were performed at the primary sequence level. The advent of tertiary structure prediction tools, such as AlphaFold2, and databases to search for tertiary structure similarities, such as Foldseek, makes it possible to identify sequence-unrelated but structurally similar proteins secreted by a specific pathogen or even proteins that are shared between different pathogens.

A β -trefoil fold was predicted to be adopted by six proteins from the 29 *D. septosporum* CEs, all of them with no obvious amino acid sequence similarity. The structures of CEs Ds69113 and Ds74283 (present only in *D. septosporum*), Ds71487 and orthologue Cm2492 from *C. minus*, as well as all Ds/Cm/FfEcp32 family members (**Table 6.2**), are similar to β -trefoil proteins, and all of these proteins, apart from Ds69113, triggered cell death responses in non-host plants. Using the Foldseek server, a database search found proteins with predicted tertiary structures from *Phytophthora* species that might also adopt this fold. These identified structures were then used to search among all predicted *P. pluvialis* proteins, based on amino acid similarity. Two *P. pluvialis* proteins were shown to adopt a β -trefoil fold, although they were not tested for cell death activity. However, these results suggest a common fold that might be important for *D. septosporum*, *C. minus* and *P. pluvialis* virulence, being shared between fungi and oomycetes (**Table 6.2**).

It remains to be determined if the β -trefoil fold is present in other secreted proteins from *D. septosporum*, *C. minus* and *P. pluvialis*, as well as in other forest pathogens. Such knowledge is needed to reveal how widespread this fold actually is in these pathogens. All the β -trefoil proteins identified in this thesis have homologues in other fungal species (**Chapter 2** and **Chapter 3**) with different lifestyles, ranging from saprophytes, to

biotrophs, hemibiotrophs and necrotrophs. This suggests that the β -trefoil fold is common among fungal effectors. However, additional work is needed to determine if these other fungi actually secrete effectors that are structurally similar to β -trefoil proteins. In a recent study, both non-pathogenic and pathogenic fungi were shown to share structurally similar CE families (Seong & Krasileva, 2022). Comparative studies between β -trefoil proteins from pathogenic and non-pathogenic species might help answer the question of why some of these proteins trigger cell death responses while others do not. This could be achieved by identifying amino acid sequence and, more importantly, tertiary structure differences between the identified proteins that adopt a β -trefoil fold, not only from the three pine pathogens, but from other fungal and even pathogenic and non-pathogenic oomycete species.

Table 6.2. Summary of β -trefoil proteins identified in *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*.

CE protein ^a	β -trefoil superfamily ^b	Thesis Section
<i>D. septosporum</i>		
Ds69113	Trypsin inhibitor	Section 3.3.3.3 of Chapter 3
Ds71487*	Trypsin inhibitor	Section 3.3.3.3 of Chapter 3
Ds74283	30K lipoprotein C-terminal domain-like	Section 3.3.3.3 of Chapter 3
DsEcp32 family	Trypsin inhibitor	Section 2.3.2 of Chapter 3
<i>C. minus</i>		
Cm2492*	Trypsin inhibitor	Section 3.3.3.3 of Chapter 3
CmEcp32 family	Trypsin inhibitor	Section 5.3.4.1.2 of Chapter 5
<i>P. pluvialis</i>		
Pp7483	30K lipoprotein C-terminal domain-like	Section 3.3.3.3 of Chapter 3
Pp12323	Ricin B-like lectin	Section 3.3.3.3 of Chapter 3

^a *Dothistroma septosporum* NZE10 (Ds) JGI protein ID; *Cyclaneusma minus* NZFS809 (Cm) ID (McDougal, unpublished); *Phytophthora pluvialis* NZFS3000 (Pp) ID (Studholme et al., 2016). Protein IDs of the DsEcp32 and CmEcp32 family are shown in **Chapter 2** and **Chapter 5**, respectively. The asterisk (*) indicates orthologous proteins.

^b Ds, Cm and Pp candidate effector (CE) protein structural predictions were made using AlphaFold2 (Jumper et al., 2021) and the predicted structure was queried against those in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using the protein structure comparison server, Dali (Holm, 2020). The first hit in PDB was used to classify the structures according to the Structure Classification of Proteins (SCOP) website (<https://scop.mrc-lmb.cam.ac.uk/>).

Interesting, all of the fungal β -trefoil proteins identified from *D. septosporum* and *C. minus* have a conserved disulphide bond in their predicted tertiary structures (**Chapter 3**). This disulphide bond was not present in the two β -trefoil proteins from *P. pluvialis*. Again, additional sampling should be performed to determine if this conserved disulphide bond is a feature present only in fungal species or is also present in β -trefoil

proteins from other oomycetes as well, as it may provide further support for an ancestral origin.

All of the *D. septosporum* β -trefoil proteins identified in this thesis are highly expressed at the Late (necrotrophic) stage of *D. septosporum* infection of *Pn. radiata*. They potentially act as necrotrophic effectors, are involved in the necrotrophic switch, or could be involved in sporulation or eruption through the pine cell wall. Deletion or disruption of the genes that encode β -trefoil proteins, using CRISPR/Cas9 technology, could help determine what impact they have on disease progression by this fungus. As mentioned above, Ds74283 is predicted to adopt a β -trefoil fold and it was one of the CEs for which the corresponding gene was disrupted using CRISPR/Cas9. Disruption of Ds74283 led to a significant decrease in *D. septosporum* biomass following infection of *Pn. radiata* when compared to the wild-type (WT) fungus. However, this decrease in fungal biomass was also observed in the complementation strain, so no clear conclusion could be made. Despite this, Ds74283 remains an important candidate virulence factor of *D. septosporum*, due to its gene high expression and predicted tertiary structure.

Another approach, based on gene expression, would be to determine if any types of protein folds are over-represented in any of three infection stages of *D. septosporum* on pine. Based on previous studies (Bradshaw et al., 2016; Kabir et al., 2015), the infection process of *D. septosporum* in *Pn. radiata* was divided into Early (biotrophic), Mid (early necrotrophic) and Late (sporulation). It would be possible to identify whether over-represented proteins secreted in each one of these stages are part of any expanded families with similar folds. This, in turn, would enable the identification of effectors involved in the biotrophic stage that are likely involved in suppressing the host defence responses, or those involved during the necrotrophic switch. For example, *Magnaporthe oryzae* secretes structurally similar effectors that are co-regulated during the biotrophic phase, providing new information on the range of effectors secreted by this pathogen at this specific infection stage (Yan et al., 2022).

Proteins with a β -trefoil fold can have different functions during host infection. This fold is present in protease inhibitors, such as trypsin inhibitors, which could suggest that they act as plant protease inhibitors and might suppress plant defence responses. Other proteins with a β -trefoil fold, like cytolytins and some lectins, are known to interact with,

and disrupt, the host plasma membrane (Juillot et al., 2016; Schubert et al., 2012; Wohlschlager et al., 2011). Ds69113, Ds71487 (and its orthologue from *C. minus*, Cm2492) and all Ds/Cm/FfEcp32 family members are structurally similar to protease inhibitors (**Table 6.2**). The structures of Ds74283 and Pp7483 are more similar to β -trefoil proteins belonging to the 30K protein family, while Pp12323 was predicted to belong to the ricin B-like lectin superfamily. It remains to be determined the exact roles these proteins have during pine infection.

If indeed the β -trefoil fold is common amongst effectors of fungal and oomycete species, it would be interesting to determine the actual biological function and/or host targets of these proteins, and if this function/target is conserved between all of them. Some proteins with conserved structural folds display different functions and, therefore, might not have a shared function (e.g., members of the ToxA family (Sarma et al., 2005)), and the structural conservation is simply providing a template for diversification of functions. This could likely be the case with the β -trefoil proteins identified here. Based on the functions of other characterised β -trefoil proteins, some could be acting as protease inhibitors, and help the pathogen to escape host recognition, acting during the biotrophic phase of these pathogens. Others, perhaps, may have a toxic effect by interacting with and disrupting the host membrane and might help the pathogen during the necrotrophic phase, which could explain the cell death responses observed in host and non-host plants (in both susceptible and tolerant pine genotypes used). There is also the possibility that these proteins have toxicity to other microorganisms found co-existing in *Pn. radiata* needles, and that the host cell death is simply the consequence of the infiltration of these proteins in high concentrations in host and non-host plants.

Overall, these results support the idea that many sequence-unrelated effector proteins share conserved folds, likely evolving from common ancestral proteins (de Guillen et al., 2015). Sequence diversification can lead to amino acid polymorphisms, while maintaining the overall structure, which is known as divergent evolution. Because of the constant arms-race between pathogens and plants, it is not surprising that pathogens would have large structurally similar families of virulence factors; a recognized effector of such family could then be lost without an associated fitness cost to the

pathogen, while other sequence-related family members acquire new virulence functions or escape host recognition.

All of this highlights the importance of identifying structural similarities between proteins when sequence homology is not enough to detect them. The advent of tertiary structure prediction tools and databases to search for structure similarities will make it possible to identify similar folds present in proteins secreted by evolutionarily different pathogens that might have similar virulence functions in the host. The results obtained in this thesis add to the growing evidence that a pathogen's effector repertoire might be finite, with the secretion of sequence-unrelated but structurally similar proteins. Considering everything that was discussed previously, structural predictions and comparisons between such structures, using tools like AlphaFold2 and Foldseek, should be incorporated into effector prediction pipelines in order to provide more insights into effector function.

6.3 Shared candidate effectors between *Dothistroma septosporum*, *Cyclaneusma minus* and *Phytophthora pluvialis*

Based on the amino acid sequence similarity between proteins of *D. septosporum*, *C. minus* and *P. pluvialis*, it was possible to identify two sets of CEs conserved between these three pine pathogens.

One set of conserved CEs, Ds69335, Cm8840 and Pp7927, has predicted structural similarity with 'cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1' (CAP) proteins and, more specifically, the pathogen-related yeast 1 (Pry1) protein from *Saccharomyces cerevisiae* (Darwiche et al., 2016). Many CAP proteins have been shown to bind to small hydrophobic molecules, and some have roles in suppressing host defence responses. CcCAP1 from the plant pathogen *Cytospora chrysosperma* localizes to the plant nucleus and is believed to modulate plant immunity (Han et al., 2021). Others were shown to have a dual role, as was recently demonstrated for a CAP protein from *Ustilago maydis*. UmPR-1La was shown to bind to phenolic compounds, protect the fungal hyphae and also suppress plant defence responses (Ma et al., 2022).

With that in mind, along with the fact that none of the orthologues in the three pine pathogens triggered any visible cell death responses in non-host plants and that

Ds69335 is highly up-regulated at the beginning of *D. septosporum*-pine infection, *Ds69335* disrupted strains were created and inoculated onto *Pn. radiata*. Gene disruption lead to a significant decrease in fungal biomass when compared to the WT fungus, but because the complementation strain did not seem to restore virulence, no clear conclusion could be made regarding the role of *Ds69335* in virulence.

Ds69335 could be functionally redundant, as shown for CAP proteins from *Candida albicans*. Deletion of one or other of the two CAP-encoding genes from this pathogen resulted in moderate attenuation of disease, while the virulence of the double mutant was dramatically reduced (Röhm et al., 2013). Nevertheless, *Ds69335* (or its orthologues) should be co-infiltrated with cell death elicitors in non-host plants to determine any cell death-suppressing activity by these proteins, and other CAP proteins secreted by *D. septosporum*.

Alternatively, since CAP proteins are also found in many other non-pathogenic species (Schneider & Di Pietro, 2013), they might also have functions not related to virulence/pathogenicity. For instance, the pine needle pathogens might secrete CAP proteins to ward off competing microorganisms. Because CAP proteins are also involved in sequestering sterols from the membranes of other microbes (Choudhary & Schneider, 2012), they could provide a defence mechanism for *D. septosporum* and *C. minus* against sterol auxotrophs such as *P. pluvialis* and other *Phytophthora* species. Future work could include *in vitro* binding assays of the CAP proteins identified in this thesis with hydrophobic molecules, including sterols. Also, co-cultivation of the *D. septosporum* and WT *Ds69335*-disrupted strains, to determine any impact on the growth of *P. pluvialis*, and possibly also *C. minus*, could also be assessed. Another possibility would be to treat these three pine pathogens, and other pathogens associated with pines, with purified CAP proteins (either *Ds69335*, *Cm8840* or *Pp79274*) to determine if any of them inhibit pathogen growth.

Ds69335, *Cm8840* and *Pp7927* were predicted to have large intrinsically disordered regions (IDRs). The IDRs of some bacteria and oomycete effectors can affect virulence levels or help the pathogen escape host recognition. For example, the IDR of AVR2 from the potato pathogen *Phytophthora infestans* confers a more virulent phenotype compared to AVR2 that do not have this disordered region (Yang et al., 2020). It remains

to be determined how these regions might impact on the virulence of pine needle pathogens. Experiments that involve mutating the IDRs, or specific disorder-promoting residues with the IDRs, in attempts to convert the disordered structure into an ordered structure, could be performed to assess any differences in virulence or host target interactions.

The second set of conserved proteins identified between the three pine pathogens in this study, Ds131885, Cm2721 and Pp10632, triggered strong and consistent cell death responses in non-host plants. In the case of Ds131885 in *N. benthamiana* in which gene expression was studied, these responses appeared to be the result of plant immune system activation. Disruption of *Ds131885* did not affect the virulence of *D. septosporum* on *Pn. radiata*, similar to that observed when deleting *VmE02*, a gene encoding the *Valsa mali* orthologue of these proteins (Nie et al., 2019).

VmE02-triggered cell death requires the *N. benthamiana* immune receptor RE02 (Nie et al., 2019; Nie et al., 2020). Due to their strong primary sequence and predicted structural similarity, it is very likely that the orthologous proteins from the three pine pathogens might also be recognized by this same receptor, so future work should test these proteins in *RE02*-silenced plants to determine if they interact with the receptor *in vitro*. RE02 is the best BLASTp hit of PsRLK, a characterized receptor from *Pinus sylvestris* (Ávila et al., 2006), suggesting the *Pinus* genus might also encode a receptor that could potentially recognize Ds131885, Cm2721 and Pp10632. The gene encoding this receptor could then be identified in *Pn. radiata* as a possible *R* gene for use in engineering disease resistance, provided the pathogens do not use this receptor to switch to, or enhance, necrotrophy. Alternatively, if recognition by this receptor facilitates pathogen infection, it will be considered a susceptibility (*S*) gene. Mutation or deletion of *S* genes from the host can limit the ability of the pathogen to trigger disease, something that would also be beneficial in breeding programmes (van Schie & Takken, 2014). This could be determined by deletion of either the host receptor gene or the pathogen effector gene.

6.4 Screening of candidate effector proteins in the host *Pinus radiata*

Expressing CE proteins in non-host plants is an established way to study their function. However, development of a method to screen these proteins in the host

Pn. radiata provides a more reliable understanding of the interactions occurring between pine pathogens and the host. The yeast *Pichia pastoris* was chosen to heterologously express the CEs identified in this thesis, and, after purification, these proteins were vacuum-infiltrated into clonal *Pn. radiata* shoots grown via tissue culture. Two pine genotypes that are relatively susceptible and one that is relatively tolerant to *D. septosporum* infection were used. Interestingly, all proteins that had previously triggered cell death responses in *Nicotiana* species also triggered cell death-like responses in pine. In the future, this cell death response should also be further characterized as mentioned above, with ROS measurements and analysis of the expression of pine defence genes following infiltration with the CE. Screening a wide range of pine genotypes might also help to find those differing in response to CE proteins. Notably, the cell death responses might be due to a dose-dependent toxicity rather than recognition by the host, which could be determined by a series of serial dilutions prior to protein infiltration.

Further optimization of the method might also provide more reliable results when analysing the differences observed between genotypes. For example, by quantifying the volume of purified protein solution that is actually being infiltrated into pine shoots; this could be performed by including a fluorescent dye whose uptake could be monitored and quantified. It is important to note, however, that these shoots, although clonal, are not identical with respect to size/weight and numbers of needles, all of which could also affect the response analysis. Other forms of protein delivery into pine, such as *Agrobacterium tumefaciens* mediated-transient transformation assays (ATTAs), could also be a good alternative for testing CEs. There are studies showing that this method works for trees, such as poplar (Zheng et al., 2012), *Pinus elliottii* (slash pine) (Tang et al., 2014) and *Pn. radiata* (Grant et al., 2015), even though agrotransformation of trees is a slow and challenging process.

Nevertheless, testing pine genotypes with CE proteins might serve as a first attempt to screen for possible avirulence factors and the host targets of these proteins. Once avirulence factors are identified, they could then serve as candidates for functional analysis in the same way as was done for the gene deletion/disruption via CRISPR/Cas9 of the three *D. septosporum* CE genes *Ds69335*, *Ds74283* and *Ds131885*. Perhaps

generating *D. septosporum* strains overexpressing the CE genes and inoculating these, as well as gene disruption strains in the same genetically identical pine tissue culture shoots used in this thesis, might provide an alternative to functionally characterize the proteins encoded by these genes. Attempts to establish CRISPR/Cas9 gene editing protocols in *C. minus* and *P. pluvialis* should also be performed.

6.5 Concluding remarks and future outlook

This thesis continued the previous work by Hunziker (2018), in which *D. septosporum* CEs were identified and characterized in non-host plants. That work determined that Ds70057 (DsEcp32-3), Ds70694 (DsEcp20-3), Ds71487 and Ds131885 had the ability to trigger cell death responses in *N. benthamiana* and *N. tabacum*, while Ds69335 did not trigger any visible plant response. In this thesis, it was possible to further characterize Ds70057 and Ds70694 and show that both proteins belong to two different families, Ecp32 and Ecp20 respectively, and that both have members that differ in their ability to trigger cell death. Both families are also present in *F. fulva*, the closest species to *D. septosporum*. Also, the Ecp32 family was shown to be present in *C. minus* and, as found in *D. septosporum* and *F. fulva*, members differed in their ability to trigger cell death responses. It was also possible to identify putative *N. benthamiana* receptors that might be involved in the recognition of some of these proteins. Moreover, it was possible to optimize the pine vacuum infiltration method first trialled by Hunziker (2018) to screen CE proteins in the host to try to identify those that are potentially recognized by the plant.

Following on from the work done for Ds131885 and Ds69335, this thesis showed that orthologues of both proteins are also present in the other pine pathogens, *C. minus* and *P. pluvialis*, and that the orthologues of Ds131885 also have the ability to trigger cell death responses. To further characterize these proteins during *D. septosporum* infection of *Pn. radiata*, Ds13885, Ds69335, along with Ds74283 (a newly identified non-host cell death elicitor in this thesis) were disrupted by CRISPR/Cas9, which was also established in *D. septosporum* in this thesis.

As mentioned above, Ds69335- and Ds74283-disrupted strains showed a decrease in *D. septosporum* biomass *in planta* compared to the WT, while disruption of Ds131885

did not convincingly alter the levels of fungal biomass. However, all complementation strains also differed from the WT in the amount of biomass, and no differences were observed between disrupted and complementation strains. These differences could be due to problems in the target gene complementation process, as outlined in **Chapter 4**, in which the Cas9 might have integrated into the fungal genome, inactivating the inserted copy of the *CE* gene. Despite these ambiguous results, these genes are still good candidate virulence factors that should be explored further. Due to the combination of information about their expression *in planta*, and the information generated based on protein tertiary structure predictions, some hypotheses can be made regarding their possible roles during pine infection.

Ds69335 is speculated to help the fungus during its biotrophic phase, either by detoxification of plant or other microbial compounds. The β -trefoil structure of Ds74283 might make the protein functionally redundant, due to *D. septosporum* secreting other structurally similar proteins. Alternatively, it could have roles in host plasma membrane disruption, based on late expression of the gene during fungal infection of pine. As for Ds131885, the host and non-host cell death triggered by this protein could be due to the pathogen exploiting a host *S* gene, highlighted by the late expression of the gene that codes for this protein. Identification and elimination of *S* genes can undermine the ability of a necrotrophic pathogen to successfully colonize its host. It remains to be determined if the *N. benthamiana* receptor identified as recognizing an orthologue of Ds131885, Cm2721 and Pp10632 might be an *S* gene that the pathogen uses for its own benefit. If identified in pine, disruption of this *S* gene, or screening for pines lacking this gene, could confer durable and broad-spectrum disease resistance, a technique that has been employed in many different plants (Zaidi et al., 2018).

Perhaps one of the most compelling results here is the identification of what seems to be a common fold between the three pine pathogens, with the indication that this fold is also present in many other microorganisms. Many proteins secreted by *D. septosporum*, *C. minus*, *F. fulva*, and likely *P. pluvialis*, were shown to be sequence-unrelated, but to adopt the same β -trefoil fold. This discovery was only possible because of advances in tertiary structure prediction tools, such as AlphaFold2, suggesting that these highly diverse pathogens might be more similar than previously thought when

infecting the host. Identifying common structural folds shared between these pine pathogens can help to better understand the mechanisms they employ to successfully infect the host. Host defences based on recognition of these shared effectors are more likely to be durable as there would be a fitness penalty associated with their mutation or loss. Thus, analysis of existing and new potential shared CEs between these three pine pathogens can lead to identification of *Pn. radiata* genes that could be used for breeding for disease resistance. The association of a putative pine receptor with resistance to oomycete and fungal pathogens will need to be evaluated in the forest and, then, molecular markers based on those receptors could be developed for breeding durable and broad-spectrum resistance.

In conclusion, the results obtained in this thesis provided a step towards better understanding pine-pathogens interactions, and hopefully can be used in the future to generate new and improved control strategies not only against *D. septosporum*, *C. minus* and *P. pluvialis*, but also against other conifer pathogens.

Appendix

Table A.1. Microorganisms and plants used in this thesis.

Organism and strain/isolate/accession	Characteristics ^a	Reference
<i>Dothistroma septosporum</i>		
NZE10	<i>D. septosporum</i> wild-type (ICMP 24376)	de Wit et al. (2012)
FJT209	NZE10 <i>Ds69335</i> T2-D; Gen ^R	This study
FJT210	NZE10 <i>Ds69335</i> T40-D; Gen ^R	This study
FJT211	NZE10 <i>Ds74283</i> T3-D; Gen ^R	This study
FJT212	NZE10 <i>Ds74283</i> T56-D; Gen ^R	This study
FJT213	NZE10 <i>Ds131885</i> T9-D; Gen ^R	This study
FJT214	NZE10 <i>Ds131885</i> T47-D; Gen ^R	This study
FJT215	FJT209 complemented <i>Ds69335</i> ; Gen ^R , Phleo ^R	This study
FJT216	FJT211 complemented <i>Ds74283</i> ; Gen ^R , Phleo ^R	This study
FJT217	FJT213 complemented <i>Ds131885</i> ; Gen ^R , Phleo ^R	This study
<i>Dothistroma pini</i>		
CBS 116487	<i>D. pini</i> wild-type	BioSample ID SAMN02254964
<i>Cyclaneusma minus</i>		
NZFS809	<i>C. minus</i> wild-type	Hunter et al., 2016
<i>Phytophthora pluvialis</i>		
NZFS3000	<i>P. pluvialis</i> wild-type	Studholme et al. (2016)
<i>Fulvia fulva</i>		
OWU	<i>F. fulva</i> wild-type	de Wit et al. (2012)
<i>Pichia pastoris</i>		
GS115	Histidine-auxotrophic strain; enables selection on histidine deficient media	Invitrogen
<i>Escherichia coli</i>		
DH5α	<i>F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ-</i>	Taylor et al. (1993)
<i>Agrobacterium tumefaciens</i>		
GV3101	Disarmed strain carrying pMP90 (pTiC58) helper plasmid; C58C1; Rif ^R , Gent ^R	Hellens et al. (2000)
<i>Nicotiana benthamiana</i>		
	Wild-type	Dr. K. Sohn
<i>Nicotiana tabacum</i>		
Wisconsin 38	Wild-type	Dr. K. Sohn
<i>Pinus radiata</i>		
S6 (clonal shoot)	Wild-type relatively susceptible to DNB	Scion
S11 (clonal shoot)	Wild-type relatively susceptible to DNB	Scion
R4 (clonal shoot)	Wild-type relatively tolerant to DNB	Scion
Seedlings	Wild-type from a cross between two parents with similarly low levels of DNB resistance	Radiata Pine Breeding Company

^a Antibiotic resistance (R). Gen: geneticin; Phleo: phleomycin; Rif: rifampicin; Gent: gentamycin. DNB: *Dothistroma* needle blight.

Table A.2. Primers used in this thesis.

Primer name	Sequence 5' – 3'
Chapter 2	
Golden Gate pICH86988 cloning	
FfEcp20-2 fw	GGTCTCGCAAGGTACCATCGGAGCTAGCCGGGCCGA
FfEcp20-2 rv	GGTCTCGAAGCCTAGGCGACACCTGTGAGCCGGTCTC
DsEcp20-1 fw	GGTCTCGCAAGGCAGCAATCGAGGCCCG
DsEcp20-1 rv	GGTCTCGAAGCTTAGAGAACAGCTGAGGAGAGCTT
DsEcp20-2 fw	GGTCTCGCAAGGCCCGCTCAGAACTAGCC
DsEcp20-2 rv	GGTCTCGAAGCCTAGACGACACGTGTCGG
DsEcp20-3 fw	GGTCTCGCAAGCTCCCCGCCGGCGTC
DsEcp20-3 rv	GGTCTCGAAGCTAAGAGCTCTTGACTTGGTACC
DsEcp20-4 fw	GGTCTCGCAAGGCACCGCCGCCGA
DsEcp20-4 rv	GGTCTCGAAGCTATGATGGGAGTTCTGTGGAGT
FfEcp32-1 fw	GGTCTCGCAAGGCAGCAACCCAGGAGC
FfEcp32-1 rv	GGTCTCGAAGCCTAGTTGTAAGTCCATGCGC
FfEcp32-2 fw	GGTCTCGCAAGGGCACAACCTGAACCACTCC
FfEcp32-2 rv	GGTCTCGAAGCCTAGACGTACTGGTATGCCTGG
FfEcp32-3 fw	GGTCTCGCAAGTCCCCAGTTCCCAAGC
FfEcp32-3 rv	GGTCTCGAAGCTTACTGGTACTGCCAAGCCG
FfEcp32-4 fw	GGTCTCGCAAGCAACGTTCTGTCTACCTCATGGC
FfEcp32-4 rv	GGTCTCGAAGCCTACGAATACTCGAAAGCGCCC
FfEcp32-5 fw	GGTCTCGCAAGCTGCCATCTCTCAAGGGCG
FfEcp32-5 rv	GGTCTCGAAGCTTACACGTATTGCCAAGCAGATACA
DsEcp32-1 fw	GGTCTCGCAAGGCAGCAACTCCAGAAGCC
DsEcp32-1 rv	GGTCTCGAAGCTTAGTTGCTGTACTGCCATGC
DsEcp32-3 fw	GGTCTCGCAAGTCTCCAGTCCCCAAACCG
DsEcp32-3 rv	GGTCTCGAAGTACTCATACTGATAAGCACTTTCCG
DsEcp32-4 fw	GGTCTCGCAAGCAACGCTCTTCTACCTGCTC
DsEcp32-4 rv	GGTCTCGAAGCCTATGAATACTCGAAAGCTCCTGGT
DsEcp32-5 fw	GGTCTCGCAAGCTTCCATCCCTCAAGACCCGT
DsEcp32-5 rv	GGTCTCGAAGCTTACTCGTACTGCCAAGCAGG
GUA2 DsEcp20-3 fw	GGTCTCGCAAGCTCCCCGCCGGCGTC
GUA2 DsEcp20-3 rv	GGTCTCGAAGCCTAAGAATTCTTGACTTCGTACCTGAAAT
COLN DsEcp32-1 fw	GGTCTCGCAAGGCAGCAACTCCAGAAGCC
COLN DsEcp32-1 rv	GGTCTCGAAGCTTAGTTGCTGTACTGCCATGC
GUA2 DsEcp32-3 fw	GGTCTCGCAAGTCCCCGTTCCCAAAACCA
GUA2 DsEcp32-3 rv	GGTCTCGAAGCCTACTCATACTGATACGCACTCTCC
DpEcp20-3 fw	GGTCTCGCAAGCTCCCCGCCGGCGTC
DpEcp20-3 rv	GGTCTCGAAGCCTACGAGCTCTTGACTTAGTACCC
DpEcp32-1 fw	GGTCTCGCAAGGCAGCGACTCCAGAAGCCG
DpEcp32-1 rv	GGTCTCGAAGCTTAGCTGCTGTACTGCCATGC
DpEcp32-3 fw	GGTCTCGCAAGTCTCCAGTCCCCAAGCCGA
DpEcp32-3 rv	GGTCTCGAAGCCTACTGGTACTGGTACGCACTTTCGT
pPic9-His₆ cloning	
BcSSP fw	CCCGGGCCCGACTACAAGGACGACGATGACAAGTGACCTATTGCCAGTGTGAGT
BcSSP rv	GGGAATTCCTATTGCTTATAACAAGGTGTTCTATCAAG
DsEcp20-3 fw	CCCGGGCCCGACTACAAGGACGACGATGACAAGTCCCCGCCGGCGTC
DsEcp20-3 rv	GGGAATTCCTAAGAGCTCTTGACTTGGTACCTG
DsEcp20-4 fw	CCCGGGCCCGACTACAAGGACGACGATGACAAGGCACCGGCCGCGG
DsEcp20-4 rv	GGGAATTCCTATGATGGGAGTTCTGTGGAG
DsEcp32-3 fw	CCCGGGCCCGACTACAAGGACGACGATGACAAGTCTCCAGTCCCCAAACCG
DsEcp32-3 rv	GGGAATTCCTACTCATACTGATAAGCACTTTCCGTC
Sequencing	
pICH86988 fw	AGGACACGCTCGAGTATAAG
pICH86988 rv	CATGCGATCATAGGCTTCTC
AOX1 fw	GACTGGTTCGAATTGACAAGC
AOX1 rv	GCAAATGGCATTCTGACATCC
TRV-VIGS constructs	
TRV:T26 fw	CGACAAGACCCTGCACACAGGGGTAGTTGGAACGA

TRV:T26 rv	GAGAAGAGCCCTGCATCAATGACAATCCACTAGCTCC
TRV:T26-2 fw	CGACAAGACCCTGCACTGCCCTATCTGCACTAGAT
TRV:T26-2 rv	GAGAAGAGCCCTGCAAGGATATGGACCTGATACCG
TRV:BAK1 fw	CGACAAGACCCTGCAAGCTCATACTGGGCAACGG
TRV:BAK1 rv	GAGAAGAGCCCTGCAGTGAGGGTGGTGAGCAGGAT
TRV:SOBIR1 fw	CGACAAGACCCTGCAAATCTTTATCCACCAGATC
TRV:SOBIR1 rv	GAGAAGAGCCCTGCACAGAAAGTTTCCAATGGC
RT-qPCR	
Niben101Scf03138g01049.1 rv	GTTATACCTCCAACAATTCAGCTG
Niben101Scf03138g01049.1 fw	CATTCCCCTTTAGGTCCAGATA
Niben101Scf01834g02009.1 fw	CCTTACATTTCTTAGGTCGCTTG
Niben101Scf01834g02009.1 rv	GGACTGAATAATCGACCATTGCT
qTRV:BAK1 fw	ATTGCTGGAGGAGTTGCTGCCGG
qTRV:BAK1 rv	CCACGTACAGCAGTGGTAACAT
qTRV:SOBIR1 fw	CTTAGAAAACTCTCTTTAGC
qTRV:SOBIR1 rv	TATGGATTGGAGTGACATTATG
Chapter 3	
Golden Gate pICH86988 cloning	
Ds19040 fw	GGTCTCGCAAGGCAGTCTTGCTAGAGCCAA
Ds19040 rv	GGTCTCGAAGCTCAGTCGAACCAGGAGGA
Ds24695 fw	GGTCTCGCAAGTCGCCCTCCTGCCCC
Ds24695 rv	GGTCTCGAAGCTCATGTACAATATACTCCACAAATCTCCC
Ds42387 fw	GGTCTCGCAAGGGCCTGCTCTTCTCCGC
Ds42387 rv	GGTCTCGAAGCTTATTGGTACTCTTTCGAAGGCGC
Ds46236 fw	GGTCTCGCAAGTTCGTTCTCCACGTAAGTATGATGACCCGGATTGTGGT
Ds46236 rv	GGTCTCGAAGCTACGCCACAGCAGTAATGTTTAT
Ds67456 fw	GGTCTCGCAAGAAAGACGTCGGTATACCGTGGC
Ds67456 rv	GGTCTCGAAGCTTAATACCCATCTCCCTTCTCCAC
Ds6745 BsaI fw	AGACAGTTGGTCACAACTCTTCCC
Ds6745 BsaI rv	GGGAAGAGTTGTGACCAACTGTCT
Ds69113 fw	GGTCTCGCAAGGCACCGGCCCAAGGCA
Ds69113 rv	GGTCTCGAAGCAATGTGCGAGTAGGTGCA
Ds69335 fw	GGTCTCGAAGTCCCGTACAACAAGGAGCAG
Ds69335 rv	GGTCTCGAAGCTACGATGAGCCGGTGTCCA
Ds69845 fw	GGTCTCGAAGGCACCATACGTCCCAAGAG
Ds69845 rv	GGTCTCGAAGCTCAAGCATAGAACCAATACTCCGC
Ds70155 fw	GGTCTCGCAAGACTACTGTCAGCTACGACGAAGG
Ds70155 rv	GGTCTCGAAGCCTATGCGTTGAATTCGATCGACC
Ds71487 fw	GGTCTCGCAAGCAGACTGTGCAAGTCCAAGCC
Ds71487 rv	GGTCTCGAAGCCTAGGCGAATCTCTCACAACC
Ds71565 fw	GGTCTCGCAAGGCACCACTCGACAGGAGACAATGCC
Ds71565 rv	GGTCTCGAAGCTTAAAGAACGCACTACTCGGAGC
Ds74283 fw	GGTCTCGCAAGTACAACAACAACACTGCCGAATAC
Ds74283 rv	GGTCTCGAAGCTCAGATGTATTGCGGAATGAGGTAC
Ds74790 fw	GGTCTCGCAAGGCGCCAGTGGCCGAC
Ds74790 rv	GGTCTCGAAGCCTACTTGGGAACGTCCTTCTTCC
Ds74875 fw	GGTCTCGAAGCGCTGCAACGTTGGTGATC
Ds74875 rv	GGTCTCGAAGCTCAATCATCAATCCTCAGCGCA
Ds75009 fw	GGTCTCGAAGACCCCAATCGCTGGCGA
Ds75009 rv	GGTCTCGAAGCTTAGAGGTCTAGCAGAGAAGAGC
Ds124808 fw	GGTCTCGCAAGTTCGTAATCATGCCCTTAATCCGC
Ds124808 rv	GGTCTCGAAGCTCACACCTCTCCACGCT
Ds129328 fw	GGTCTCGCAAGCAAACCGACTACCACGCGA
Ds129328 rv	GGTCTCGAAGCTTACGCTTTAGCAGCACCACA
Ds131088 fw	GGTCTCGCAAGCTGAGCCCAGGAACGAAGAG
Ds131088 rv	GGTCTCGAAGCTCAACGTCCAGTAGCTGGTCT
Ds131290 fw	GGTCTCGCAAGACACCAATTGCCATGCCCCG
Ds131290 rv	GGTCTCGAAGCCTAAGCCTTACACAGACCCAATA
Ds131885 fw	GGTCTCGCAAGACTTTGGACCCTGCGACC
Ds131885 rv	GGTCTCGAAGCCTATGTACAGCTAGAAGTCTCCG
Ds153323 fw	GGTCTCGAAGCTCTACATTACACGTAATGCCTGGA
Ds153323 rv	GGTCTCGAAGCTCAGCTGAATACCACCGCCT

Ds158381 fw	GGTCTCGCAAGGCAGTCCTCAGCCCGAGGTTC
Ds158381 rv	GGTCTCGAAGCTCACTCGTCGTTCTCGGGTTATACG
COLN Ds69335 fw	GGTCTCGCAAGGTCCCGTACAACAAGGAGCAGAAGCG
COLN Ds69335 rv	GGTCTCGAAGCCTACGATGAGCCGGTGTTCAGTTCTG
COLN Ds69335 intron fw	CAACGTTGAGGAGGGTGGAGGCG
COLN Ds69335 intron rv	ACCCTCCTCAACGTTGTGTGCGTAGA
Dp69335 fw	GGTCTCGAAGGTCCCGTACAACAAGGAGCAGAAGC
Dp69335 rev	GGTCTCGAAGCCTACGATGAGCCCGTGTTCAGTTC
Dp69335 intron fw	CAACACTGAGCAGGGTGGAGGTG
Dp69335 intron rev	TGCTCAGTGTGTGTGCGTAGACG
Pp7927 fw	GGTCTCGCAAGGACAGCTTCACCGCCGAG
Pp7927 rv	GGTCTCGAAGCTTAAATGATCCGGATGCCGCC
Pp10632 fw	GGTCTCGCAAGACACTCGACCCGGCCAGC
Pp10632 rev	GGTCTCGAAGCTTACACGCAGCTGCTGCTGTCG
Pp10632 intron fw	TGGAGTGGCAACGGCACCGA
Pp10632 intron rv	TGCCACTCCAGAAGAACGTC
Cm835 fw	GGTCTCGCAAGTCTCCCATCTTCAAGCGCG
Cm835 rv	GGTCTCGAAGCTTACTCATACTGGAACGCCCC
Cm2492 fw	GGTCTCGCAAGCAGTACGACACCCAATCTCCCTCCTTCTA
Cm2492 rv	GGTCTCGAAGCTCAGACGAACACTCGATGCAAGTCCAC
Cm2721 fw	GGTCTCGCAAGAATGTAGACTCATGCTCCTCCA
Cm2721 rv	GGTCTCGAAGCTCAAGTGCAGCTGTCTCG
Cf189800 fw	GGTCTCGCAAGTACAACAACACGACGGCAGAA
Cf189800 rev	GGTCTCGAAGCTCAGATGTACTGCGGCATGAGGTAC
Cf192236 fw	GGTCTCGCAAGGTGCCCTACAACCAGGAGC
Cf192236 rev	GGTCTCGAAGCCTACGACGAGCCCGTGTC
Vi17736 fw	GGTCTCGCAAGCTTCCCTCTCCACAAGCACCA
Vi17736 rev	GGTCTCGAAGCTCAAGAAGAACCACTCGCCGAA
Vi23981 fw	GGTCTCGCAAGCTGCCAGCAGCAGAACC
Vi23981 rev	GGTCTCGAAGCCTATTCTGTGTCGATTGGCAGT
Vi13495 fw	GGTCTCGCAAGACCCTCTACCAGCAACCTC
Vi13495 rev	GGTCTCGAAGCCTACACGCAATCATCCCTCCC
pPic9-His₆ cloning	
Ds69335 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGGTCCCGTACAACAAGGAGCAGAAGCGC
Ds69335 rv	GAATTCCTACGATGAGCCGGTGTTCGAATTCGCG
Ds71487 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGCAGACTGTGCAGTCCAAGCCTTTC
Ds71487 rv	GGGAATTCTTAGGCGAACTTCTCTACAACCTCG
Ds74283 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGTACAACAACAACACTGCCGAATACA
Ds74283 rv	GGGAATTCTCAGATGTATTGCGGAATGAGGTAC
Ds131885 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGACTTTGGACCTTGCGACCTC
Ds131885 rv	GGGAATTCTTATGTACAGCTAGAACTTCCCGCTAT
Ds158381 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGGCAGTCTCAGCCCGA
Ds158381 rv	GGGAATTCTCACTCGTCGTTCTCTCGG
Pp7927 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGGACAGCTTCACCGCCGAGGACCAG
Pp7927 rv	GGGAATTCTTAAATGATCCGGATGCCGCCGTCTCGC
Pp10632 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGACACTCGACCCGGCCAGCTCCAAC
Pp10632 rv	GGGAATTCTTACACGCAGCTGCTGCTGTCGGCG
Cm2721 fw	CCCGGGCCCCGACTACAAGGACGACGATGACAAGAATGTAGACTCATGCTCCTCCA
Cm2721 rv	GGGAATTCTCAAGTGCAGCTGTCTCGC
Sequencing	
pICH86988 fw	AGGACACGCTCGAGTATAAG
pICH86988 rv	CATGCGATCATAGGCTTCTC
AOXI fw	GACTGGTTCCAATTGACAAGC
AOXI rv	GCAAATGGCATTCTGACATCC
RT-qPCR	
NbActin fw	TGGTCGTACCACCGGTATTGTGTT
NbActin rv	TCACTTGCCCATCAGGAAGCTCAT
qRT-NbHIN1 fw	CCAACCTGAACGGAGCCTATTA
qRT-NbHIN1 rv	AGGCATCCAAAGAGACAACACTAC

qRT-NbHSR203J fw	ACGCAGATTTCACCGAGTAT
qRT-NbHSR203J rv	GCCAGTCGATTGGAGATAA
qRT-NbPR1a fw	CCGCCTTCCCTCAACTCAAC
qRT-NbPR1a rv	GCACAACCAAGACGTAAGT
qRT-NbPR2 fw	AGGTGTTTGCTATGGAATGC
qRT-NbPR2 rv	TCTGTACCCACCATCTTGC
qRT-NbPR4 fw	GGCCAAGATTCTGTGGTAGAT
qRT-NbPR4 rv	CACTGTTGTTTGAGTTCCTGTTCT
qRT-NbLOX fw	AAAACCTATGCCTCAAGAAC
qRT-NbLOX rv	ACTGCTGCATAGGCTTTGG
qRT-NbERF1 fw	GCTCTTAACGTCGGATGGTC
qRT-NbERF1 rv	AGCCAAACCCTAGCTCCATT
qRT-NbAcre31 fw	AATTCGGCCATCGTGATCTTGGTC
qRT-NbAcre31 rv	GAGAACTGGGATTGCCTGAAGGA
qRT-NbWRKY7 fw	CACAAGGGTACAAACAACACAG
qRT-NbWRKY7 rv	GGTTGCATTTGGTTCATGTAAG
qRT-NbWRKY8 fw	AACAATGGTGCCAAATAATGC
qRT-NbWRKY8 rv	TGCATATCCTGAGAAACCAT
Chapter 4	
sgRNA synthesis	
Ds69335 sgRNA7 fw	GTCACTCCGTCTCTGACATCGCG
Ds69335 sgRNA7 rv	AACCGCGATGTCAGAGACGGAGT
Ds74283 sgRNA3 fw	GTCGTTGTTGTAGGCAGAGACGA
Ds74283 sgRNA3 rv	AACTCGTCTCTGCTACAACAAC
Ds131885 sgRNA21 fw	GTCCTTGCTGTTGGAGGTCGCA
Ds131885 sgRNA21 rv	AACTGCGACCTCCAACAGCAAGG
donor DNA flank amplification	
5'flank Ds69335 fw	GGGTTTTCCAGTCACGACCGATGGGACTTCGACGCGG
5'flank Ds69335 rv	GGAACAACCTGGCATGAATTCTCAGAGACGGAGTGATTGGCAC
3'flank Ds69335 fw	AGGTAATCCTCTTTCTAGATCGGACGACGGTCTCGC
3'flank Ds69335 rv	ATTTACACAGGAAACAGCTTAGGGCGCTTAGAGCCTTGA
5'flank Ds74283 fw	GGGTTTTCCAGTCACGACGTGACGGCTGCGATACGAGA
5'flank Ds74283 rv	GAACAACCTGGCATGAATTCAAGCAACGAGGGCCAGAAAGA
3'flank Ds74283 fw	GGTAATCCTTCTTTCTAGATGCCTACAACAACAACACTGC
3'flank Ds74283 rv	ACAATTTACACAGGAAACAGCTGTACACGAGCTGCACGA
5'flank Ds131885 fw	AGGGTTTTCCAGTCACGACTCTCTTGCTGGACCTTCGAG
5'flank Ds131885 rv	GGGAACAACCTGGCATGAATTGAGTCCAAAGTGGCATTTCGC
3'flank Ds131885 fw	AGAGGTAATCCTTCTTTCTAGATCCAACAGCAAGGGCAAG
3'flank Ds131885 rv	TTCACACAGGAAACAGCCCAAATACTGCTTTCTATCGGA
nptII cassette amplification	
nptII fw	GAATTCATGCCAGTTGTTC
nptII rv	TCTAGAAAGAAGGATTACCTCTAAAC
pAN-7 backbone amplification	
pRS427 fw	GTCGTGACTGGGAAAACCC
pRS427 rv	GCTGTTTCCTGTGTGAAATTG
Sequencing	
Cas9HygAMAcclB fw	TTTTCTCTCCATTACGC
nptII seq fw	TTTACCCAGAATGCACAGGTACA
nptII seq rv	TAGGCCGAATAACTGCACAAATTG
Screening CRISPR/Cas9 transformants	
MT91 fw	ATGCGCTCCACTCTTGC
MT92 rv	CTACGATGAGCCGGTGTTC
MT93 fw	CAGGGCTGATGAGGTGAAGT
MT94 rv	GTCAATGTTATAGAGATGTGCGGT
MT95 fw	ATGAAGTACCTTGCCCTCTTCT
MT96 rv	TCAGATGTATTGCGGAATGAGGT
MT97 fw	GAGGAATTGCTGATGAACAAGGTC
MT98 rv	AGAAGCTCGCTCATTATGTTTCCA
MT99 fw	ATGCAGTTCCGTACTTTCATTGG
MT100 rv	CTATGTACAGCTAGAACTTCCCGC
MT101 fw	TGCCGGAACCTCTGCTTC
MT102 rv	ATACGCCGGCATGATTTGAAC

Southern hybridization probe amplification	
Ds69335 probe fw	CAACTTTGAGCTTTTCGGT
Ds69335 probe rv	AGCAACGGGACAAGTCA
Ds74283 probe fw	GACAAACATCACCGCAAATG
Ds74283 probe rv	TGTACACGAGCTGCACGAAA
Ds131885 probe fw	CAAACGTTTCGAGCTTTGA
Ds131885 probe rv	CCAAAATACTGCTTTCTATCGGATTCC
Complementation sequence amplification	
Ds69335 fw	ATGCATGGGCCCCACATCACACACGTCACGGA
Ds69335 rv	ATGCATTCTAGAGTTATAGAGATGTGCGGTGCG
Ds74283 fw	ATGCATGGGCCCCGGAGGTGGAATTGAACCG
Ds74283 rv	ATGCATTCTAGATGTACACGAGCTGCACGAAA
Ds131885 fw	ATGCATGGGCCCCCTCCTCGAGCACCATCTGG
Ds131885 rv	ATGCATTCTAGACCAAAATACTGCTTTCTATCGGATT
RT-qPCR	
Ds69335 comp fw	CAGAACATTGCAGCTGGTGT
Ds69335 comp rv	GTCTGTCTGGGTCGTCCTT
Ds74283 comp fw	GACAAACATCACCGCAAATG
Ds74283 comp rv	GTTGATGAAGAAGCCGGAGA
Ds131885 comp fw	GACCTTCACGTCCGACTCAG
Ds131885 comp rv	TGTTCTCACAGCCACACTCG
DsAfIR fw	GGAAGAGTAGTGTACCATTGT
DsAfIR rv	CATCTATTCAACGACCTACA
DsPksA fw	CTGTCTTCCTCGACCTGTT
DsPksA rv	AGCACACCTGGAAAGAATGA
CAD fw	CAGCAAGAGGATTGGACCTA
CAD rv	TTCAATACCCACATCTGATCAAC
Chapter 5	
Morphotype characterization	
Cm simile fw	CCGGGCCTTATGGTCCGC
Cm simile rv	CAGGCGCCAGCCCAGCG
Cm verum fw	CCGGGCCTTCGGGCCTAC
Cm verum rv	CAGGCACCAACCCAGGC
Golden Gate pICH86988 cloning	
Cm3871 fw	GGTCTCGCAAGTCTCTCTAAGTTCTCTACTGCCTCGTCAA
Cm3871 rv	GGTCTCGAAGCTCAGTCATACGCATACGCACTCGGC
Cm4059 fw	GGTCTCGCAAGTCTCTCTCCAAAACAAACGCC
Cm4059 rv	GGTCTCGAAGCTCAAATATACTGATAAGCCCCATGAACC
Cm9849 fw	GGTCTCGCAAGTCTCCCTCGCCAAGC
Cm9849 rv	GGTCTCGAAGCTTAGTCGTAAGTGGTAGGCGG

Table A.3. Plasmids used in this thesis.

Plasmid	Characteristics	Antibiotic ^a	Reference
Chapter 2			
pTWISTamp	Shuttle vector with <i>Bsa</i> I sites, PR1a secretion signal from <i>N. tabacum</i> , 3xFLAG-tag, and candidate effector (synthesized)	Amp	de la Rosa (2022)
pPR1α-3xFLAG	Entry vector holding fused PR1α and 3xFLAG tag; N-terminal universal secretion signal and tag for immunoblotting	Amp	Y. Guo
p3xFLAG	Entry vector holding fused 3xFLAG tag; N-terminal tag for immunoblotting	Amp	Y. Guo
pICH86988	Binary vector, 35S CaMV promoter and NosT terminator	Kan	Weber et al. (2011)
pPic9-His ₆	Expression vector for <i>Pi. pastoris</i> , complements histidine auxotrophy, methanol-inducible promoter, His ₆ tag for purification	Amp	Invitrogen
pINF1	pBC302-3 containing INF1	Kan	Bos et al. (2006)
pFfEcp20-1	pICH86988 containing 5'PRIα-3xFLAG-FfEcp20-1	Kan	This study
pFfEcp20-2	pICH86988 containing 5'PRIα-3xFLAG-FfEcp20-2	Kan	This study
pFfEcp20-3	pICH86988 containing 5'PRIα-3xFLAG-FfEcp20-3	Kan	This study
pFfEcp20-4	pICH86988 containing 5'PRIα-3xFLAG-FfEcp20-4	Kan	This study
pDsEcp20-1	pICH86988 containing 5'PRIα-3xFLAG-DsEcp20-1	Kan	This study
pDsEcp20-2	pICH86988 containing 5'PRIα-3xFLAG-DsEcp20-2	Kan	This study
pDsEcp20-3	pICH86988 containing 5'PRIα-3xFLAG-DsEcp20-3	Kan	This study
pDsEcp20-4	pICH86988 containing 5'PRIα-3xFLAG-DsEcp20-4	Kan	This study
pFfEcp32-1	pICH86988 containing 5'PRIα-3xFLAG-FfEcp32-1	Kan	This study
pFfEcp32-2	pICH86988 containing 5'PRIα-3xFLAG-FfEcp32-2	Kan	This study
pFfEcp32-3	pICH86988 containing 5'PRIα-3xFLAG-FfEcp32-3	Kan	This study
pFfEcp32-4	pICH86988 containing 5'PRIα-3xFLAG-FfEcp32-4	Kan	This study
pFfEcp32-5	pICH86988 containing 5'PRIα-3xFLAG-FfEcp32-5	Kan	This study
pDsEcp32-1	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-1	Kan	This study
pDsEcp32-3	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-3	Kan	This study
pDsEcp32-4	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-4	Kan	This study
pDsEcp32-5	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-5	Kan	This study
pDsEcp20-3-GUA2	pICH86988 containing 5'PRIα-3xFLAG-DsEcp20-3-GUA2	Kan	This study
pDsEcp32-1-COLN	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-1-COLN	Kan	This study
pDsEcp32-3-GUA2	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-3-GUA2	Kan	This study
pDpEcp20-3	pICH86988 containing 5'PRIα-3xFLAG-DpEcp20-3	Kan	This study
pDpEcp32-1	pICH86988 containing 5'PRIα-3xFLAG-DpEcp32-1	Kan	This study
pDpEcp32-3	pICH86988 containing 5'PRIα-3xFLAG-DpEcp32-3	Kan	This study
pFfEcp20-2 noSP	pICH86988 containing 5'-3xFLAG-pFfEcp20-2	Kan	This study
pDsEcp20-3 noSP	pICH86988 containing 5'-3xFLAG-pDsEcp20-3	Kan	This study
pFfEcp32-1 noSP	pICH86988 containing 5'-3xFLAG-pFfEcp32-1	Kan	This study
pFfEcp32-2 noSP	pICH86988 containing 5'-3xFLAG-pFfEcp32-2	Kan	This study
pFfEcp32-3 noSP	pICH86988 containing 5'-3xFLAG-pFfEcp32-3	Kan	This study
pFfEcp32-5 noSP	pICH86988 containing 5'-3xFLAG-pFfEcp32-5	Kan	This study
pDsEcp32-1 noSP	pICH86988 containing 5'-3xFLAG-pDsEcp32-1	Kan	This study
pDsEcp32-3 noSP	pICH86988 containing 5'-3xFLAG-pDsEcp32-3	Kan	This study
pDsEcp32-5 noSP	pICH86988 containing 5'-3xFLAG-pDsEcp32-5	Kan	This study
pDsEcp20-3-GUA2 noSP	pICH86988 containing 5'-3xFLAG-pDsEcp20-3-GUA2	Kan	This study
pDsEcp32-1-COLN noSP	pICH86988 containing 5'-3xFLAG-pDsEcp32-1-COLN	Kan	This study
pDsEcp32-3-GUA2	pICH86988 containing 5'-3xFLAG-pDsEcp32-3-GUA2	Kan	This study
pDpEcp20-3 noSP	pICH86988 containing 5'-3xFLAG-pDpEcp20-3	Kan	This study
pDpEcp32-1 noSP	pICH86988 containing 5'-3xFLAG-pDpEcp32-1	Kan	This study
pDpEcp32-3 noSP	pICH86988 containing 5'-3xFLAG-pDpEcp32-3	Kan	This study
pBcSSP	pPic9-His ₆ containing 5'-FLAG-BcSSP	Amp	This study
pDsEcp20-3	pPic9-His ₆ containing 5'-FLAG-DsEcp20-3	Amp	This study
pDsEcp20-4	pPic9-His ₆ containing 5'-FLAG-DsEcp20-4	Amp	This study

pDsEcp32-3	pPic9-His ₆ containing 5'-FLAG-DsEcp32-3	Amp	This study
Chapter 3			
pTWISTamp	Shuttle vector with <i>Bsa</i> I sites, PR1a secretion signal from <i>N. tabacum</i> , 3xFLAG-tag, and candidate effector (synthesized)	Amp	de la Rosa (2022)
pPR1α-3xFLAG	Entry vector holding fused PR1α and 3xFLAG tag; N-terminal universal secretion signal and tag for immunoblotting	Amp	Y. Guo
p3xFLAG	Entry vector holding fused 3xFLAG tag; N-terminal tag for immunoblotting	Amp	Y. Guo
pICH86988	Binary vector, 35S CaMV promoter and NosT terminator	Kan	Weber et al. (2011)
pPic9-His ₆	Expression vector for <i>Pi. pastoris</i> , complements histidine auxotrophy, methanol inducible promoter, His ₆ tag for purification	Amp	Invitrogen
pINF1	pBC302-3 containing INF1	Kan	Bos et al. (2006)
pDs19040	pICH86988 containing 5'PRIα-3xFLAG-Ds19040	Kan	This study
pDs24695	pICH86988 containing 5'PRIα-3xFLAG- Ds24695	Kan	This study
pDs42387	pICH86988 containing 5'PRIα-3xFLAG-Ds42387	Kan	This study
pDs46236	pICH86988 containing 5'PRIα-3xFLAG-Ds46236	Kan	This study
pDs67456	pICH86988 containing 5'PRIα-3xFLAG-Ds67456	Kan	This study
pDs69113	pICH86988 containing 5'PRIα-3xFLAG-Ds69113	Kan	This study
pDs69335	pICH86988 containing 5'PRIα-3xFLAG-Ds69335	Kan	This study
pDs69845	pICH86988 containing 5'PRIα-3xFLAG-Ds69845	Kan	This study
pDs70155	pICH86988 containing 5'PRIα-3xFLAG-Ds70155	Kan	This study
pDs71487	pICH86988 containing 5'PRIα-3xFLAG-Ds71487	Kan	This study
pDs71565	pICH86988 containing 5'PRIα-3xFLAG-Ds71565	Kan	This study
pDs74283	pICH86988 containing 5'PRIα-3xFLAG-Ds74283	Kan	This study
pDs74790	pICH86988 containing 5'PRIα-3xFLAG-Ds74790	Kan	This study
pDs74875	pICH86988 containing 5'PRIα-3xFLAG-Ds74875	Kan	This study
pDs75009	pICH86988 containing 5'PRIα-3xFLAG-Ds75009	Kan	This study
pDs124808	pICH86988 containing 5'PRIα-3xFLAG-Ds124808	Kan	This study
pDs129328	pICH86988 containing 5'PRIα-3xFLAG-Ds129328	Kan	This study
pDs131088	pICH86988 containing 5'PRIα-3xFLAG-Ds131088	Kan	This study
pDs131290	pICH86988 containing 5'PRIα-3xFLAG-Ds131290	Kan	This study
pDs131885	pICH86988 containing 5'PRIα-3xFLAG-Ds131885	Kan	This study
pDs153323	pICH86988 containing 5'PRIα-3xFLAG-Ds153323	Kan	This study
pDs158381	pICH86988 containing 5'PRIα-3xFLAG-Ds158381	Kan	This study
pDsEcp32-1	pICH86988 containing 5'PRIα-3xFLAG-DsEcp32-1	Kan	This study
pPp7927	pICH86988 containing 5'PRIα-3xFLAG-Pp7927	Kan	This study
pPp10632	pICH86988 containing 5'PRIα-3xFLAG-Pp10632	Kan	This study
pCm835	pICH86988 containing 5'PRIα-3xFLAG-Cm835	Kan	This study
pCm2492	pICH86988 containing 5'PRIα-3xFLAG-Cm2492	Kan	This study
pCm2721	pICH86988 containing 5'PRIα-3xFLAG-Cm2721	Kan	This study
pCm3871	pICH86988 containing 5'PRIα-3xFLAG-Cm3871	Kan	This study
pCm4059	pICH86988 containing 5'PRIα-3xFLAG-Cm4059	Kan	This study
pCm9849	pICH86988 containing 5'PRIα-3xFLAG-Cm9849	Kan	This study
pCm937-110	pICH86988 containing 5'PRIα-3xFLAG-Cm937	Kan	This study
pCm3003-3305	pICH86988 containing 5'PRIα-3xFLAG-Cm3003	Kan	This study
pCm4060-809	pICH86988 containing 5'PRIα-3xFLAG-Cm4060	Kan	This study
pCm4577-3305	pICH86988 containing 5'PRIα-3xFLAG-Cm4577	Kan	This study
pCm5237-3305	pICH86988 containing 5'PRIα-3xFLAG-Cm5237	Kan	This study
pCm5316-110	pICH86988 containing 5'PRIα-3xFLAG-Cm5316	Kan	This study
pCm5317-110	pICH86988 containing 5'PRIα-3xFLAG-Cm5317	Kan	This study
pCm6323-110	pICH86988 containing 5'PRIα-3xFLAG-Cm6323	Kan	This study
pCm7338-3305	pICH86988 containing 5'PRIα-3xFLAG-Cm7338	Kan	This study
pCm7980-3305	pICH86988 containing 5'PRIα-3xFLAG-Cm7980	Kan	This study
pCm8527-110	pICH86988 containing 5'PRIα-3xFLAG-Cm8527	Kan	This study
pCm9109-110	pICH86988 containing 5'PRIα-3xFLAG-Cm9109	Kan	This study
pCmScf53-110	pICH86988 containing 5'PRIα-3xFLAG-Cm Scf53	Kan	This study
pFf189800	pICH86988 containing 5'PRIα-3xFLAG-Cf189800	Kan	This study
pFf192236	pICH86988 containing 5'PRIα-3xFLAG-Cf192236	Kan	This study

pVi17736	pICH86988 containing 5'PRL α -3xFLAG-Vi17736	Kan	This study
pVi23981	pICH86988 containing 5'PRL α -3xFLAG-Vi23981	Kan	This study
pVi13495	pICH86988 containing 5'PRL α -3xFLAG-Vi13495	Kan	This study
pDs71487 noSP	pICH86988 containing 5'-3xFLAG-Ds71487	Kan	This study
pDs74283 noSP	pICH86988 containing 5'-3xFLAG-Ds74283	Kan	This study
pDsEcp32-1 noSP	pICH86988 containing 5'-3xFLAG-DsEcp32-1	Kan	This study
pPp10632 noSP	pICH86988 containing 5'-3xFLAG-Pp10632	Kan	This study
pCm835 noSP	pICH86988 containing 5'-3xFLAG-Cm835	Kan	This study
pCm2492 noSP	pICH86988 containing 5'-3xFLAG-Cm2492	Kan	This study
pCm2721 noSP	pICH86988 containing 5'-3xFLAG-Cm2721	Kan	This study
pDs69335	pPic9-His ₆ containing 5'-FLAG-Ds69335	Amp	This study
pDs71487	pPic9-His ₆ containing 5'-FLAG-Ds71487	Amp	This study
pDs74283	pPic9-His ₆ containing 5'-FLAG-Ds74283	Amp	This study
pDs131885	pPic9-His ₆ containing 5'-FLAG-Ds131885	Amp	This study
pDs158381	pPic9-His ₆ containing 5'-FLAG-Ds158381	Amp	This study
pPp7927	pPic9-His ₆ containing 5'-FLAG-Pp7927	Amp	This study
pPp10632	pPic9-His ₆ containing 5'-FLAG-Pp10632	Amp	This study
pCm2721	pPic9-His ₆ containing 5'-FLAG-Cm2721	Amp	This study
Chapter 4			
pCas9HygAMAcadb	sgRNA and <i>cas9</i> expression vector	Amp	Rocafort et al. (2022)
pII9	Contains the <i>nptII</i> cassette (<i>P_{trpC}-nptII-T_{trpC}</i>) used as selective marker	Amp/Gen	Lara-Ortiz et al. (2003)
pAN-7	Backbone of dDNA, Gibson Assembly compatible	Amp	Punt et al., 1990
pBC-phleo	Backbone to generate the complementation plasmid	Chl/Phleo	Silar, 1995
pDs69335-sgRNA	pCas9HygAMAcadb containing sgRNA targeting <i>Ds69335</i>	Amp	This study
pDs74283-sgRNA	pCas9HygAMAcadb containing sgRNA targeting <i>Ds74283</i>	Amp	This study
pDs131885-sgRNA	pCas9HygAMAcadb containing sgRNA targeting <i>Ds131885</i>	Amp	This study
pdonorDs69335	pAN-7 containing 5'flank- <i>nptII</i> cassette-3'flank from <i>Ds69335</i> for homologous recombination	Gen	This study
pdonorDs74283	pAN-7 containing 5'flank- <i>nptII</i> cassette-3'flank from <i>Ds74283</i> for homologous recombination	Gen	This study
pdonorDs131885	pAN-7 containing 5'flank- <i>nptII</i> cassette-3'flank from <i>Ds131885</i> for homologous recombination	Gen	This study
pDs69335comp	pBC-phleo containing 5'flank- <i>Ds69335</i> -3'flank for complementation	Chl/Phleo	This study
pDs74283comp	pBC-phleo containing 5'flank- <i>Ds74283</i> -3'flank for complementation	Chl/Phleo	This study
pDs131885comp	pBC-phleo containing 5'flank- <i>Ds131885</i> -3'flank for complementation	Chl/Phleo	This study

^a Antibiotic resistance. Amp: ampicillin; Kan: kanamycin; Chl: chloramphenicol; Gen: geneticin; Phleo: phleomycin.

Table A.4. Percentage pairwise amino acid identity between Ecp20 and Ecp32 family members from *Fulvia fulva* 0WU, *Dothistroma pini* CBS 116487 and *Dothistroma septosporum* NZE10.

ID ^a	FfEcp20-1	FfEcp20-2	FfEcp20-3	FfEcp20-4	DpEcp20-1	DpEcp20-2	DpEcp20-3	DpEcp20-4	DsEcp20-1	DsEcp20-2	DsEcp20-3	DsEcp20-4
FfEcp20-1		14.9	11.2	13.2	47.6	13.1	9.5	10.8	45.8	12.5	9.5	10.8
FfEcp20-2	14.9		43.8	13.2	17.9	73.5	42.1	12.6	19	68.2	41.4	13.8
FfEcp20-3	11.2	43.8		13.1	13.7	42.2	86.3	11.9	15.5	42.2	83	12.5
FfEcp20-4	13.2	13.2	13.1		14.3	10.7	14.5	84	14.9	11.3	15.1	85.4
DpEcp20-1	47.6	17.9	13.7	14.3		16	13.7	14.3	88.6	17.8	13.7	14.3
DpEcp20-2	13.1	73.5	42.2	10.7	16		39.2	11.9	17.2	83.4	37.9	13.2
DpEcp20-3	9.5	42.1	86.3	14.5	13.7	39.2		13.2	15.5	39.9	88.8	13.2
DpEcp20-4	10.8	12.6	11.9	84	14.3	11.9	13.2		14.3	14.5	13.8	93.8
DsEcp20-1	45.8	19	15.5	14.9	88.6	17.2	15.5	14.3		17.8	14.9	14.9
DsEcp20-2	12.5	68.2	42.2	11.3	17.8	83.4	39.9	14.5	17.8		40.5	14.5
DsEcp20-3	9.5	41.4	83	15.1	13.7	37.9	88.8	13.8	14.9	40.5		13.8
DsEcp20-4	10.8	13.8	12.5	85.4	14.3	13.2	13.2	93.8	14.9	14.5	13.8	

	FfEcp32-1	FfEcp32-2	FfEcp32-3	FfEcp32-4	FfEcp32-5	DpEcp32-1	DpEcp32-2	DpEcp32-3	DpEcp32-4	DsEcp32-1	DsEcp32-2	DsEcp32-3	DsEcp32-4	DsEcp32-5
FfEcp32-1		38	49.8	23	31.1	71.9	43.1	22.1	69.5	44.5	20.7	31.1		
FfEcp32-2	38		42.1	21.7	29.5	36.7	38.8	20.7	34.6	39.3	19.2	26.7		
FfEcp32-3	49.8	42.1		25.7	35.1	49.5	71.5	25.7	48.6	70.4	23.4	37.1		
FfEcp32-4	23	21.7	25.7		25.6	25.7	23.9	82.4	24.3	25.7	83.9	24.3		
FfEcp32-5	31.1	29.5	35.1	25.6		30.4	31.6	22.7	30.8	32.7	21.8	80.1		
DpEcp32-1	71.9	36.7	49.5	25.7	30.4		44.3	23.8	83	43.3	23.8	30.4		
DpEcp32-2	43.1	38.8	71.5	23.9	31.6	44.3		25.7	45.5	80.6	23.9	33.2		
DpEcp32-3	22.1	20.7	25.7	82.4	22.7	23.8	25.7		22.4	25.2	91.7	23.8		
DsEcp32-1	69.5	34.6	48.6	24.3	30.8	83	45.5	22.4		43.5	23.4	30.8		
DsEcp32-2	44.5	39.3	70.4	25.7	32.7	43.3	80.6	25.2	43.5		24.3	34.3		
DsEcp32-3	20.7	19.2	23.4	83.9	21.8	23.8	23.9	91.7	23.4	24.3		22.9		
DsEcp32-4	31.1	26.7	37.1	24.3	80.1	30.4	33.2	23.8	30.8	34.3	22.9			

^a Ff: *Fulvia fulva*, Dp: *Dothistroma pini*, Ds: *Dothistroma septosporum*.

Table A.5. Protein structural hits of each *Dothistroma septosporum* and *Fulvia fulva* Ecp20 and Ecp32 protein in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB).

Available at:

<https://www.frontiersin.org/articles/10.3389/fmicb.2022.964851/full#supplementary-material>

Table A.6. JGI hits of each *Dothistroma septosporum* Ecp20 and Ecp32 family member.

Available at:

<https://www.frontiersin.org/articles/10.3389/fmicb.2022.964851/full#supplementary-material>

Table A.7. Summary of allelic variation of *Dothistroma septosporum* NZE10 Ecp20 and Ecp32 proteins across 18 isolates from different locations.

Available at:

<https://www.frontiersin.org/articles/10.3389/fmicb.2022.964851/full#supplementary-material>

Table A.8. Features of *Dothistroma septosporum* NZE10 candidate effector proteins.

Protein ID ^a	<i>D. septosporum</i> expression ^b				EffectorP ^c			Prediction
	FM	Early	Mid	Late	Cytoplasmic	Apoplastic	Non-effector	
19040	46.2	66.0	50.8	48.4	-	Y (0.833)	-	Apoplastic
24695	1222.3	83.1	529.0	339.3	Y (0.521)	Y (0.928)	-	Dual
42387	497.9	514.5	584.2	363.7	Y (0.681)	-	-	Cytoplasmic
43416	72.8	233.1	192.5	3732.0	-	Y (0.855)	-	Apoplastic
46236	47.1	5.9	606.3	171.4	-	Y (0.871)	-	Apoplastic
51158	10.0	17.0	57.0	84.0	Y (0.901)	Y (0.679)	-	Dual
51311	19.0	62.3	52.7	57.2	Y (0.507)	Y (0.849)	-	Dual
67456	69.6	102.3	60.2	51.3	Y (0.672)	-	-	Cytoplasmic
69113	53.2	83.3	256.9	342.1	-	Y (0.641)	-	Apoplastic
69335	425.9	1448.3	1012.5	553.6	-	Y (0.612)	-	Apoplastic
69845	81.0	94.0	81.4	118.0	-	Y (0.502)	-	Apoplastic
70057	310.5	467.1	2569.2	3312.4	-	Y (0.745)	-	Apoplastic
70155	256.7	70.8	507.3	1302.5	-	Y (0.975)	-	Apoplastic
70694	4.6	4.2	205.0	114.5	-	Y (0.834)	-	Apoplastic
71487	27.2	239.3	144.6	981.6	-	Y (0.776)	-	Apoplastic
71565	205.8	157.4	74.7	154.1	Y (0.737)	Y (0.692)	-	Dual
74283	40.6	26.7	141.2	243.2	-	Y (0.717)	-	Apoplastic
74790	30.2	18.4	48.7	158.6	-	Y (0.697)	-	Apoplastic
74875	1.2	9.8	0.0	132.7	-	Y (0.845)	-	Apoplastic
75009	9234.4	455.1	6494.0	4861.6	-	Y (0.844)	-	Apoplastic
75860	53.2	45.5	34.4	67.2	Y (0.626)	Y (0.554)	-	Dual
124808	40.6	38.2	54.6	28.3	-	Y (0.599)	-	Apoplastic
129328	872.1	27.7	52.9	193.9	Y (0.681)	Y (0.9)	-	Dual
131088	35.9	17.3	138.8	53.2	Y (0.562)	-	-	Cytoplasmic
131290	399.0	636.6	89.2	174.2	-	Y (0.755)	-	Apoplastic
131885	1.4	9.0	369.2	202.6	-	Y (0.797)	-	Apoplastic
153323	172.7	61.1	107.8	110.5	Y (0.889)	-	-	Cytoplasmic
158381	1.3	7.7	243.6	17.9	-	Y (0.923)	-	Apoplastic
174276	14.0	17.0	536.0	101.0	-	Y (0.833)	-	Apoplastic

^a *Dothistroma septosporum* protein IDs in the Joint Genome Institute (JGI) database.^b *D. septosporum* expression during infection stages of *Pinus radiata* in culture (fungal mycelium, FM) and *in planta* (Early, Mid, Late) (Bradshaw et al, 2016) as RNA-seq Reads Per Million per Kilobase (RPMK) values.^c Protein sequences were queried at <https://github.com/JanaSperschneider/EffectorP-3.0>. Dual prediction: apoplastic/cytoplasmic prediction.**Table A.9.** BLASTp results of *Dothistroma septosporum* candidate effectors against other pine pathogens/endophytes.Available at: [appendix A9.xlsx](#)

Table A.10. Searches for *Phytophthora* β -trefoil proteins using the Foldseek server.

Ds ID ^a	Foldseek hit ^b	Organism	Uniprot ID ^c	Pp hit ID ^d	RCSB PDB hit ^e
Ds69113	AF-W2XDZ5-F1-model_v3	<i>Phytophthora parasitica</i>	W2XDZ5	Pp12323	Beta-galactoside-specific lectin 1 - 4eb2 (19.0)
Ds71487	AF-A0A329SBG6-F1-model_v3	<i>Phytophthora cactorum</i>	A0A329SBG6	Pp7483	Ubiquitin-like protein ATG12 - 4gdk (20.4)
Ds74283	AF-A0A329SBG6-F1-model_v3	<i>Phytophthora cactorum</i>	A0A329SBG6	Pp7483	Ubiquitin-like protein ATG12 - 4gdk (20.4)
Ds75860	AF-A0A225WF42-F1-model_v3	<i>Phytophthora megakary</i>	A0A225WF42	Pp7483	Ubiquitin-like protein ATG12 - 4gdk (20.4)

^a *Dothistroma septosporum* (Ds) protein IDs in the Joint Genome Institute (JGI) database.

^b The AlphaFold2-predicted tertiary structure of each *D. septosporum* protein was queried against the Foldseek server (<https://search.foldseek.com/search>) using *Phytophthora* species as the taxonomic filter. Only the top hit is shown.

^c Uniprot ID of each protein identified by Foldseek.

^d The amino acid sequence of each protein identified by the Foldseek server was used in BLASTp (E-value < 10⁻⁵) searches against predicted proteins of *Phytophthora pluvialis* (Studholme et al., 2016)

^e *P. pluvialis* protein structure predictions were made using AlphaFold2 (Jumper et al., 2021) and the predicted structures were queried against those in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB), using the protein structure comparison server, Dali (Holm, 2020). The top hit is shown, next to the RCSB PDB accession ID, and in parenthesis the Z-score is shown. A hit was considered to be significant if the Z-score was >2.

Table A.11. Summary of *Pinus radiata* needles with *Dothistroma* needle blight lesions from virulence assays with *Dothistroma septosporum* transformants.

CE gene ^a	Strain name/rep ^b	Healthy needles ^c	Needles with DNB lesions (total lesions) ^d	Total needles ^e
Week 1				
<i>Ds69335</i>	WT A	372	83 (83)	455
	WT B	438	61 (65)	499
	WT C	644	78 (101)	722
	<i>Ds69335</i> T2-D A	584	44 (46)	628
	<i>Ds69335</i> T2-D B	344	60 (60)	404
	<i>Ds69335</i> T2-D C	378	68 (73)	446
	<i>Ds69335</i> T2-D D	370	52 (57)	422
	<i>Ds69335</i> T40-D A	362	61 (61)	423
	<i>Ds69335</i> T40-D B	614	72 (72)	686
	<i>Ds69335</i> T40-D C	503	60 (60)	563
	<i>Ds69335</i> T40-D D	168	70 (70)	238
	<i>Ds69335</i> T2-Co A	465	47 (51)	512
	<i>Ds69335</i> T2-Co B	213	71 (71)	284
	<i>Ds69335</i> T2-Co C	587	53 (56)	640
	<i>Ds69335</i> T2-Co D	175	104 (104)	279
<i>Ds74283</i>	<i>Ds74283</i> T3-D A	355	71 (74)	426
	<i>Ds74283</i> T3-D B	322	107 (107)	429
	<i>Ds74283</i> T3-D C	258	112 (112)	370
	<i>Ds74283</i> T3-D D	335	80 (81)	415
	<i>Ds74283</i> T56-D A	370	79 (79)	449
	<i>Ds74283</i> T56-D B	176	113 (113)	289
	<i>Ds74283</i> T56-D C	549	82 (82)	631
	<i>Ds74283</i> T56-D D	243	85 (85)	328
	<i>Ds74283</i> T3-Co A	393	62 (62)	455
	<i>Ds74283</i> T3-Co B	220	92 (92)	312
	<i>Ds74283</i> T3-Co C	254	105 (105)	359
	<i>Ds74283</i> T3-Co D	452	70 (72)	522
<i>Ds131885</i>	<i>Ds131885</i> T9-Co A	564	50 (50)	614
	<i>Ds131885</i> T9-Co B	407	65 (65)	472
	<i>Ds131885</i> T9-Co C	568	80 (80)	648
	<i>Ds131885</i> T9-Co D	452	105 (105)	557
Week 2				
<i>Ds131885</i>	WT D	337	129 (129)	466
	WT E	486	121 (121)	607
	WT F	306	114 (114)	420
	<i>Ds131885</i> T9-D A (L)	430	52 (55)	482
	<i>Ds131885</i> T9-D B (L)	425	150 (150)	575
	<i>Ds131885</i> T9-D C (L)	388	104 (104)	492
	<i>Ds131885</i> T9-D D (M)	232	142 (142)	374
	<i>Ds131885</i> T9-D E (M)	246	99 (99)	345
	<i>Ds131885</i> T9-D F (M)	538	82 (89)	620
	<i>Ds131885</i> T47-D A	553	40 (50)	593
	<i>Ds131885</i> T47-D B	481	48 (57)	529
	<i>Ds131885</i> T47-D C	587	50 (52)	637

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* transformants (T) with a targeted gene disruption are indicated by "D"; complementation transformants are indicated by "Co". WT: wild-type. A, B, C and D indicate the values are from replicate seedlings. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings according to **Table 4.7**.

^c Number of needles that did not show any *Dothistroma* needle blight (DNB) lesions from each seedling.

^d Number of needles that showed characteristic *Dothistroma* needle blight (DNB) lesions from each seedling. In parenthesis, the total number of lesions from these needles.

^e Sum of healthy needles and needles with DNB lesions.

Table A.12. Results for relative quantification of *Dothistroma septosporum* biomass in pine needles.

CE gene ^a	Strain name/rep ^b	Ct tar ^c	Ct ref ^d	Tar/Ref ^e	DNA conc (ng/μL) ^f	Total DNA (ng) ^g	Fungal DNA (ng) ^h
Week 1							
<i>Ds69335</i>	WT A	20.0	27.8	57.6	5.2	156.0	153.3
	WT B	20.5	28.0	51.1	4.6	137.4	134.8
	WT C	19.9	27.7	61.6	5.9	175.8	173.0
	<i>Ds69335</i> T2-D A	25.0	29.2	11.6	2.8	56.8	52.3
	<i>Ds69335</i> T2-D B	23.6	27.2	8.7	12.1	242.0	217.1
	<i>Ds69335</i> T2-D C	22.2	27.7	16.6	5.6	166.8	157.3
	<i>Ds69335</i> T2-D D	24.9	28.8	6.2	3.0	91.2	78.5
	<i>Ds69335</i> T40-D A	24.2	32.3	103.4	0.8	16.9	16.7
	<i>Ds69335</i> T40-D B	22.5	30.5	78.2	3.1	62.0	61.2
	<i>Ds69335</i> T40-D C	23.5	29.1	18.4	3.2	96.0	91.0
	<i>Ds69335</i> T40-D D	21.0	28.1	41.9	6.7	201.6	196.9
	<i>Ds69335</i> T2-Co A*	25.7	31.0	16.1	0.6	19.3	18.2
	<i>Ds69335</i> T2-Co B*	22.5	25.7	6.3	1.3	50.8	43.8
	<i>Ds69335</i> T2-Co C	24.0	27.7	5.2	4.6	138.0	115.9
	<i>Ds69335</i> T2-Co D	25.8	30.1	9.3	2.0	60.6	54.7
	<i>Ds74283</i> T3-D A	29.3	34.8	26.5	0.4	10.6	10.2
	<i>Ds74283</i> T3-D B	22.7	28.4	17.8	3.1	93.0	88.1
	<i>Ds74283</i> T3-D C	23.7	28.6	11.2	2.3	69.6	63.9
	<i>Ds74283</i> T3-D D	22.8	28.1	13.5	3.6	108.6	101.1
	<i>Ds74283</i> T56-D A	23.3	28.9	18.5	2.9	85.8	81.4
	<i>Ds74283</i> T56-D B	25.3	31.1	21.5	1.5	45.0	43.0
	<i>Ds74283</i> T56-D C	23.6	28.8	13.8	2.5	73.8	68.8
	<i>Ds74283</i> T56-D D	22.9	28.5	16.0	3.4	103.2	97.1
	<i>Ds74283</i> T3-Co A*	24.8	30.6	22.6	0.7	20.4	19.5
	<i>Ds74283</i> T3-Co B*	21.4	24.2	3.2	5.0	201.6	153.4
	<i>Ds74283</i> T3-Co C	26.2	31.0	13.2	0.7	22.3	20.7
	<i>Ds74283</i> T3-Co D	24.6	28.1	4.5	4.6	139.2	113.9
<i>Ds131885</i>	<i>Ds131885</i> T9-Co A*	26.3	30.9	14.6	0.2	6.2	5.8
	<i>Ds131885</i> T9-Co B*	26.3	31.9	29.4	0.5	10.0	9.6
	<i>Ds131885</i> T9-Co C	23.1	27.8	9.4	5.0	150.6	136.1
	<i>Ds131885</i> T9-Co D	30.9	35.1	10.0	0.4	11.3	10.3
Week 2							
<i>Ds131885</i>	WT D	23.7	28.8	14.2	2.4	73.2	68.4
	WT E	25.2	30.3	15.1	1.2	36.6	34.3
	WT F	25.3	29.6	8.9	1.4	42.6	38.3
	<i>Ds131885</i> T9-D A (L)	23.7	27.9	7.2	3.2	96.6	84.8
	<i>Ds131885</i> T9-D B (L)	24.3	29.9	21.0	1.2	35.7	34.1
	<i>Ds131885</i> T9-D C (L)	23.4	28.2	11.1	3.2	96.6	88.6
	<i>Ds131885</i> T9-D D (M)	22.8	22.8	0.4	1.8	53.4	14.9
	<i>Ds131885</i> T9-D E (M)	22.5	22.5	0.4	3.9	116.4	31.9
	<i>Ds131885</i> T9-D F (M)	22.3	22.3	0.4	4.1	122.4	33.2

Table A.12. Continued.

CE gene ^a	Strain name/rep ^b	Ct tar ^c	Ct ref ^d	Tar/Ref ^e	DNA conc (ng/μL) ^f	Total DNA (ng) ^g	Fungal DNA (ng) ^h
Week 2							
<i>Ds131885</i>	<i>Ds131885</i> T47-D A	24.77	28.0	4.1	3.6	107.4	86.4
	<i>Ds131885</i> T47-D B	28.81	28.6	0.5	3.3	100.2	33.9
	<i>Ds131885</i> T47-D C	31.18	38.5	102.0	2.8	82.8	82.0

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* transformants (T) with a targeted gene disruption are indicated by :D"; complementation transformants are indicated by "Co". WT: wild-type. A, B, C and D indicate the values are from replicate seedlings. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings according to **Table 4.7**. The asterisk * indicated the samples that were extracted using the CTAB method (Doyle & Doyle, 1987) then further purified using GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific).

^c Cycle threshold (Ct) for amplification of the target (tar) gene *DsPksA* from *D. septosporum*. Regression line equation: $y = -3.7597x + 26.877$.

^d Cycle threshold (Ct) for amplification of the reference (ref) gene *CAD* from *Pn. radiata*. Regression line equation: $y = -3.365x + 27.84$.

^e Ratio indicating relative amounts of *DsPksA* to *CAD*, calculated from each regression equation.

^f DNA concentration in each sample quantified by Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific) in a Invitrogen™ Qubit™ 3 Fluorometer (Invitrogen).

^g Total DNA was calculated by multiplying the DNA concentration (ng/μL) by the volume (μL) of the extracted DNA and the dilution factor used (these values are shown in **Appendix Table A.13**).

^h Fungal biomass (ng).

Table A.13. Results for absolute quantification of *Dothistroma septosporum* biomass in pine needles.

CE gene ^a	Strain name/rep ^b	Ct tar ^c	Fungal DNA (ng/μL) ^d	Volume DNA (μL) ^e	DF ^f	Fungal DNA (ng) ^g	DW used ^h	Lesions used ⁱ
Week 1								
<i>Ds69335</i>	WT A	20.0	66.0	30	1	1981.3	19.2	59.9
	WT B	20.5	48.3	30	1	1447.5	20.6	53.1
	WT C	19.9	69.9	30	1	2096.8	25.7	103.4
	<i>Ds69335</i> T2-D A	25.0	3.1	20	1	62.8	40.4	46.0
	<i>Ds69335</i> T2-D B	23.6	7.4	20	1	148.1	54.6	60.0
	<i>Ds69335</i> T2-D C	22.2	17.7	30	1	530.2	44.2	56.1
	<i>Ds69335</i> T2-D D	24.9	3.3	30	1	98.9	13.9	15.2
	<i>Ds69335</i> T40-D A	24.2	5.1	20	1	101.6	31.7	61.0
	<i>Ds69335</i> T40-D B	22.5	14.4	20	1	287.9	43.0	72.0
	<i>Ds69335</i> T40-D C	23.5	7.9	30	1	235.5	35.0	33.0
	<i>Ds69335</i> T40-D D	21.0	35.6	30	1	1067.4	26.6	27.5
	<i>Ds69335</i> T2-Co A	25.7	2.0	30	1	60.4	60.9	51.0
	<i>Ds69335</i> T2-Co B	22.5	14.5	40	1	580.2	53.9	71.0
	<i>Ds69335</i> T2-Co C	24.0	5.7	30	1	172.3	27.3	33.1
	<i>Ds69335</i> T2-Co D	25.8	1.9	30	1	58.5	22.6	17.8
	<i>Ds74283</i> T3-D A	29.3	0.2	30	1	6.8	23.2	42.3
	<i>Ds74283</i> T3-D B	22.7	13.1	30	1	392.7	39.9	72.6
	<i>Ds74283</i> T3-D C	23.7	6.9	30	1	208.0	27.9	58.1
	<i>Ds74283</i> T3-D D	22.8	12.1	30	1	361.6	22.2	42.6
	<i>Ds74283</i> T56-D A	23.3	9.1	30	1	273.2	28.6	35.9
	<i>Ds74283</i> T56-D B	25.3	2.6	30	1	78.8	29.6	49.0
	<i>Ds74283</i> T56-D C	23.6	7.3	30	1	219.2	28.8	43.2
	<i>Ds74283</i> T56-D D	22.9	11.1	30	1	333.9	23.1	29.7
	<i>Ds74283</i> T3-Co A	24.8	3.6	30	1	108.2	38.8	62.0
	<i>Ds74283</i> T3-Co B	21.4	28.1	40	1	1124.2	61.2	92.0
	<i>Ds74283</i> T3-Co C	26.2	1.6	30	1	46.7	25.7	49.1
	<i>Ds74283</i> T3-Co D	24.6	4.1	30	1	122.1	41.0	44.5
<i>Ds131885</i>	<i>Ds131885</i> T9-Co A	26.3	1.4	30	1	43.0	38.0	50.0
	<i>Ds131885</i> T9-Co B	26.3	1.5	20	25.6	745.4	87.1	65.0
	<i>Ds131885</i> T9-Co C	23.1	10.0	30	1	300.0	29.8	39.5
	<i>Ds131885</i> T9-Co D	30.9	0.1	30	1	2.5	25.5	33.7
Week 2								
<i>Ds131885</i>	WT D	23.7	7.2	30	1	216.5	25.3	81.2
	WT E	25.2	2.8	30	1	84.6	23.4	76.3
	WT F	25.3	2.6	30	1	78.3	21.3	70.8
	<i>Ds131885</i> T9-D A (L)	23.7	7.1	30	1	213.9	17.8	34.5
	<i>Ds131885</i> T9-D B (L)	24.3	5.0	30	1	149.9	30.2	92.6
	<i>Ds131885</i> T9-D C (L)	23.4	8.6	30	1	257.8	19.8	36.9
	<i>Ds131885</i> T9-D D (M)	22.8	12.3	30	1	367.7	32.7	86.0
	<i>Ds131885</i> T9-D E (M)	22.5	15.0	30	1	448.7	49.8	73.1
	<i>Ds131885</i> T9-D F (M)	22.3	16.8	30	1	504.1	33.6	46.3
	<i>Ds131885</i> T47-D A	24.8	3.6	30	1	109.0	26.7	38.5
	<i>Ds131885</i> T47-D B	28.8	0.3	30	1	9.2	16.2	22.3
	<i>Ds131885</i> T47-D C	31.2	0.1	30	1	2.2	17.7	27.6

^a Joint Genome Institute (JGI) protein ID corresponding to the *Dothistroma septosporum* NZE10 (*Ds*) candidate effector (CE) gene of interest that was disrupted.

^b *D. septosporum* transformants (T) with a targeted gene disruption are indicated by "D"; complementation transformants are indicated by "Co". WT: wild-type. Low (L) and moderate (M) concentration of spores used for inoculation of *Pinus radiata* seedlings according to **Table 4.7**.

^c Cycle threshold (Ct) for amplification of the target (tar) gene *DsPksA* from *D. septosporum*. Regression line equation: $y = -3.7597x + 26.877$

^d Concentration of fungal DNA (ng/μL) calculated based on the regression equation using the Ct tar value obtained.

^e Total of volume of the DNA used.

^f Dilution factor (DF).

^g Fungal biomass (ng).

^h Dry-weight (DW) of needles with lesions used for DNA extraction.

ⁱ Number of lesions used for DNA extraction based on the DW proportion compared to the total lesion number (see **Appendix Table A.11**).

Table A.14. *Cyclaneusma minus* isolates genome sequencing results.

NZFS ^a	Clean reads	Clean bases	Read length	Q20(%)	Complete BUSCOs (%)	Missing BUSCOs (%)
110	48,163,694	7,224,554,100	150	97.22	1268 (96.6)	23 (1.8)
725	41,918,188	6,287,728,200	150	98.16	1271 (96.9)	21 (1.6)
1800	41,659,540	6,248,931,000	150	97.97	1274 (97.1)	20 (1.5)
3276	45,743,630	6,861,544,500	150	97.96	1277 (97.3)	22 (1.7)
759	48,122,680	7,218,402,000	150	97.21	1265 (96.3)	26 (1.9)
809	48,611,346	7,291,702,900	150	97.15	1276 (97.3)	12 (0.8)
3305	48,146,488	7,221,973,200	150	97.97	1280 (97.6)	11 (0.8)
3325	49,479,662	7,421,949,300	150	96.92	1281 (97.7)	13 (0.9)

^a Scion Forest Health Reference Laboratory Culture Collection number (NZFS).

Table A.15. Protein sequences for the predicted candidate effectors from *Cyclaneusma minus* isolates NZFS110, 725, 1800, 3276 (morphotype verum), NZFS759, 809 (morphotype simile) and NZFS3305, 3325 (morphotype novus).

Available at:

<https://masseyuni->

my.sharepoint.com/:x/g/personal/mtarallo_massey_ac_nz/EauWrsMUSNBHjQAEhCosgm4BI4I_9YpKcxUxEZncoFDmFg?e=oPmmzj

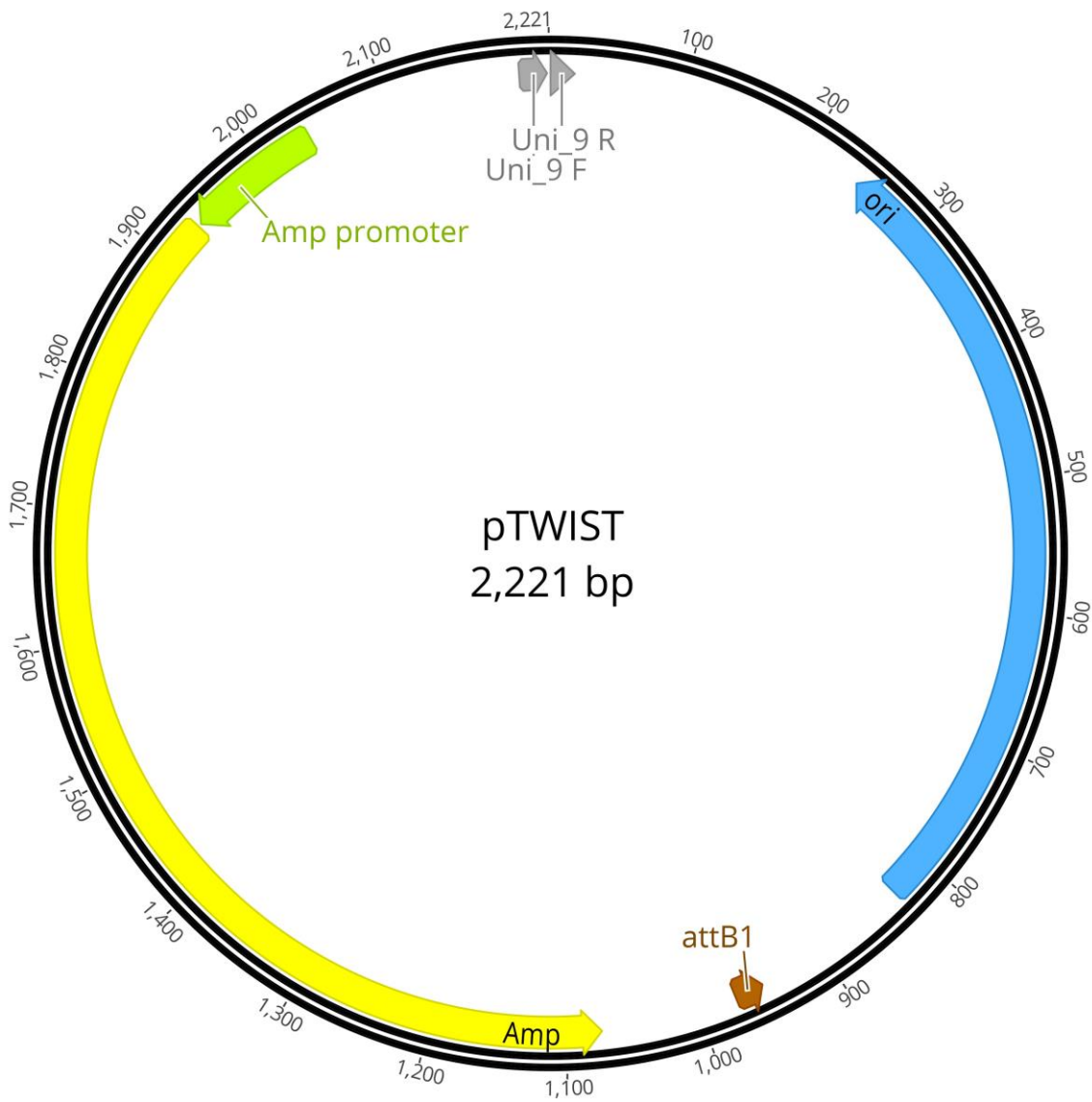


Figure A.1. Schematic of pTWISTamp (de la Rosa, 2022). Key features of interest: *Amp*, ampicillin resistance gene; bp, base pairs.

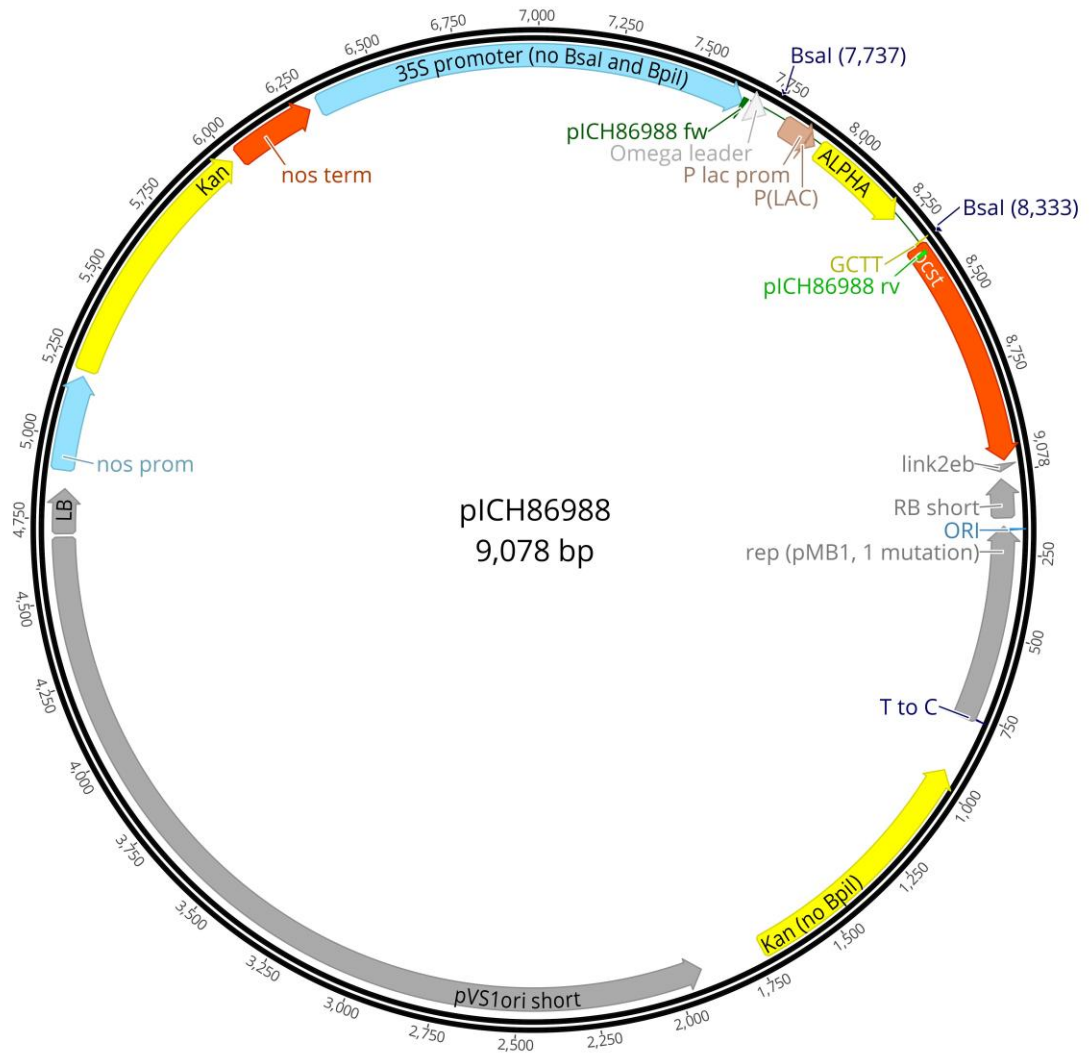


Figure A.2. Schematic of pICH86988 (Weber et al., 2011). Key features of interest: 35S cauliflower mosaic virus (CaMV) 35S promoter region; *ocst*, octopine synthase gene (enhancer of transcription in plant cells); NosT terminator; LacZ alpha; *Kan*, kanamycin resistance gene; pICH86988 forward (fw) and reverse (rv) primer-binding sites for verification of sequencing inserts by polymerase chain reaction; BsaI restriction sites for Golden Gate Assembly; bp, base pairs.

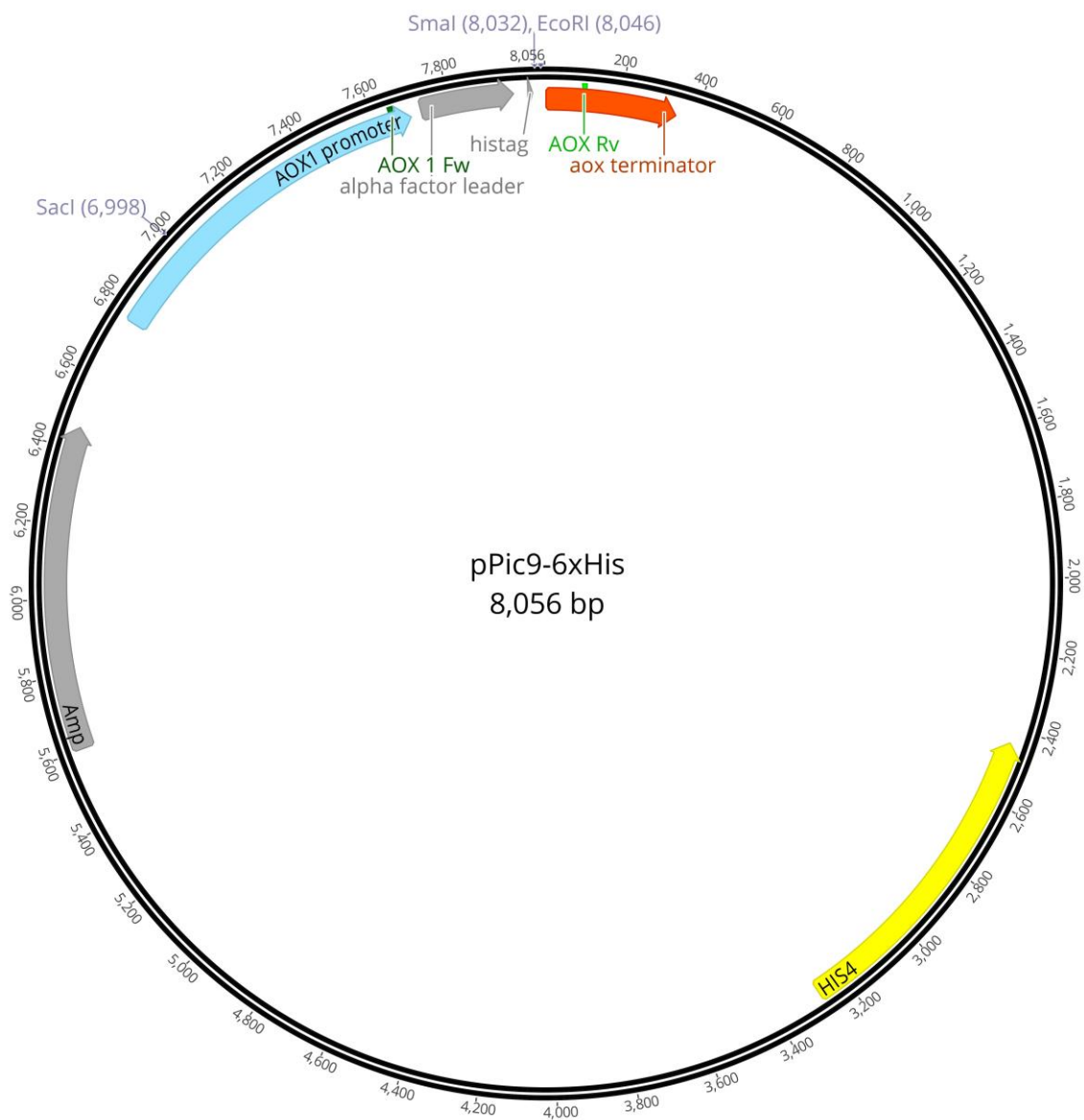


Figure A.3. Schematic of pPic9-His₆ (Invitrogen). The gene of interest, added with a 3xFLAG-tag for detection by Western blot, is directionally cloned between restriction sites (SmaI and EcoRI). Histidine-tag (histag) for purification of the produced protein; SP, *Pi. pastoris* signal peptide sequence; Amp, ampicillin resistance gene; AOX1 promoter, with the restriction enzyme site that will be used for linearization; bp, base pairs.

>FfEcp20-1
MFSNVASTLAFSLGLASMASAAVAAPQSDSGDNLWHISGFDARCANARCQYSFQITSYGDDIPGFSASCQTTGISRNDQTRPCT
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>FfEcp20-2
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>FfEcp20-3
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SKLYAFISKDDNLLQLRYEVTDVATSTVYNYGKAVYAATSDYADLQTPFNVTEISGTSKSA*

>FfEcp20-4
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>FfEcp32-1
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>FfEcp32-2
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LKQVDAVVDGGPSQAYQYV*

>FfEcp32-3
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GTLSTMTVPGGQYVYVNDTGRLRFTTAHSAHQDGTPEAKGFSIVHDAKFQFEKKDWIACPEGPKGTYSVYAAAYKKADKKCL
SNAGEGCLGFTWRVQVGDETASAWQYQ*

>FfEcp32-4
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PSKSCGANLLASNTSGPGAFEYS*

>FfEcp32-5
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VPGGQYVYVNDTGRLRFTTAHSAHQDGTPEAKGFSIVHDAKFQFEKKDWIACPEGPKGTYSVYAAAYKKADKKCL
DNTTVSAWQYV*

>DsEcp20-1
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>DsEcp20-2
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>DsEcp20-3
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>DsEcp20-4
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>DsEcp32-1
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CLGFAWKVQLDDSVPSAWQYSN*

Figure A.4. Legend on the next page.

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AGDDCLEFSWRTVQVADGTESAYQE*

>DsEcp32-4
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QCLGANLLASNTSGPGAFEYS*

>DsEcp32-5
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RAIEADNTTIPAWQE*

>DpEcp20-1
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>DpEcp20-2
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>DpEcp20-3
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>DpEcp20-4
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CGPYVISSNWSGQFGDGNFQTLAVVNGKQIIPAYTDKQLVSGQVVTDPQSYTPQNLPS*

>DpEcp32-1
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CLGFTWKVIQLDDSVPTAWQYSS*

>DpEcp32-3
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GQSTLSLDTMVPGGQQVYVTEGDDATGQLAGQLKFTAHSVATSGPALYDGFISIGDDEELQFEGKDWFACPIDDFNSGYGIWAVSRV
AGSNAGADCLSFTWRAVQVGDETKSAYQYQ*

>DpEcp32-4
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SKQCLGANLLASNTSGPGAFEYS*

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Figure A.4. Amino acid sequences of Ecp20 and Ecp32 family members from *Fulvia fulva* 0WU (Ff), *Dothistroma septosporum* NZE10 (Ds) and *Dothistroma pini* (Dp).

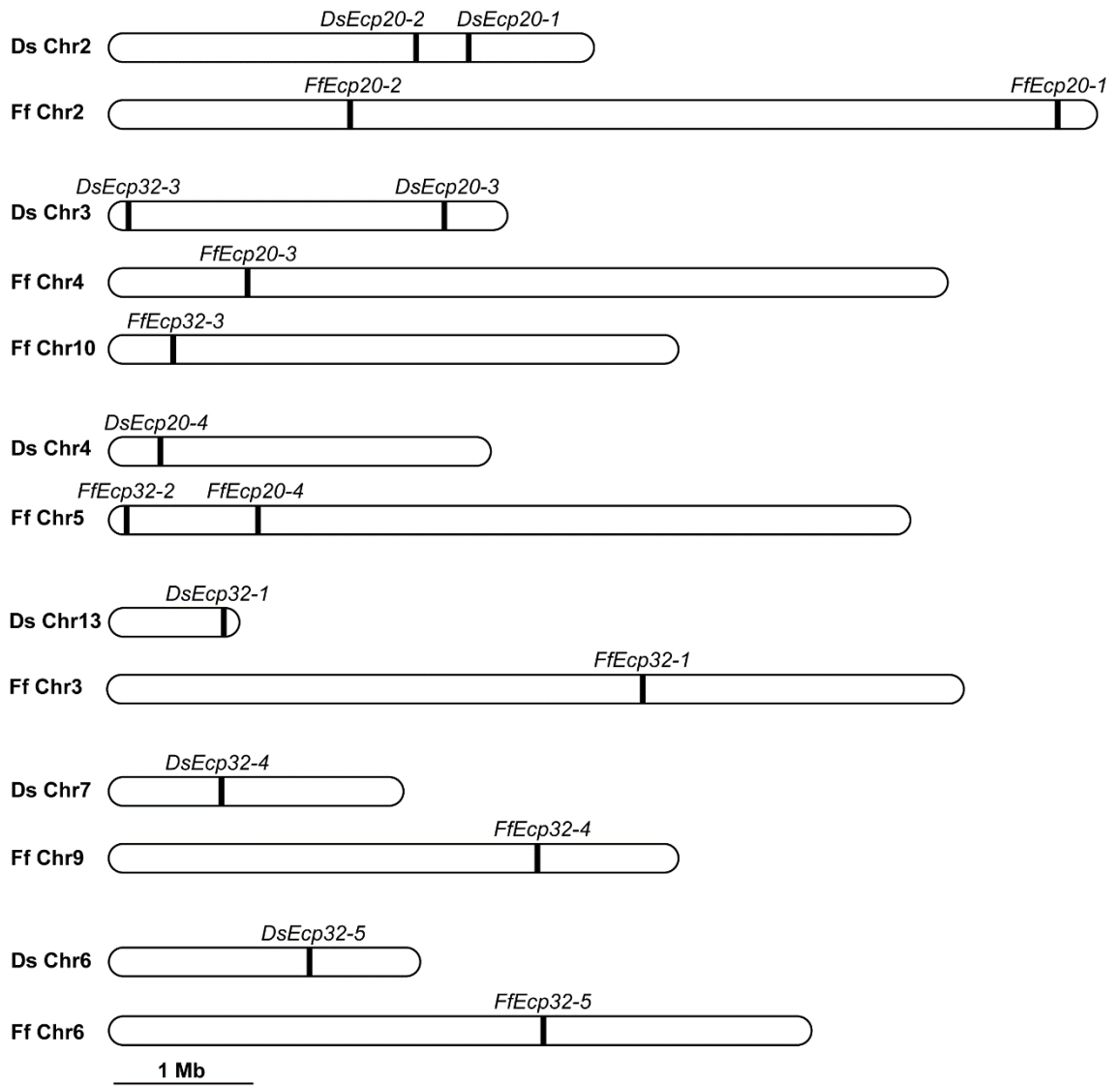


Figure A.5. Chromosome locations of *Dothistroma septosporum* NZE10 (Ds) and *Fulvia fulva* Race 5 (Ff) *Ecp20* and *Ecp32* genes. Matching chromosomes in the two species, as identified in the comparative genome analysis of Zaccaron et al (2022) are grouped together, with Ff Chr4 and Ff Chr10 both matching with Ds Chr3 from *D. septosporum* (Zaccaron et al., 2022). The matching chromosomes show mesosynteny (the same gene content is largely conserved, but not necessarily the order); the *F. fulva* chromosomes are longer due to their higher repeat content (Zaccaron et al., 2022).

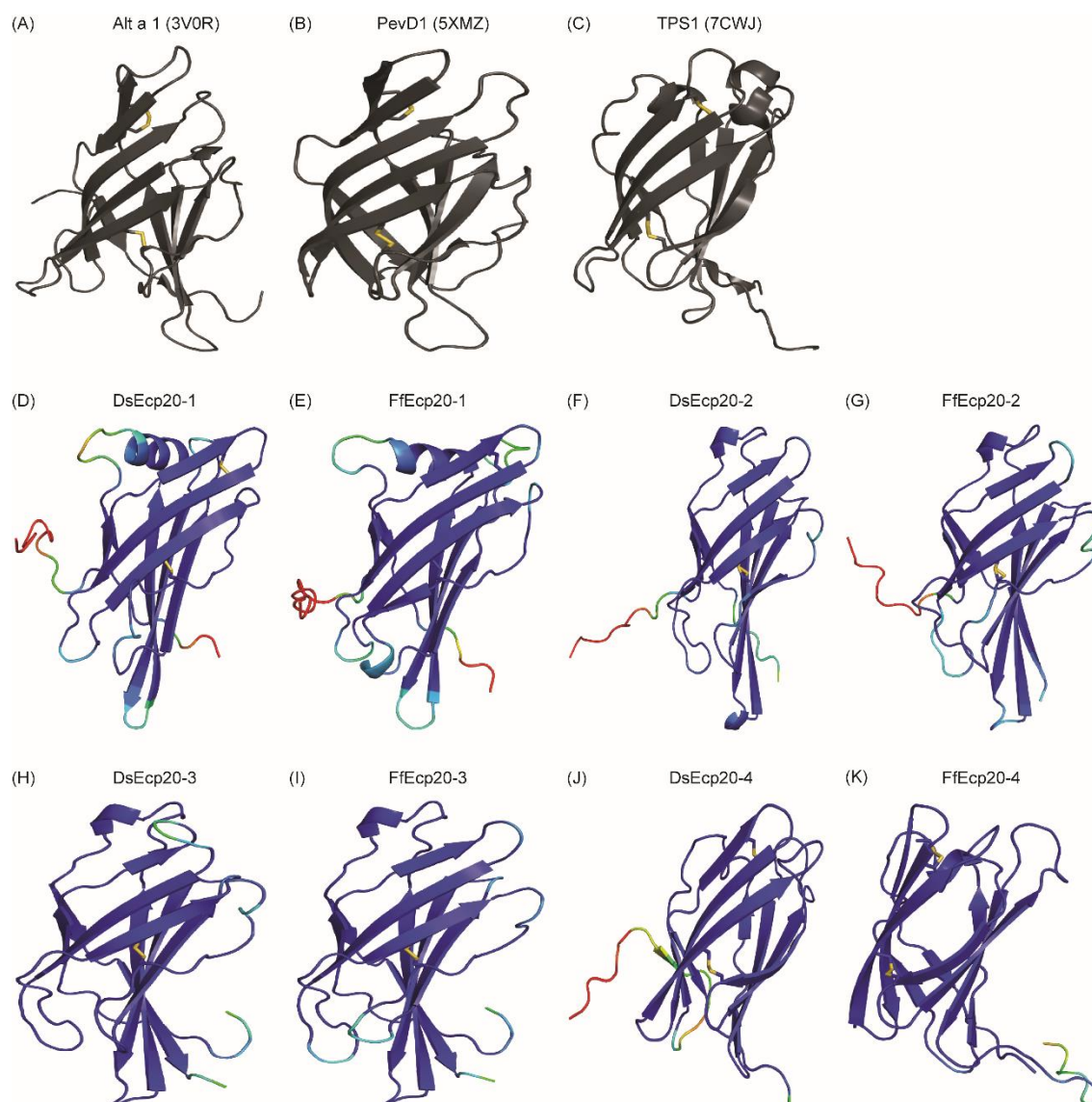


Figure A.6. Predicted protein tertiary structures of Ecp20 family members from *Dothistroma septosporum* and *Fulvia fulva*. **(A)** Solved crystal structure of Alt a 1 from *Alternaria alternata* (Research Collaboratory for Structural Bioinformatics protein data bank [RCSB PDB] ID: 3V0R) (Chruszcz et al., 2012). **(B)** Solved crystal structure of PevD1 from *Verticillium dahliae* (RCSB PDB ID: 5XMZ) (Zhou et al., 2017), **(C)** Solved crystal structure of TSP1 from *Trichoderma virens* (RCSB PDB ID: 7CWJ) (Gupta et al., 2021). **(D–K)** Predicted structures of **(D)** DsEcp20-1, **(E)** FfEcp20-1, **(F)** DsEcp20-2, **(G)** FfEcp20-2, **(H)** DsEcp20-3, **(I)** FfEcp20-3, **(J)** DsEcp20-4 and **(K)** FfEcp20-4. Disulphide bonds are shown as yellow sticks. Protein structures were predicted with AlphaFold2 and rendered in PyMol v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022). Structures D-K were coloured in PyMol according to their AlphaFold2 pLDDT score: dark blue for highly confident predicted regions, light blue and green for regions of low confidence, and red for very low confidence regions.

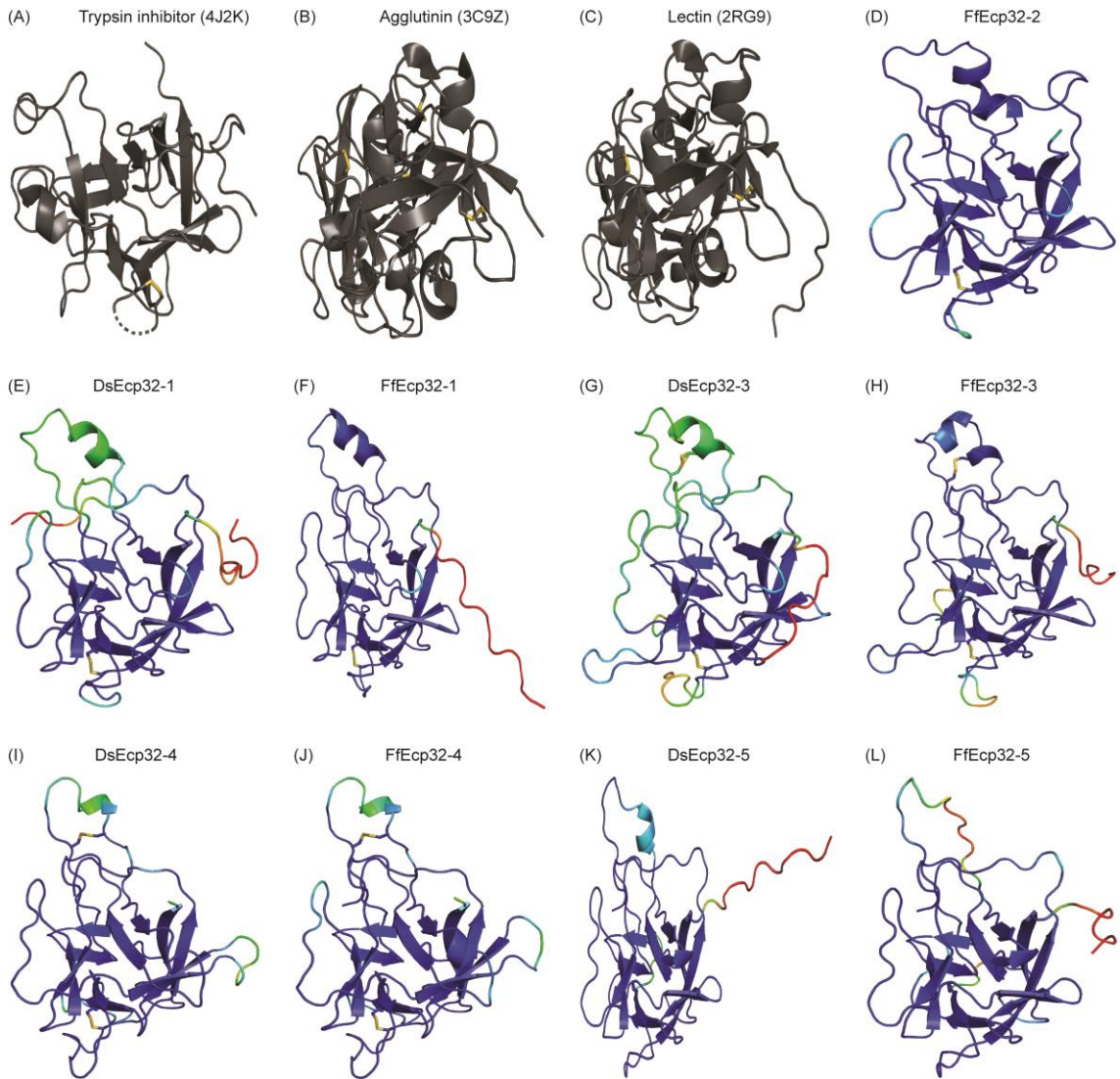


Figure A.7. Predicted protein tertiary structures of Ecp32 family members from *Dothistroma septosporum* and *Fulvia fulva*. **(A)** Solved crystal structure of trypsin inhibitor from *Enterolobium contortisiliquum* (Research Collaboratory for Structural Bioinformatics protein data bank [RCSB PDB] ID: 4J2K) (Zhou et al., 2013). **(B)** Solved crystal structure of agglutinin from *Sambucus nigra* (RCSB PDB ID: 3C9Z) (Maveyraud et al., 2009). **(C)** Solved crystal structure of lectin from *Viscum album* (RCSB PDB ID: 2RG9) (Karpechenko et al., unpublished). **(D–L)** Predicted structures of **(D)** FfEcp32-2, **(E)** DsEcp32-1, **(F)** FfEcp32-1, **(G)** DsEcp32-3, **(H)** FfEcp32-3, **(I)** DsEcp32-4, **(J)** FfEcp32-4, **(K)** DsEcp32-5 and **(L)** FfEcp32-5. Disulphide bonds are shown as yellow sticks. Protein structures were predicted with AlphaFold2 and rendered in PyMol v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022). Structures D–L were colored in PyMol according to their AlphaFold2 pLDDT score: dark blue for highly confident predicted regions, light blue and green for regions of low confidence, and red for very low confidence regions.

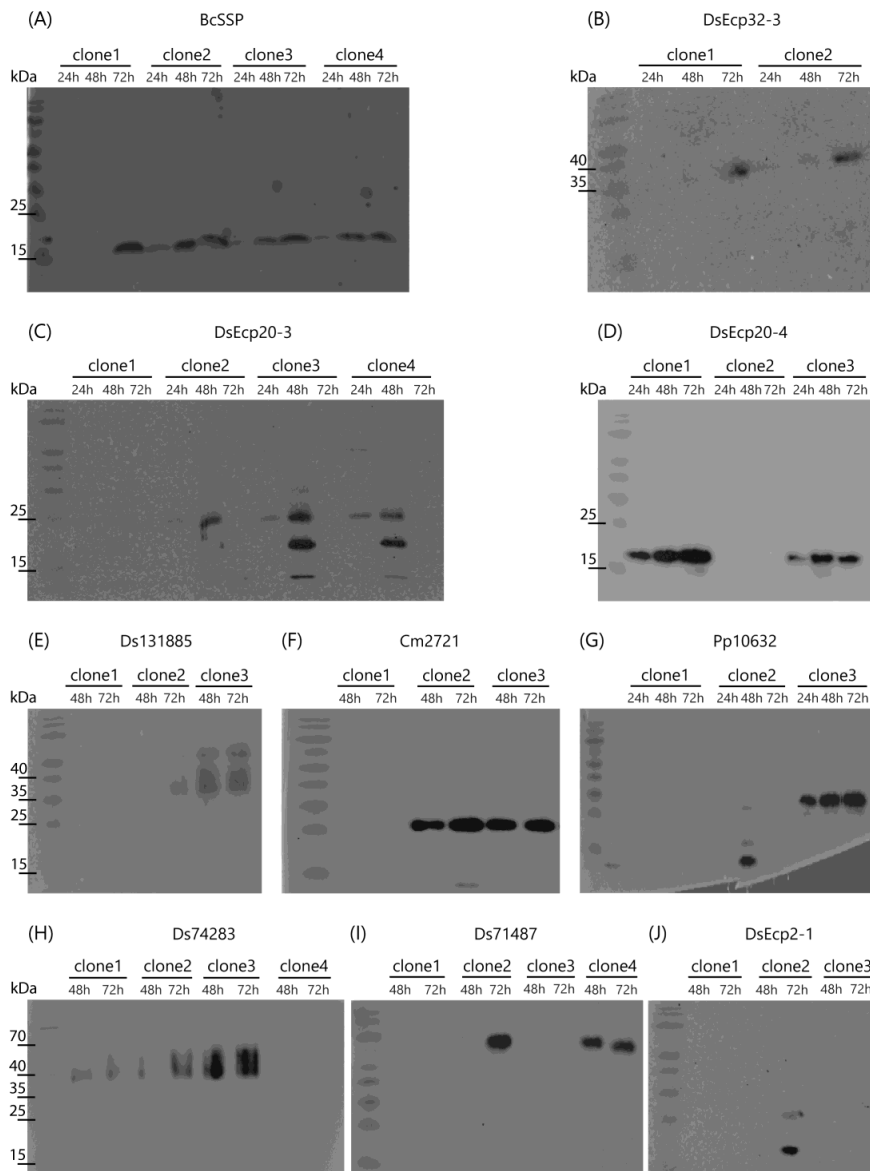


Figure A.8. Western blots of *Fulvia fulva*, *Dothistroma septosporum* and *Dothistroma pini* Ecp20 and Ecp32 proteins. Western blot detection of Ecp20 and Ecp32 proteins that did not trigger a cell death response or triggered only a weak cell death response when expressed in *Nicotiana benthamiana* and from Ecp20 and Ecp32 proteins that previously triggered a cell death response in *N. benthamiana*, but that now lack the PR1 α signal peptide (Δ sp). Expected sizes (kDa): FfEcp20-1: 17.3; FfEcp20-4: 16.2; DsEcp20-2: 17.1; DsEcp20-4: 16.2; FfEcp32-1: 22.6; FfEcp32-2: 21.6; FfEcp32-4: 22.5; DsEcp32-4: 22.6; DsEcp20-1: 19; Δ sp DsEcp32-3: 22.8; Δ spFfEcp20-2: 17.2; Δ spDsEcp32-1: 22.8; Δ spDsEcp32-5: 22.2; Δ spFfEcp32-1: 22.4; Δ spFfEcp32-2: 21.4; Δ spFfEcp32-3: 22.6; Δ spFfEcp32-5: 21.7; Δ spDsEcp20-3: 17.5; Δ spDpEcp32-3: 22.8; Δ spGUA2 Ecp32-3: 22.8; Δ spDpEcp32-1: 22.8; Δ spCOLN Ecp32-1: 22.6. * Δ spDpEcp20-3 and Δ spGUA2 Ecp20-3 proteins could not be detected. Anti-FLAG antibody was used for immuno-detection. Marker on the far left of membranes was the PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific). Bands that are not accompanied by a label do not relate to this study.

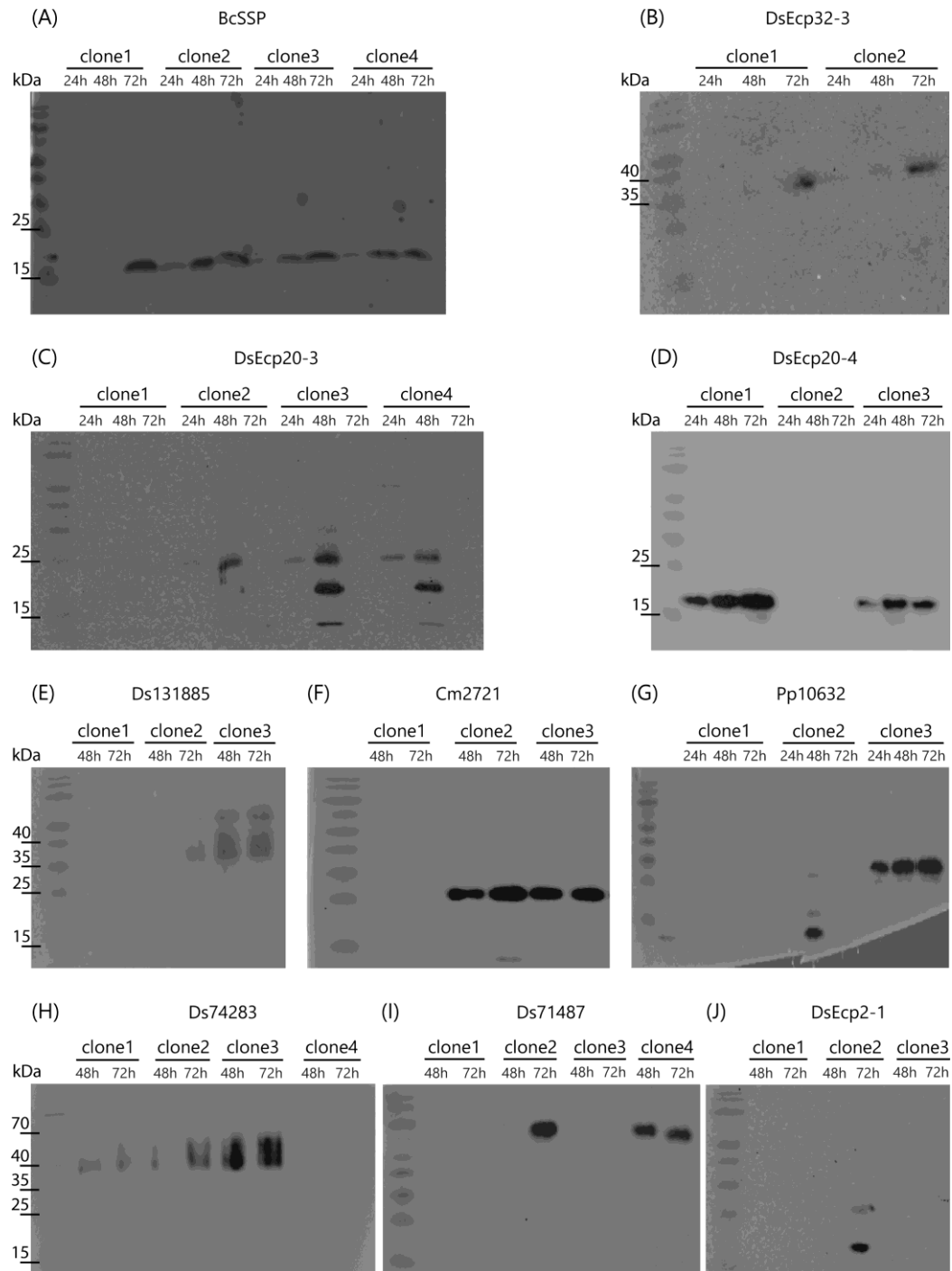


Figure A.9. Western blots of candidate effector proteins expressed in *Pichia pastoris*. Western blot detection of proteins **(A)** BcSSP, **(B)** DsEcp32-3, **(C)** DsEcp20-3, **(D)** DsEcp20-4, **(E)** Ds131885, **(F)** Cm2721, **(G)** Pp10632, **(H)** Ds74283, **(I)** Ds71487 and **(J)** DsEcp2-1 heterologously expressed in *Pi. pastoris* for 24, 48 or 72 h. Expected sizes (kDa): BcSSP: 10.5; DsEcp32-3: 23.2; DsEcp20-3: 17.9; DsEcp20-4: 16.5; Ds131885: 16.1; Cm2721: 16.7; Pp10632: 16.6; Ds74283: 23.0; Ds71487: 22.6; DsEcp2-1: 19.1. Anti-FLAG antibody was used for immuno-detection. Marker on the far left of membranes was the PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific). Ds: *Dothistroma septosporum*; Cm: *Cyclaneusma minus*; Pp: *Phytophthora pluvialis*; Bc: *Botrytis cinerea*.

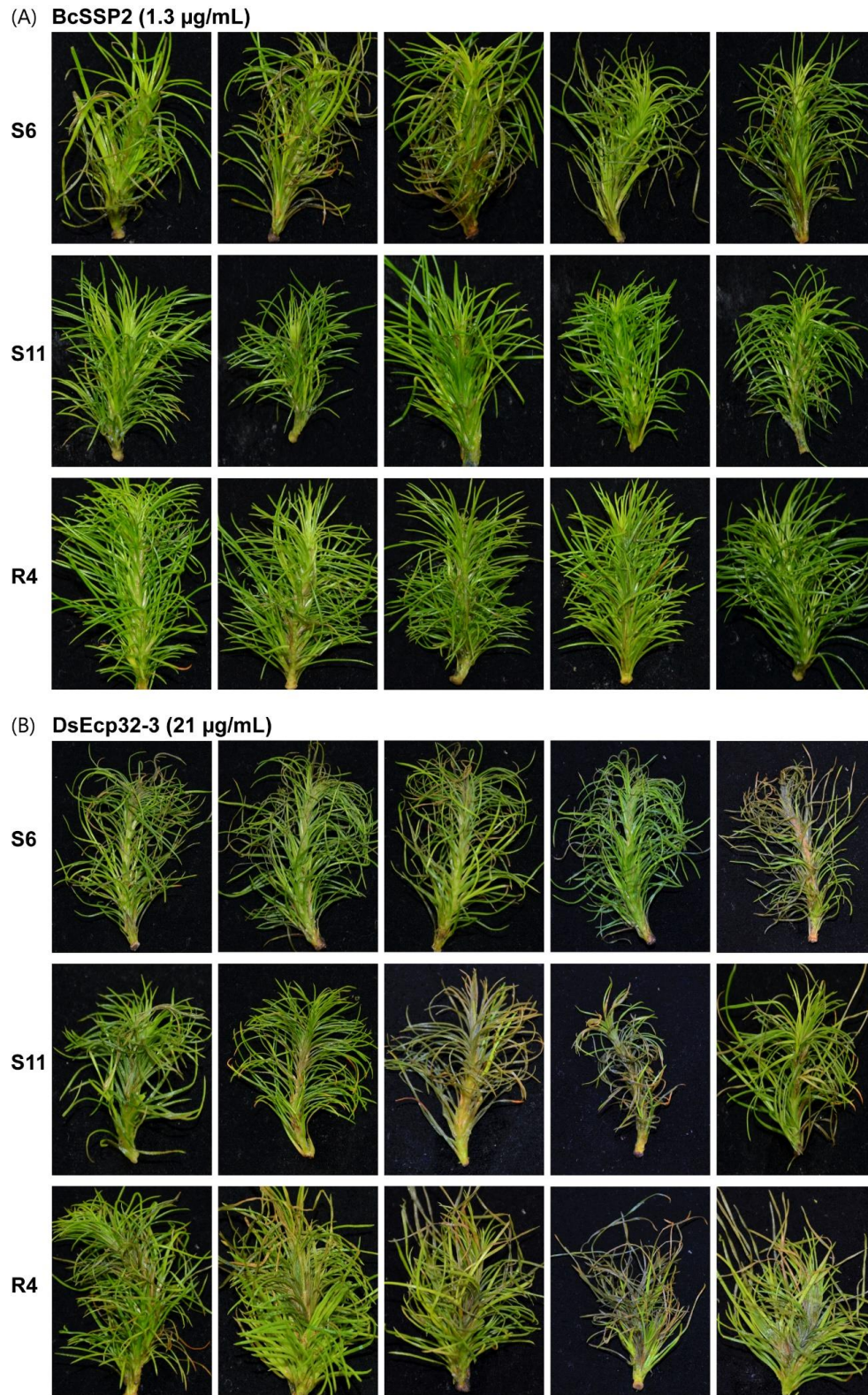


Figure A.10. Replicates of *Pinus radiata* shoot tissue infiltrated with purified BcSSP and DsEcp32-3 proteins. Whole shoots of *Pn. radiata* derived from family seedlots that are relatively susceptible (S6 and S11) or tolerant (R4) to *Dothistroma septosporum* infection were infiltrated with purified BcSSP **(A)** and DsEcp32-3 **(B)** proteins produced by heterologous expression in *Pichia pastoris*. Photos were taken 7 days after infiltration. Ds: *D. septosporum*.

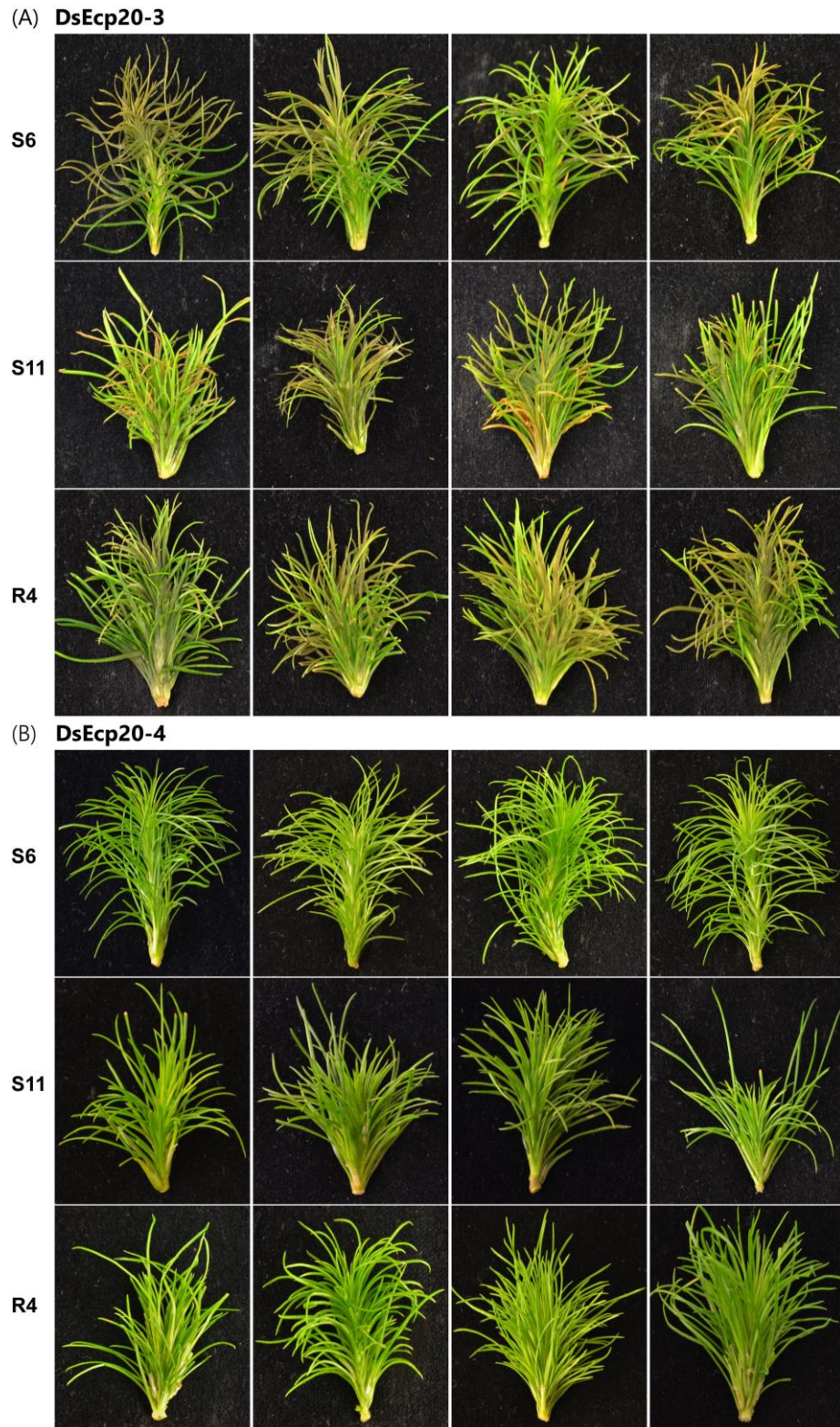


Figure A.11. Replicates of *Pinus radiata* shoot tissue infiltrated with purified DsEcp20-3 and DsEcp20-4 proteins. Whole shoots of *Pn. radiata* derived from family seedlots that are relatively susceptible (S6 and S11) or tolerant (R4) to *Dothistroma septosporum* infection were infiltrated with purified DsEcp20-3 **(A)** and DsEcp20-4 **(B)** proteins produced by heterologous expression in *Pichia pastoris*. Photos were taken 7 days after infiltration. Ds: *D. septosporum*.

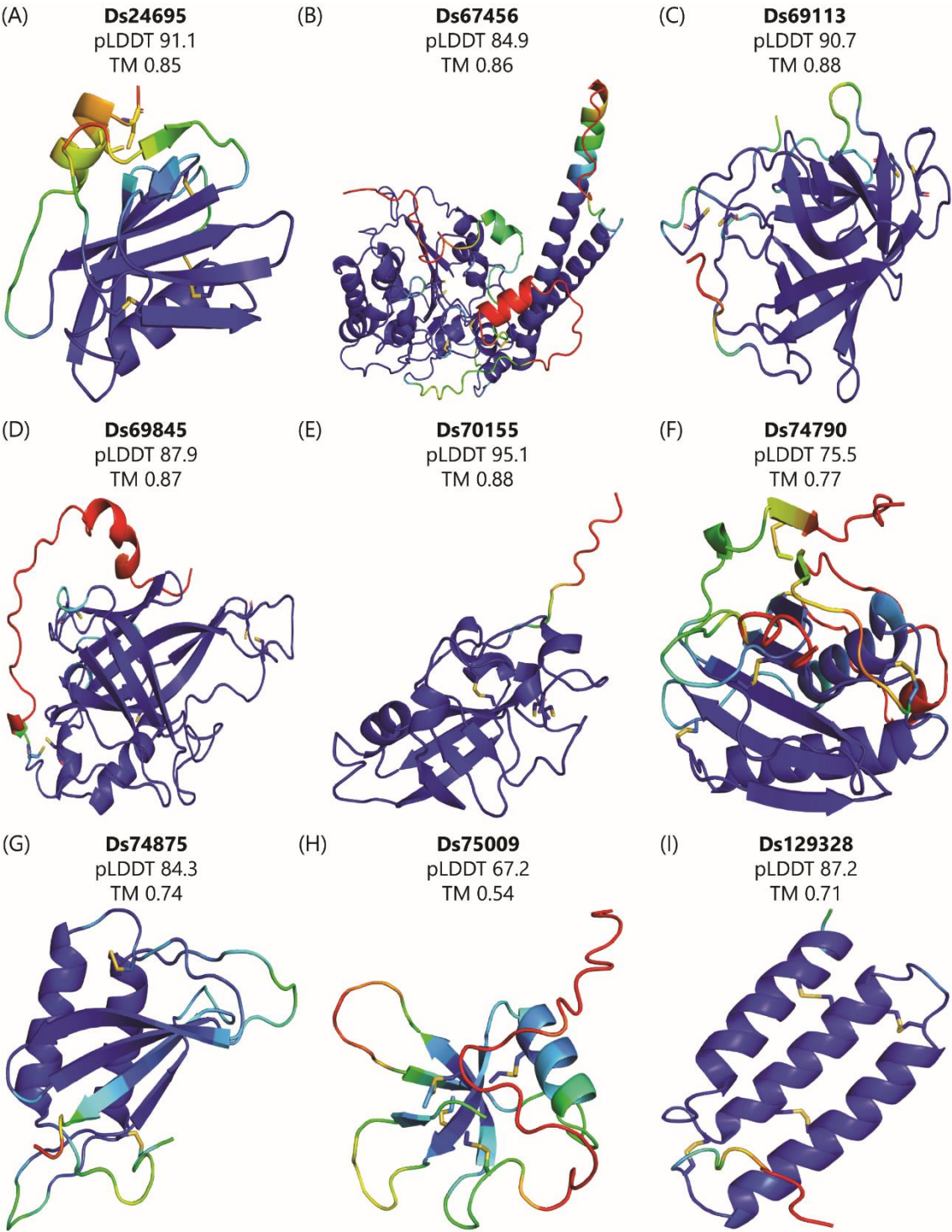


Figure A.12. Legend on the next page.

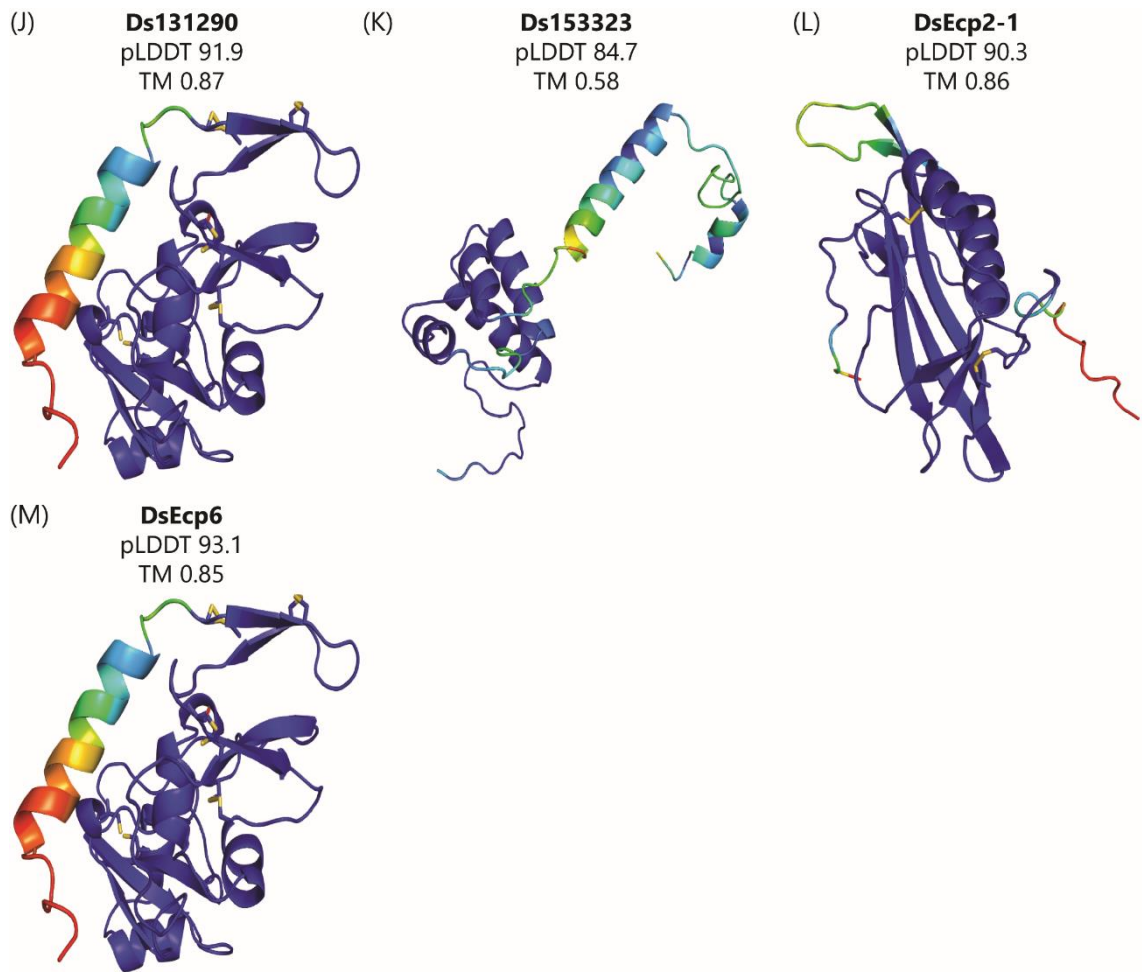


Figure A.12. Predicted protein tertiary structures of candidate effector proteins from *Dothistroma septosporum*. **(A–M)** Predicted structures of **(A)** Ds24695, **(B)** Ds67456, **(C)** Ds69113, **(D)** Ds69845, **(E)** Ds70155, **(F)** Ds74790, **(G)** Ds74875, **(H)** Ds75009, **(I)** Ds121390, **(J)** Ds131290, **(K)** Ds153323, **(L)** DsEcp2-1 and **(M)** DsEcp6. Disulphide bonds and/or cysteine residues are shown as yellow sticks. Protein structures were predicted with AlphaFold2 and rendered in PyMol v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022). Structures A–M were coloured in PyMol according to their AlphaFold2 pLDDT score: dark blue for highly confident predicted regions, light blue and green for regions of low confidence, and red for very low confidence regions. Ds: *D. septosporum*.

>Ds19040
MKTPLVLFFGYTAAAVLPRANVDRTPTAAATPGAVVVRRELSGTVTASWTSNGNSTTSTASSSVITAAPTDLVGPDDGGSPCTPHSICVD
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>Ds24695
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CVQKSAITEQLAYAASADAQSAVLVKGPNQFEQDFGEICGVYCT*

>Ds42387
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PEEFDAAGKRKSGRVVVVRVGDGTGVGLEPVDLTPAYQQTEGDRFQSIDLERIGGLVEKARNAPSKEYQ*

>Ds46236
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TIGQIKALNNNVDPESLAIGQQIVLPQNCMNITAVA*

>Ds63725
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>Ds67456
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>Ds69113
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>Ds69845
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>Ds70155
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>Ds71487
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WVAGKYPPQNPSCEKVEVVRKFA*

>Ds71565
MKSTFFAAAVAVVSLAGTSFAAPLDRRQCQIDTDGVTCTSTPTCTTQEDGSVECMRRSDNELVKRQCQIDADGKQTCSTTPVCTTR
EDGSVACVLKRAESEELEKRCYIDAQGEQHCNDTPVCTTHDDGTVCVMKRSENLSKRQCYINEDEQICSTTPVCKTQEDGTLACT
MKRSEDLSKRQCYVNEEGEQVCATEPQCYKNDDGSEYCVLKRDEHLSKRQCYINEDGEQICATEPQCYKNDEGSEYCVL*

>Ds74283
MKYLALFLALVAFVSAYNNTTAAYKLQTRLKVADVSKQEYDGLYLSSYHTGAGLNDVVFSDPEPLGNATLAATNITANGTTYNQFLDL
GNDFPWGLVMESNIEFYSAWQPVNELNAGASGSNYDVSGFFINETGLQWTSNPGQVGTDDDEFEGGWLVCDWWHGVPLFFRISYYS
SSVYQAPASCADVYLIPQYI*

Figure A.13. Legend on page 286.

>Ds74790
MRCPGALWSTLLALHSCSTVNGAPVADTAVMMFDGQPQPSRWMLYDLEMPFGPLNQSEIMETSIERRTQVTACERVAFAAASCFVI
GNSLKPIVITYLGNVIINARDQQRSCGTFGTGAVSGVTFRYQANGKNCDDTAAEEATIQQGAIYHLSTVDNGQLCATECLDLTHGGTWDGYLLI
GPTNSFDSSAYCGPQLTKDMFSKSSGGKKDVPK*

>Ds74875
MAPLWRTLLTLLAAGPLVFARCNVGDQKPARADDVAAISQLVQRGGNCGPLAYGSAKVLVVNSAQIVGVNVGTNQQATSASCA
GVARAVGLIMDECTTDDIVNGKTVSGRHTVPGNDNFNIALRIDD*

>75009
MQFAAFFAGLAAISTATPIAGEEPGISGAGTYGPGQVSDGDAAGICTALYTPQCCETSVLGVADLACNTPSVSVDSEQSLINDCAKTGA
TAECCVLPVAGQALLCYDL*

>Ds124808
VFIPAIVAAALYGIGAFVIMPLIRQHRNRYSQLPLESISQHTNSFRDLATAVTRLVVPNRVVIDASESRRGSTSGEEFVFGDAEGDSMV
GFDVGRSDRQVRGANIGSNRSVEEV*

>Ds129328
MRSSALIVLAATGIALAQTDYHATSGCGSQIDDIIANCLGTQTAQLQSCAVNDWACMCEQNNVLTTCYNNCPADPRKPAAESQSVS
WCGAAGA*

>Ds131088
MTSFITLSTIVLIAIFAGYIYVGLSPGTRKRMEEKALETMGEASHPFTLTNKASYVTKDMLGKVPDSDQQLKDLKGLSNAAGGALQ
NPLGKLAGDVADEGTRPATGR*

>Ds131290
MQYTLLTISALAATACTPFAMPAGFSAELDNGLETRQASNCTEYCSNSAGCVCTVRPSDCTAFYTVQPSDTCLSIAKSYNNFTVTQFYK
WNPSIGQTCFGLQAYVPVICIDTPWYQYTPPVQPDFGTHYTPDQTPVPVMPNIIDSCESYELVEPGKRVDELAENGFDSEMFPEWNG
NATTAWAAYWVCVKA*

>Ds131885
MQFRFTIGLAITLAYANATLDPATSNKSGKCPKTIACSASDVSTAIQAAECSHNTRTPGTQTFAVFETDHSYDSSKGAPYGTCSAYTCAVP
TTFTSDSDCWTFWWSGSGTASGVGTGCISSPTTGECGCENSSGQFIAGSSSCT*

>Ds153323
MAFLFYTISFAMILIATLYITRANWKPYAPPYITAPGTIPEPLYDISERIQNYVDSWRYSRVPTNFQEDAEAGLHSSNFNLAENIEAGDS
RQGLDEAAKREIYVIMKRRGVVFDEARRMWIEEFRRREGIADGRPLDRKAVVFS*

>Ds158381
MHFNAAVLAALLPALSLAAVLSPRFRNAGNTPGSNMCDASTFNDGTTYDIPQAPVADCRNLVNSIDTSQTFTAHSWSRPWTYGRG
AFNVRVIAGSDAGLVGGADAADLLNDSSRKFGESGSYSCRGSYGVVSAEGEVDCNAENGGRVVRVWVADSSYNPRNDE*

>Cm835
MFTPTLLTALLPALAAASPIFKRDDYVPPYTTSLLSLRASPIHFSAINASHGHFSIGEPSTSYCPVSDCSGFPNITLISIYEASGTAYMSTNVP
GGQNVVYAESGALSFTPHQEYVFPDGSYTTGFAVSNNGNGVDLLNFTQANTFTACPPCTGGEFPYQVFLDVPGFNDSSVPSGSTAD
CLPFGIAINNYTTDAGAFQYE*

>Cm2492
MKSAVFASIFAFAASSALAQYDTQSPSYLQLISDNSTINGSYLTSCHSAGAAIESLCLYGPEAADPDMAETFKLNTTSPYVANATGLITVW
LQGSNFNESEILNFYNNPTSNIAMPLFEPSETGQAIAFDCEDNAYISAYLNYSTSPSYYGEDGASVQAFYNWYLCRTNDEGYTYETVWW
VTAGEPVTDCSAVDLHRVVF*

>Cm2721
MASLLKSLLLAATLVSANVDSCSSNTKGKCPGTLKCSASASSAIQAAECSHNTRTSETQTFAVFVTDHEYDSSHGAPYGTCEAYTCTP
PTEDEMTOVDEDCWTFWWSGEGEESGVGTNCIEDPTTGECGCEDSSGTFIAGSDSCT*

>Cm3176
MLRSDGAMANLKRFLPILALVTVAGLVFMFLDLNVSETLQSLPLERIGLDIKAPSLDTAQPVDSVVLPESPVGKGDKEPSKKGKNDGSE
GYKGTNSGLDNMKPGKTKPVGERYTKNLIIPRIKEEDVSWIDENFGGDNKIPVIYVADDPAPNHPPKNKGHEVMIYLYIIDHYDELAD
VNIFMHSRYAWHNDELLGQDAVQMISRLSAERVQREGYMNMRCHWSPGCPDWMHPGNIVEDAQKQEETMLAQSWSELFLDPI
PQVLAQPCCAQFAISRDRIRSIPLARFVYRDWLLRTSLSDYISGRVWEYVWQVFTAQTVYCPKEHICYCDGFGVCFGNEEKYRLWWES
MWAKRALDAELSDWKEKDEAIKEATENGKPDKLEVPFEGKNVELEDKIKVLEEWLERERTEAMQRGDVAKNRALECGREWHGDG
GF*

Figure A.13. Legend on page 286.

>Cm3415*
MIDLLIALMLLIFSLILVAALYIVRKVRRSRQMKHQSLPQYNDHSNHRSHQRRLTISTSASHGRKSSIIDYKHHGSGESSPCLSPENIPEIH
ITFPDEHDEGDGTRKSGRVVVRVGEHGNVGLKDDQLPAYEKESGERFQSLDLERMGGGLKEKEEYA*

>Cm4412
MQFSLLSLAFLAGAAVASPLALEHQFERRDNCTTYSAAAGTVCYHTPSDCSATYLVQPNDTCTSAIYNNFTLSQFYWNPDPVGQTC
FGLRAYVPVICIDTPWYTFVPPVQAAEGTVEAASAVPVPVMEITSNCTKFELAGAGYVAEIVSQNNISEDDFLNWNAYIDKTNPAWE
GYWVCVGV*

>Cm7313
MPKAATPPGDMTILGDYFNMLASKVSEMTMSEAPKCDLSNAVLVPASPTPLPIVSEGLSLKHVAIGRGTQNYTCDLSNSTAIPVATGA
LATLYNASCVASSYPRIALLPNTTLQFADPAYNTNSLFPTDMVVS GHYFENSTTPTFNMDTELMQLGFAHASKNNSVSAPADASPG
VNGEGDGAVALWKLARADATGGLQEIVRVNTAGGSPPATCVGAEEAFVQYAAEYWFFQN*

>Cm7343
MRFSILSLLPLAASGVFAHPHIEQRDGVAVVRADACPATTS DPCRTISNYTVVSGDTLTITAEKYNSSVDALVYVNHIA DRDLIYVDQVLQI
PAPECTPCAIVGTYYTVKGDTLNIAIKEENTTVAIAIYVNP SITNINSISPGQVFNIPSYECDTVAPLKN TTGTACVDP SKSNGTYTVVK
GDTLTITADGFGITLASLEADNSQIENFDEIEIGQQINIPICMTTRKRGYLEFGKF*

>Cm8683
MFMKLPRAAFDWSSLQPPDPYIPHPAPETSATAKGAKMSKTSLLKTLFIPALISLVLYLVLYFALIPFWRRYRNRY SQYLPLDAISNRTQS
LRQRAFDAARLLIPSSWRTDFGGGRFAISAQDGS GSDFDDEGEELYSVDENRRRAALSLDERRRRQDDIRQQDEGRRLSRELEEVFRD
ESDEEEDNRLSRSGSIASLSTRFR*

>Cm8840
MQYATIFGALCAAGLAAAAPLQERAVVYDVVTDVIVVTVDSE PASTYAPAPVYTPVSVSTPPALVSVAS PSSSVVVSTAPASSYTPPA
SSYTPPKSSGAKAAIANTVNTVASTSTGGYAAPTDFESTAIYHHNLHRANH SVSDMTYNSTLAGYAATVAATCVFAHDLSEGGGGYGQ
NIASFGETGDVEALGASSMLAGAITDMWYNGELGLFLPEYGEANPDFTNFEGWGHFSQLVWKNSNQVGCASQFCPQSTGLFDFDG
WFTVCNYGPAGNMGGEYGDNIMKPLGHPSITA*

>Pp7927
MQVSAAISFVAAA VAVVNADSFTAEDQAVWVDRHNYFR TTGLPWSAGDMRRVGVWSSDLATTAATAAKCSATTAAGVNAYQSAA
SNSSAVVDEAIQQWVETSMTTLKTLAQPGASGV DVGTYNAYSQVLWASTTSVGCATASCSSGDLVVC EYSPAGNDGSSAWYVH
ADTASDCPSGTTAQTGLCLVEGDAANDQIAAISAGKLT YEVPAYVADMQTVLIEAARAIANGATS NATRSTTQATTATTATPTATTAA
PTSTTATPTATTAAPTSSSKETPSSKETSSSKETSSSKET FSSKEDSSGATKTGSSKKTASSSSDTSSSAKSSTDS SADSSTKQSSQTASNS
DESAPGTVKASSDNGSTFTNQETSTTGSSQTSGSTSATNTAN TESKTASTASSSGGATSINNQSSTKSGGGISAAGLAGIIVLAIVGI
AAFGVVMYSYKRNNQQRQREIMRDGGIRII*

>Pp10632
MHFSVFVALVAAPALVSATLDPASSNSKGKCPSTYSCSATKVS KAIQAAEC SHNTRTSTTQTFAVFVTDHQYDGNNGYPYGTCSAYTC
DPPTSAEMEDNDDCWTFWWSGNGTDSGEGAGCIKDP TTGDCGCENS DGT FVADSSSCV*

Figure A.13. Amino acid sequences of candidate effector proteins from *Dothistroma septosporum* NZE10, *Cyclaneusma minus* NZFS809 and *Phytophthora pluvialis* NZFS3000. Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*. The asterisk * following protein ID indicates the protein was wrongly predicted in the original annotation and the correct sequence was used.

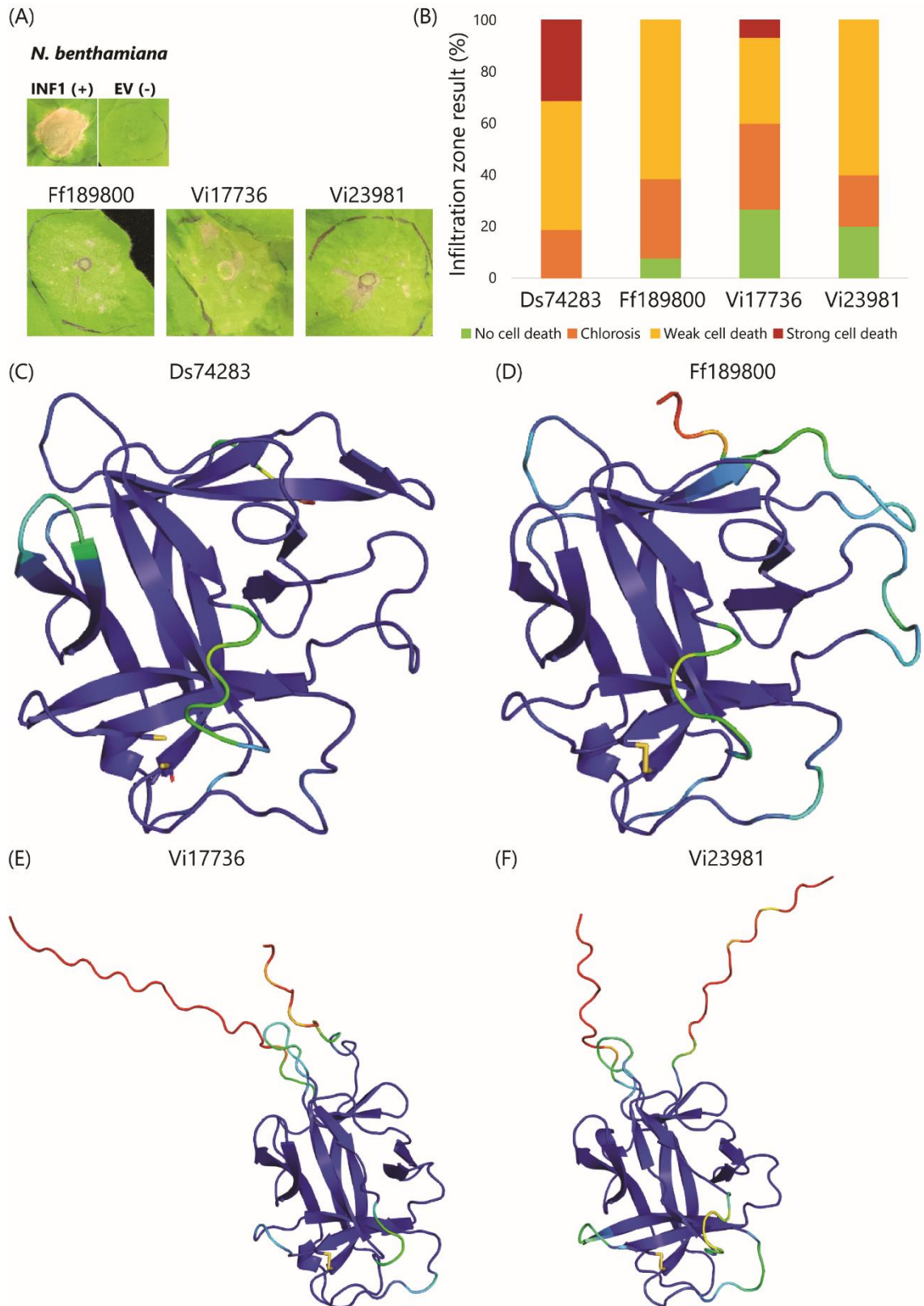


Figure A.14. Orthologues of Ds74283 from *Dothistroma septosporum* NZE10 in other fungal pathogens also trigger chlorosis/cell death in *Nicotiana benthamiana* and adopt a β -trefoil fold.

(A) Protein Ff189800 (Joint Genome Institute (JGI) ID) from *Fulvia fulva* and proteins Vi17736 and Vi23981 (JGI ID) from *Venturia inaequalis* were expressed in *N. benthamiana* using an

Agrobacterium tumefaciens-mediated transient expression assay to assess their ability to elicit chlorosis or cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. **(B)** Graphs display the percentages of infiltration zones that showed chlorosis or cell death in response to each protein, divided into four categories: strong cell death, weak cell death, chlorosis, and visible response. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no cell death control. Photos were taken 7 days after infiltration. **(C)** Predicted structure of Ds74283. **(D)** Predicted structure of Ff189800. **(E)** Predicted structure of Vi17336. **(F)** Predicted structure of Vi23981. Protein structures were predicted with AlphaFold2, rendered in PyMOL v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Disulphide bonds and/or cysteine residues are shown as yellow sticks. Ds: *D. septosporum*; Ff: *F. fulva*; Vi: *V. inaequalis*.

>Ds69335 COLN
MRSTLAIAAFAAGALAVPYNKEQKRDVVTIDYVTAYNVVTVTAGQPAATPEAPKHYGHHNNYNNPVAYTTVTTSSAAPATYSPPAV
EQPATTSSAPAYSASSASGPEPTDYAGKCVYHHNLHRANHSVSDIAWDDGLASIAQTIAESCVYAHNVEEGGGYGQNIAGVDAAN
VSAIITDLFYNGEEPYFADQYGKDDPDMTNFELFGHFTQIVWKDTISVGCATVQCPNGLANTGDGVEPVFTVCNYKNPGNYAGEYGA
NVLQPLGHPTANWNTGSS*

>Dp69335
MRSTLAIAAFAAGALAVPYNKEQKRDVVTTHWDYVTAYNVVTVTAGQPAATPEAPKHYGHHNNYNNPVAHTTVTTSPSTPATYSPA
AEEQAAEEPATTSSAPAYSASATNGPEPTDYAGKCVYHHNLHRANHSVSDIAWDDGLASIAQTIAESCVYAHNTEQGGGGYGQNIAG
GVDAANISAIITDLFYNGEEPYFADQYGKDDPDMTNFELFGHFTQIVWKDTISVGCATVQCPNGLANTGNDVEPVFTVCNYKNPGNY
AGEYGANVLKPLGHPTANWNTGSS*

>Ds71487 GUA1
MKTVAASAALLSLASVATAQTVQSKPFYLVLDCAKYNGSALVSCHEGAGIESLCVAATLGSNVKGSTYMLNTTTGEVGDKNAGTPG
TLVYKLPISGGVGESEPMQLSYNEASNVALPLFEPSTTATLVAFDKENHMNIQSYIDDTVVPKTEGAVYRWYVCKSNYEGTQYSTLN
WVAGKYPPQNPSCEKVEVVRKFA*

>Dp71487
MKTFAASAALLSLASVATAQTTQSKPFYLVLDCAQDYNALVACHGAGIEGLCVSATPGTNLNGSTYMLNTTSGQVADKNAGLPG
TLVYNLVLSSGGQTESEPMQLYNNPASNVALALFEPSTATQVAFDKDNHMNIQSYVDDTAAPAKTGTVAYYRWYVCKSYEGYQYTA
LNWVAGKYPPENPTCEKVEVVRKFV*

>Ds74283 COLN
MKYLALFLALVAFVSAYNNTTAEYKLQTRLKVADVSKQEYDGLYLSSYHTGAGLNDVVFSDPEPLGNATLAATNITANGTTYYNQLFDL
GNDFPWGLVMESNIEFYSAWQPVELNAGASGSNDVSGFFINETGLQWTSNPGQVGTDDDDQFGGWLVCDDWWHGVPQLFFRISYYS
SSVYQAPASCADVYLIPQYI*

>Dp74283
MKYLAPFLALVAVVTAYNNTTVEYKLQTKLVADLSKQEYDGLYLSSYHTGAGLNDVVFSDPEPLGNASLAATNITANGTTYYNQLFDL
GNDFPWGLVMESNIEFYSAWQPVELNAGASGSNDVSGFFINETGLQWTSNPGQVGTDDDEFGGWLVCDDWWHGVPQLFFRISYYS
SSAYEAPASCADVYLIPRYI*

>Ds131885 COLN
MQFRFTFIGLAITLAYAHATLDPATSNKKGKCPKTIACSASDVSTAIQAAECSHNTRTPGTQTFAVFETDHSYDSSKGAPYGTCSAYTCAVP
TTFTSDSDCWTFWWSGSGTESGVGTGCIRSPSTGECGCENSSGQFIAGSSSCT*

>Dp131885
MQFRFTVGLAITLACANATLDPATSNKKGKCPKSIACSATDVSTAIQAAECSHNTRTPGTQTFAVFETDHSYDSSKGGPYGTCSAYTCAV
PTSFTSDSDCWTFWWSGSGTSGSGVGTGCIRSPSTGECGCENSSGQFIAGSDSCT*

Figure A.15. Amino acid sequences of homologues of candidate effector proteins from *Dothistroma septosporum* NZE10 in other *D. septosporum* isolates and *Dothistroma pini*. Dp: *D. pini*; COLN: *D. septosporum* Colombian isolate; GUA1: *D. septosporum* Guatemalan isolate.



Figure A.16. Western blot of candidate effector Ds69335 from *Dothistroma septosporum*. Western blot detection of Ds69335 protein that did not trigger chlorosis or cell death when expressed in *Nicotiana benthamiana*. Expected size: 30.8 kDa. Anti-FLAG antibody was used for immunodetection. Marker on the far left of membranes was the PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific). Ds: *D. septosporum*.

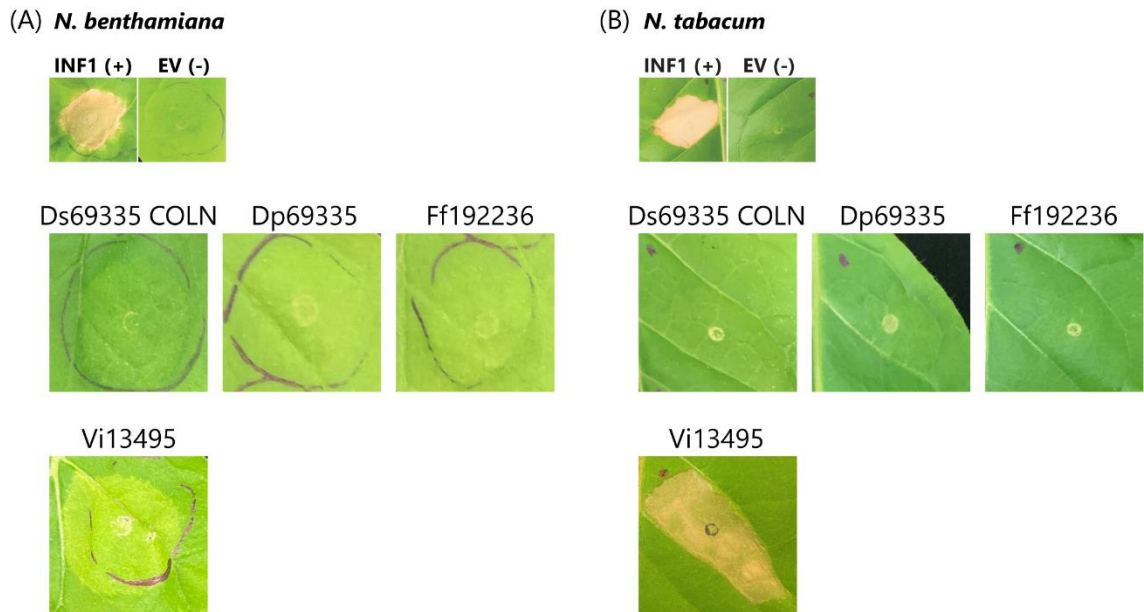


Figure A.17. Orthologues of Ds69335 and Ds131885 from *Dothistroma septosporum* NZE10 in other pathogens screened for their ability to trigger chlorosis or cell death in non-host plants. Protein Ds69335 from *D. septosporum* Colombian isolate (COLN), Dp69335 from *Dothistroma pini*, Ff192236 (Joint Genome Institute (JGI) ID) from *Fulvia fulva*, and Vi13495 (JGI ID) from *Venturia inaequalis* (Ds131885 orthologue) were expressed in *Nicotiana benthamiana* **(A)** and *N. tabacum* **(B)** using an *Agrobacterium tumefaciens*-mediated transient expression assay to assess their ability to elicit chlorosis or cell death. Representative images are shown (n = 12–24 infiltration zones), from at least three independent experiments. INF1, *Phytophthora infestans* elicitor positive cell death control; EV, empty vector negative no cell death control; Ds: *D. septosporum*; Dp: *D. pini*; Ff: *F. fulva*; Vi: *V. inaequalis*. Photos were taken 7 days after infiltration.

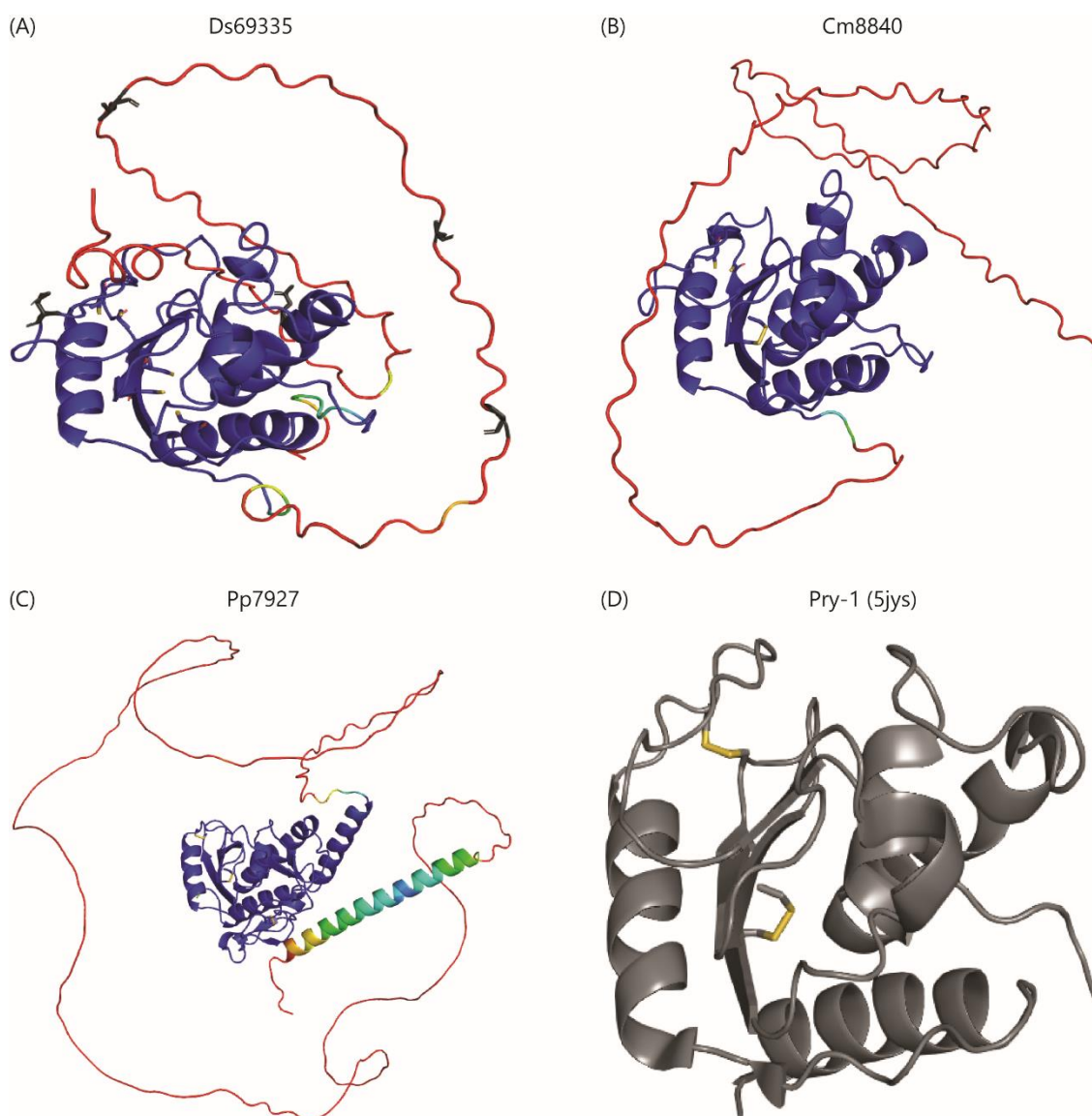


Figure A.18. Predicted protein tertiary structure of Ds69335 from *Dothistroma septosporum*, Cm8840 from *Cyclaneusma minus* and Pp7927 from *Phytophthora pluvialis*. **(A)** Predicted structure of Ds69335. The position of five non-synonymous changes in surface-exposed amino acids (N38S, V67A, A82T, P91S and Q157E) in Ds69335 of *D. septosporum* NZE10 when compared with 18 other *D. septosporum* isolates are shown as black sticks. **(B)** Predicted structure of Cm8840. **(C)** Predicted structure of Pp7927. Protein structures were predicted with AlphaFold2, rendered in PyMol v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022), and coloured according to their AlphaFold2 pLDDT score: dark blue for regions predicted with high confidence, light blue and green for regions of low confidence, and red for very low confidence regions. Disulphide bonds are shown as yellow sticks. **(D)** Characterized structure of Pry1 from *Saccharomyces cerevisiae* (Research Collaboratory for Structural Bioinformatics protein data bank [RCSB PDB] ID: 5jys) (Darwiche et al., 2016). Ds: *D. septosporum*; Cm: *C. minus*; Pp: *P. pluvialis*.

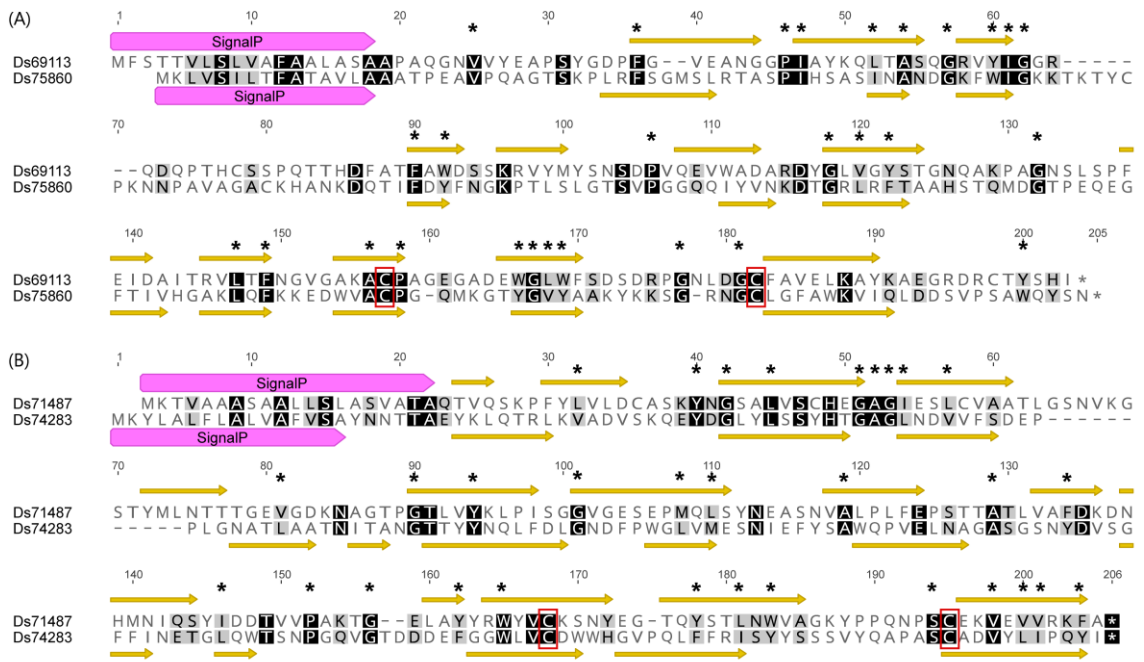


Figure A.19. Amino acid sequence alignment between predicted β -trefoil proteins of *Dothistroma septosporum*. **(A)** Amino acid sequence alignment between Ds69113 and Ds75860. **(B)** Amino acid sequence alignment between Ds71487 and Ds74283. Alignments are shown between the two sets of proteins that had higher pairwise identity. SignalP: signal peptide; yellow arrows: position of β -sheets in the predicted structure of each protein (Ds69113 – **Appendix Figure A.12**; Ds74283 – **Figure 3.4**; Ds71487 – **Figure 3.10**; Ds75860 – **Figure 3.11**). Conserved cysteine residues are highlighted in the red box. These residues form the predicted disulphide bond from **Figure 3.16**. The asterisk * shows conserved hydrophobic residues. Alignments were performed using the Geneious Alignment tool from Geneious v9.1.8 (Kearse et al., 2012) with default parameters.

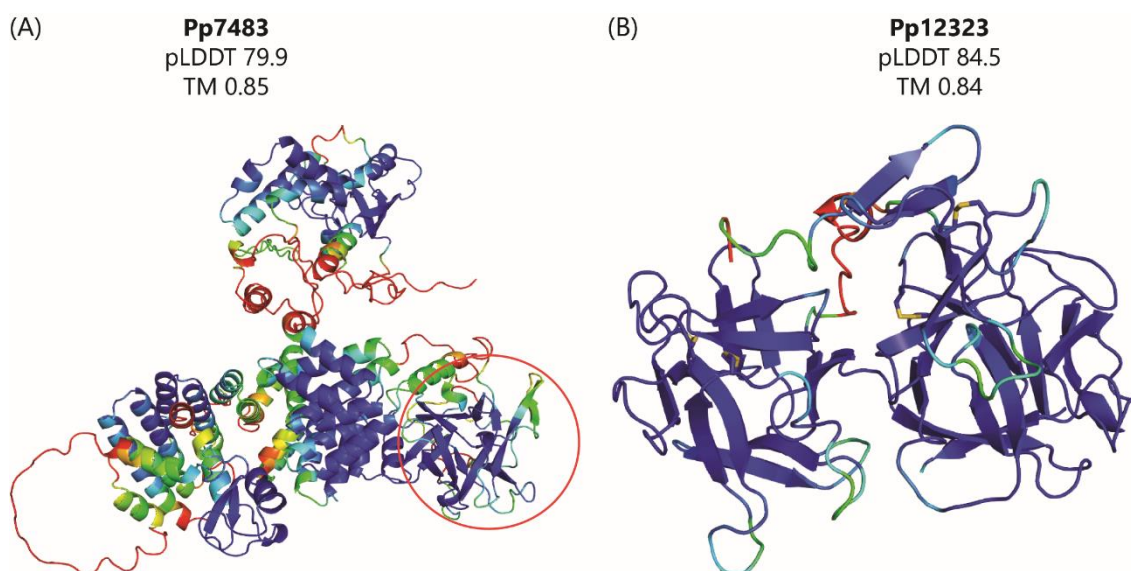


Figure A.20. Predicted tertiary structures of β -trefoil proteins from *Phytophthora pluvialis*. Predicted structure of **(A)** Pp7483 (the β -trefoil domain is circled in red) and **(B)** Pp12323. Disulphide bonds are shown as yellow sticks. Protein structures were predicted with AlphaFold2 and rendered in PyMol v2.5 (DeLano, 2002; Jumper et al., 2021; Mirdita et al., 2022). Structures were coloured in PyMol according to their AlphaFold2 pLDDT score: dark blue for highly confident predicted regions, light blue and green for regions of low confidence, and red for very low confidence regions. Pp: *P. pluvialis*.

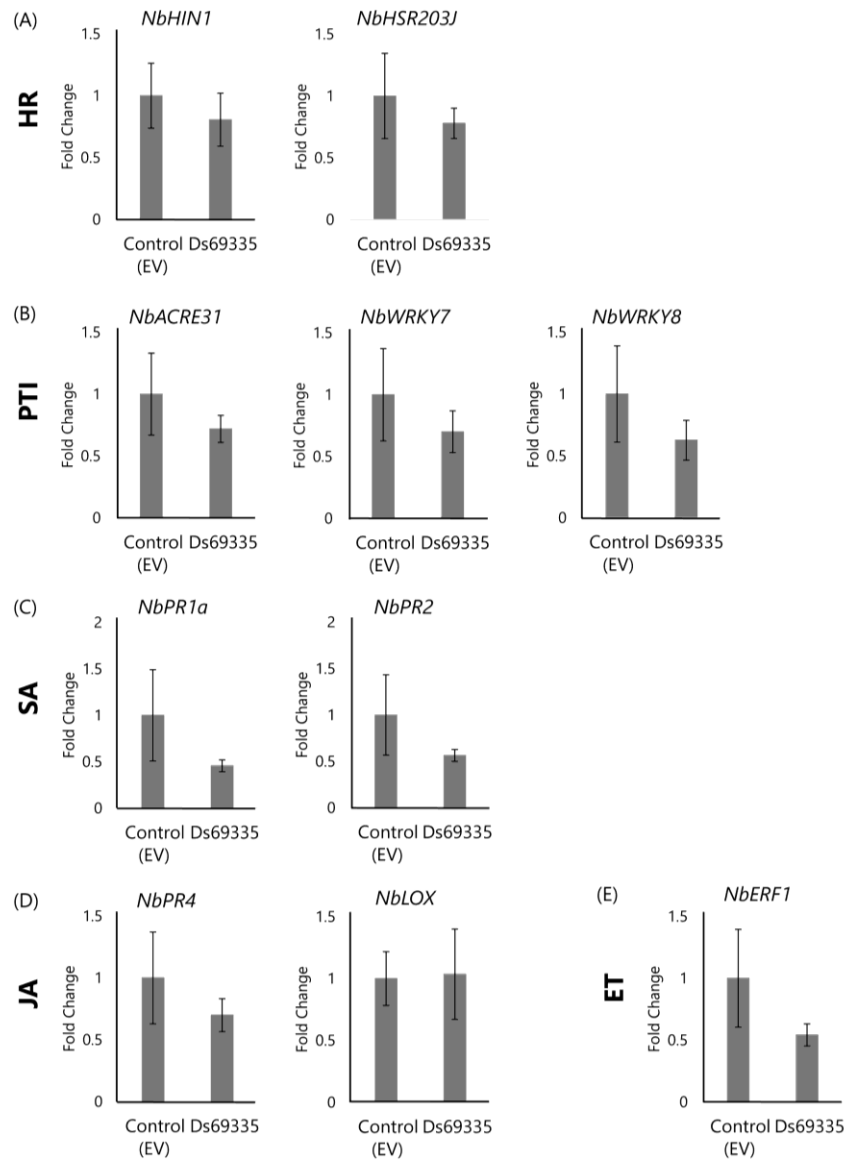


Figure A.21. *Dothistroma septosporium* candidate effector Ds69335 does not modulate expression of defence-related genes in *Nicotiana benthamiana* 24 hours after infiltration (hai). Expression of **(A)** hypersensitive response (HR)-specific marker genes (*NbHIN1* and *NbHSR203J*), **(B)** pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes (*NbACRE31*, *NbWRKY7* and *WRKY8*), and defence-related marker genes **(C)** *NbPR1a* and *NbPR2*, salicylic acid (SA)-dependent immunity, **(D)** *NbPR4* and *NbLOX*, jasmonic acid (JA)- dependent immunity and **(E)** *ERF1*, ethylene-dependent immunity) in *N. benthamiana*. Ds69335 and empty pICH86988 vector (EV) were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay and leaves were sampled 24 h after infiltration. Transcript levels were normalized to reference gene *NbActin* and compared to the level of the control (set as 1). Means and standard errors were calculated from at least three biological replicates. *P*-value calculated using Student's *t*-test. Ds: *D. septosporium*. See **Figure 3.21** for results at 48 hai when significant differences in expression were seen.

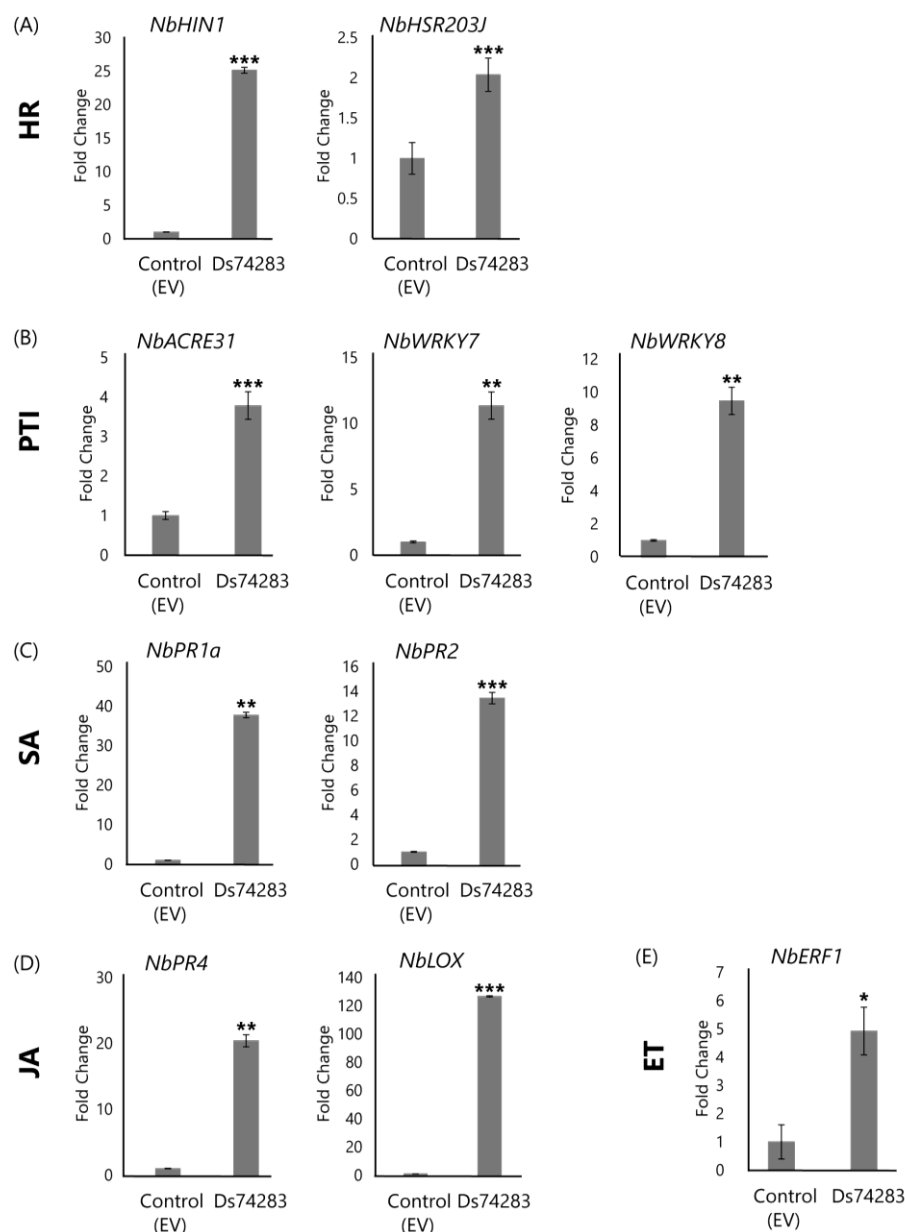


Figure A.22. *Dothistroma septosporum* candidate effector Ds74283 triggers plant defence responses in *Nicotiana benthamiana*. Expression of **(A)** hypersensitive response (HR)-specific marker genes (*NbHIN1* and *NbHSR203J*), **(B)** pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes (*NbACRE31*, *NbWRKY7* and *WRKY8*), and defence-related marker genes **(C)** *NbPR1a* and *NbPR2*, salicylic acid (SA)-dependent immunity, **(D)** *NbPR4* and *NbLOX*, jasmonic acid (JA)- dependent immunity and **(E)** *ERF1*, ethylene-dependent immunity) in *N. benthamiana*. Ds74283 and empty pICH86988 vector (EV) were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay and leaves were sampled after 48 h after infiltration. Transcript levels were normalized to reference gene *NbActin* and compared to the level of the control (set as 1). Means and standard errors were calculated from at least three biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ using Student's *t*-test. Ds: *D. septosporum*.

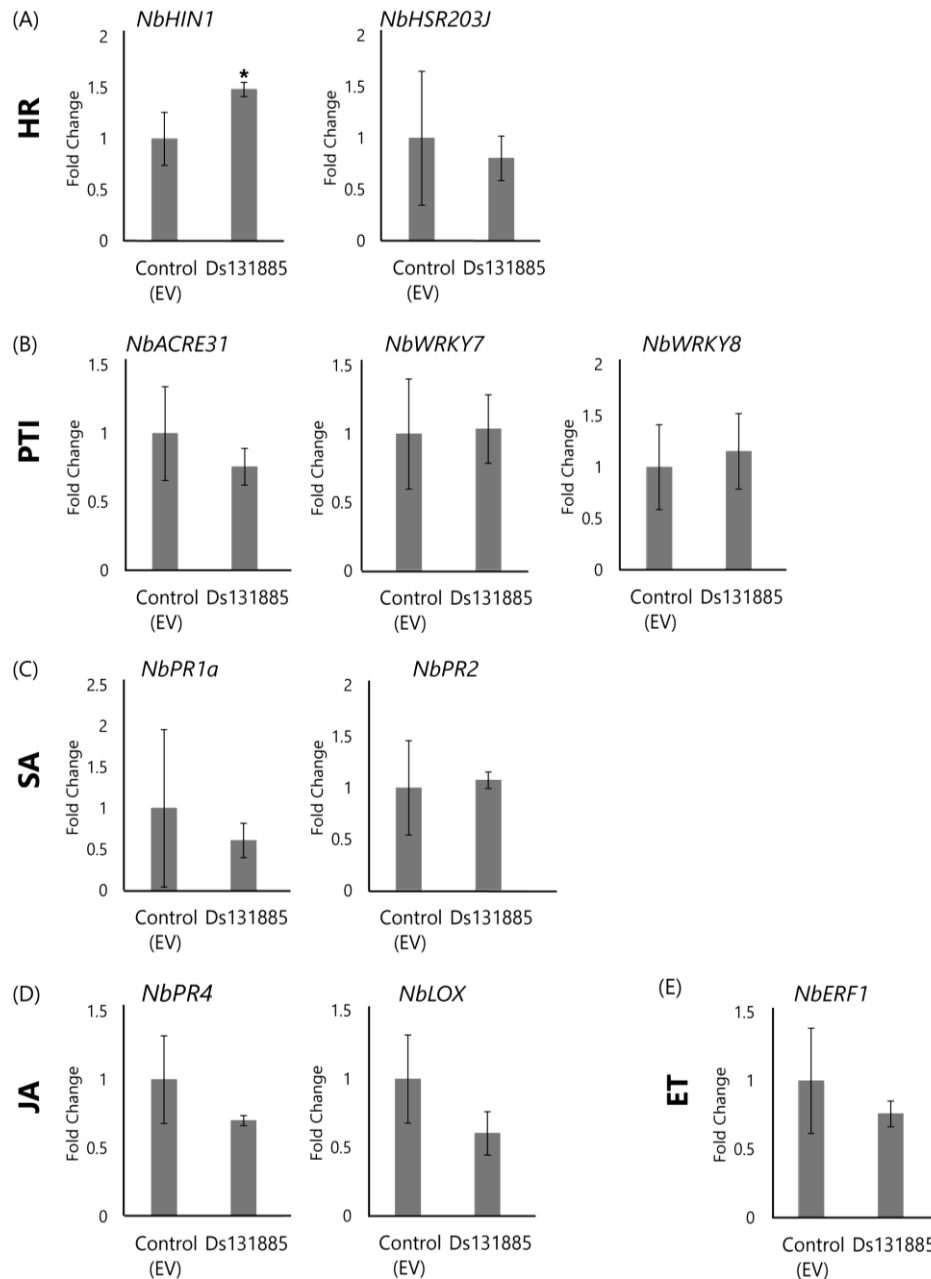


Figure A.23. *Dothistroma septosporum* candidate effector Ds131885 triggers a hypersensitive response gene in *Nicotiana benthamiana*. Expression of **(A)** hypersensitive response (HR)-specific marker genes (*NbHIN1* and *NbHSR203J*), **(B)** pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) marker genes (*NbACRE31*, *NbWRKY7* and *WRKY8*), and defence-related marker genes **(C)** *NbPR1a* and *NbPR2*, salicylic acid (SA)-dependent immunity, **(D)** *NbPR4* and *NbLOX*, jasmonic acid (JA)- dependent immunity and **(E)** *ERF1*, ethylene-dependent immunity) in *N. benthamiana*. Ds131885 and empty pICH86988 vector (EV) were expressed in *N. benthamiana* using an *Agrobacterium tumefaciens*-mediated transient expression assay and leaves were sampled 24 h after infiltration. Transcript levels were normalized to reference gene *NbActin* and compared to the level of the control (set as 1). Means and standard errors were calculated from at least three biological replicates. *, $P < 0.05$. Ds: *D. septosporum*.

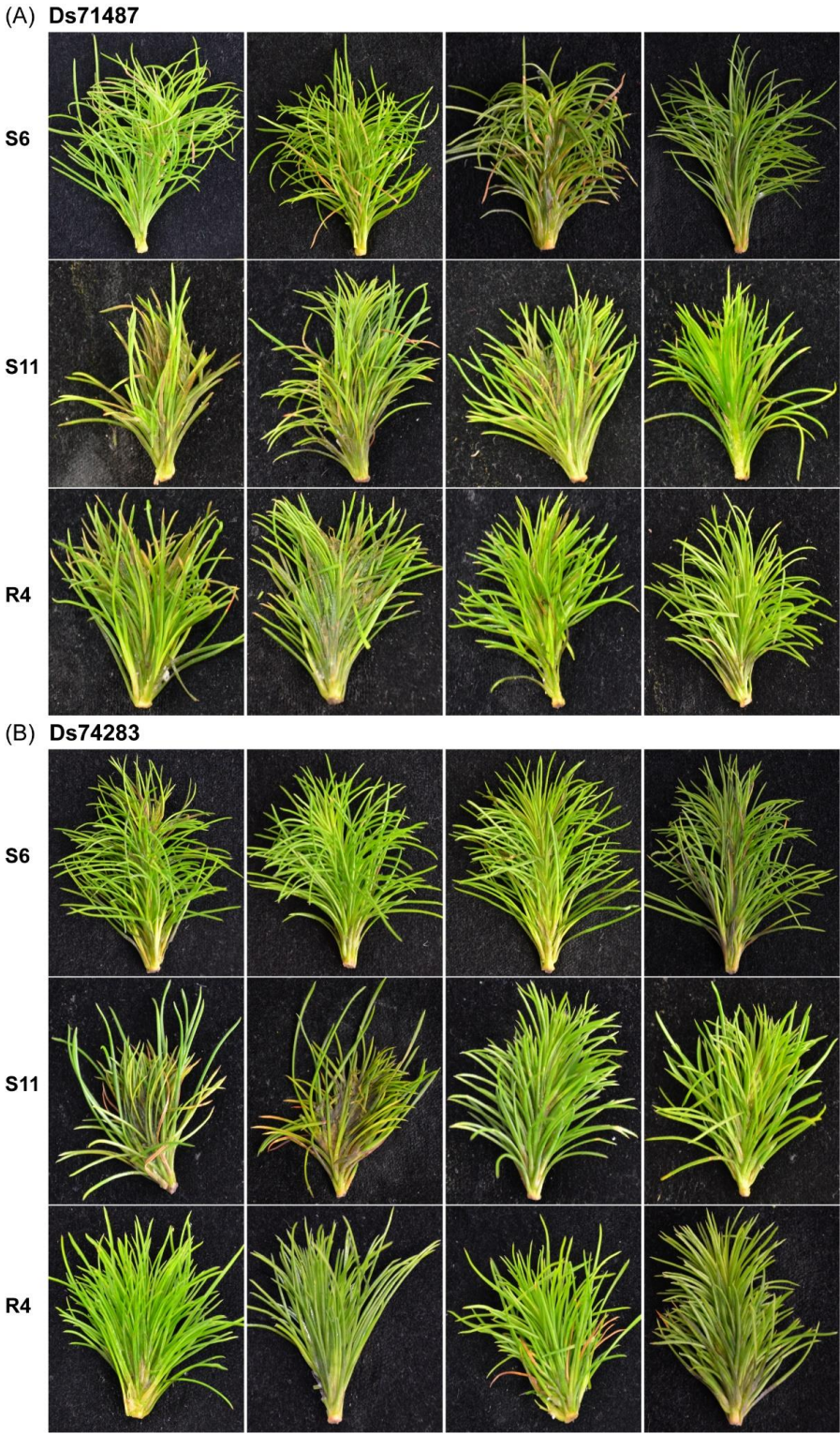
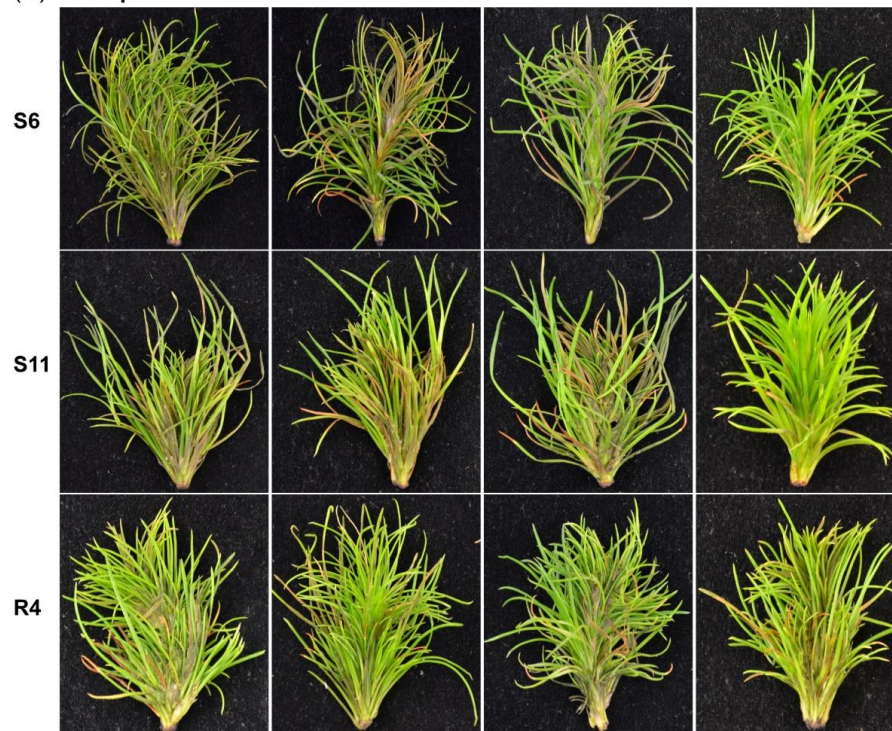


Figure A.24. Legend on page 300.

(C) **DsEcp2-1**



(D) **Ds131885**

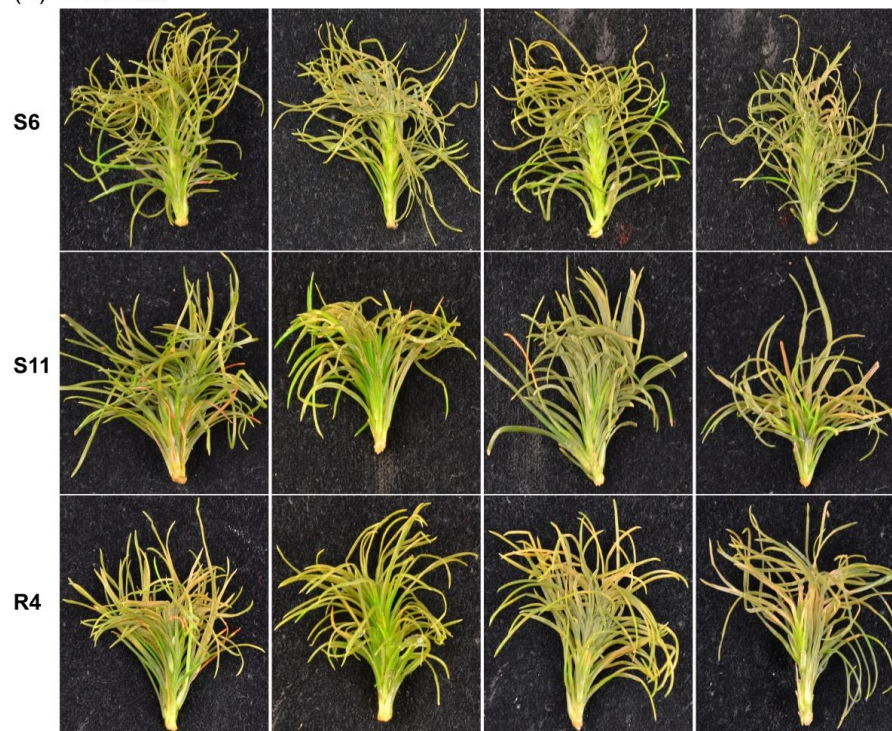


Figure A.24. Legend on page 300.

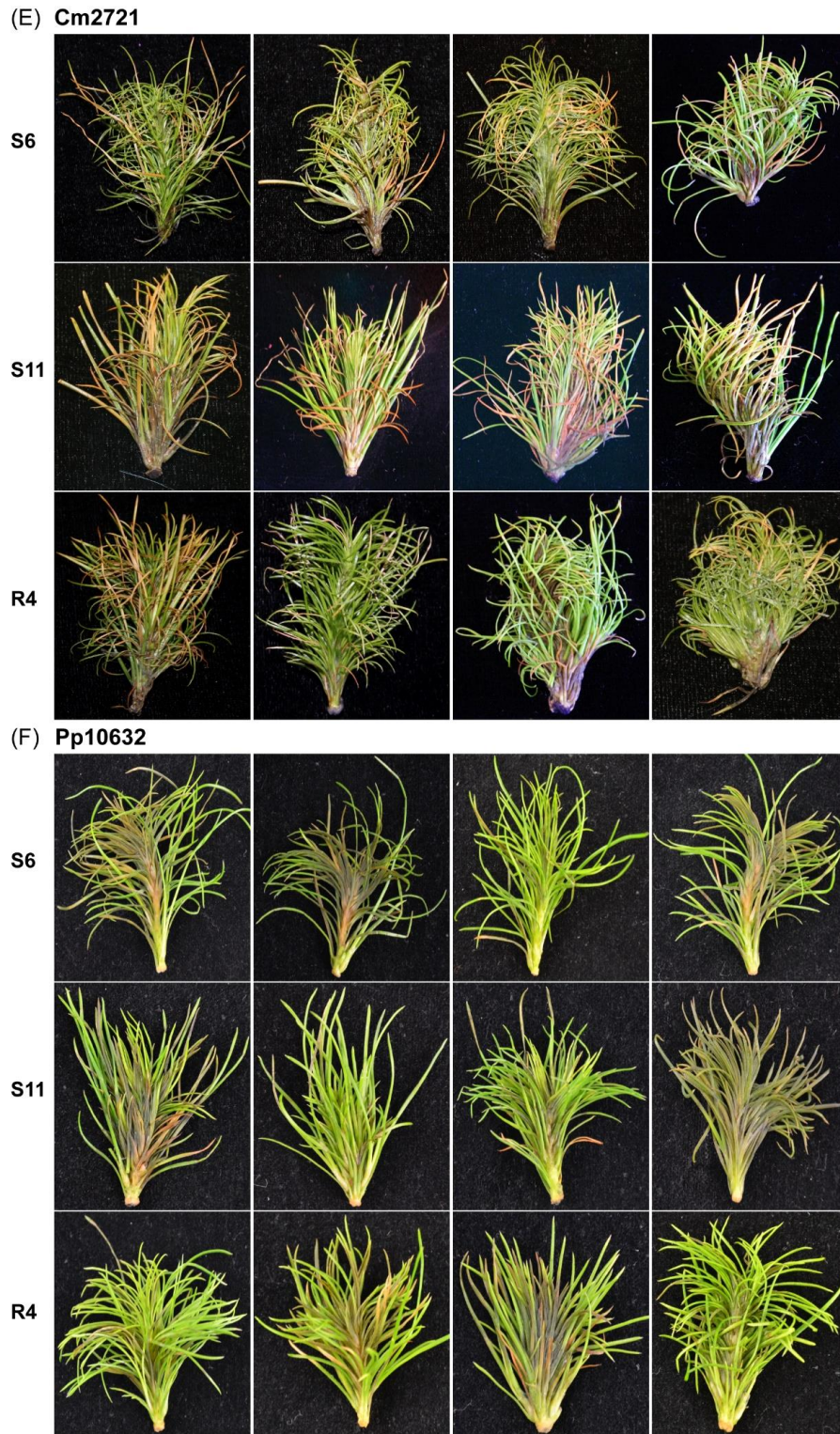


Figure A.24. Replicates of *Pinus radiata* shoot tissue infiltrated with purified candidate effector proteins. Whole shoots of *Pn. radiata* derived from family seedlots that are relatively susceptible (S6 and S11) or tolerant (R4) to *Dothistroma septosporum* infection were infiltrated with purified Ds71487 (**A**), Ds74283 (**B**), DsEcp2-1 (**C**), Ds131885 (**D**), Cm2721 (**E**) and Pp10632 (**F**) proteins produced by heterologous expression in *Pichia pastoris*. Photos were taken 7 days after infiltration. Ds: *D. septosporum*.

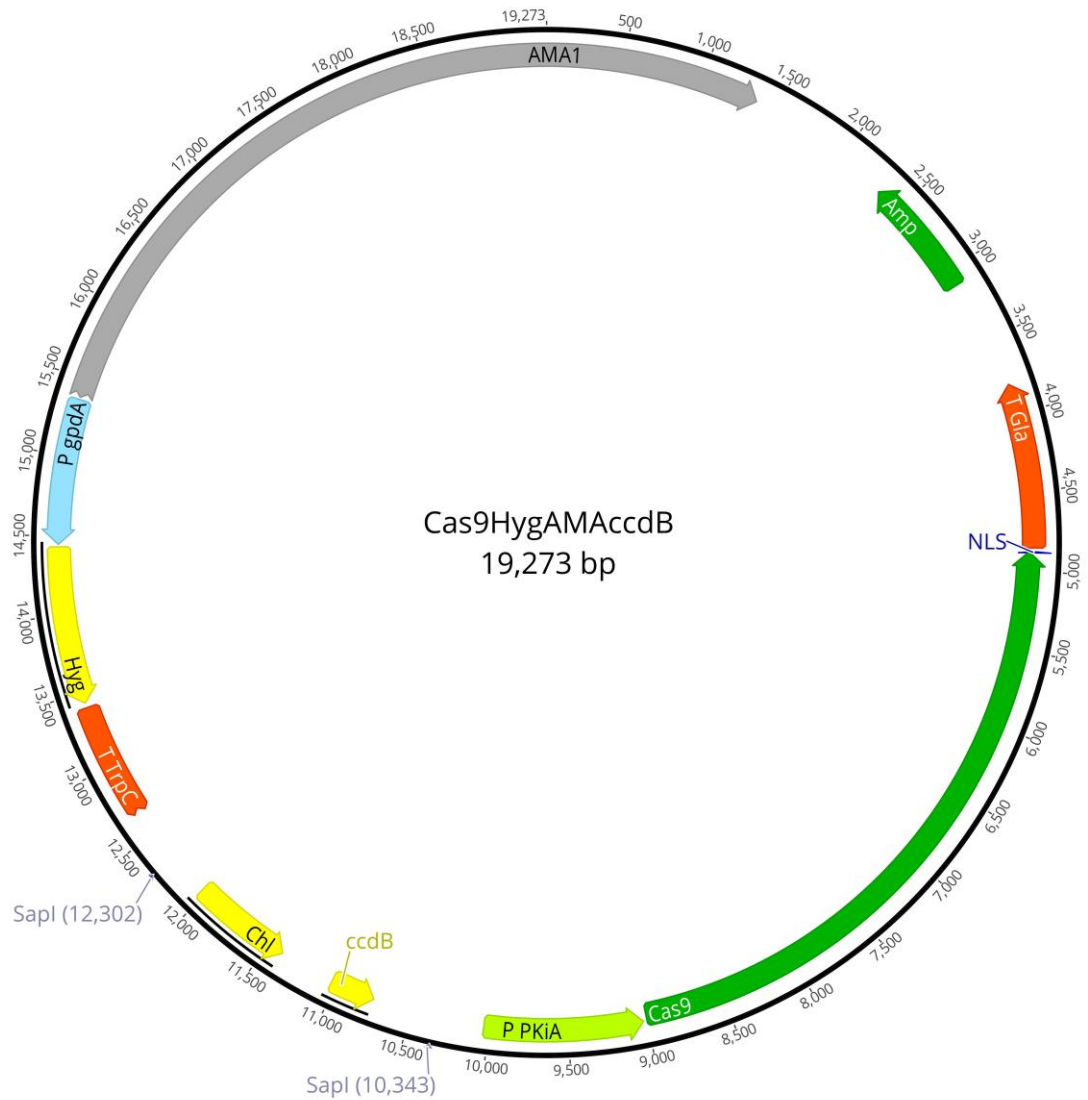


Figure A.25. Schematic of Cas9HygAMAccdB (Rocafort et al., 2022). Key features of interest: autonomously replicating sequence (AMA1) from *Aspergillus nidulans*; *cas9* gene from *Streptococcus pyogenes* (codon-optimized based on *Aspergillus niger*), flanked by the *A. niger* pyruvate kinase gene promoter (P *PkIA*) and the *A. niger* glucoamylase gene terminator (T *Gla*); NLS, nuclear localization signal; *Hyg*, hygromycin resistance gene, flanked by the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase gene promoter (P *gpdA*) and the *A. nidulans* tryptophan C gene terminator (T *TrpC*). *SapI* restriction sites for introduction of a sgRNA cassette by Golden Gate cloning (through replacement the control of cell death B (*ccdB*) gene); *Amp*, ampicillin resistance gene; *Chl*, chloramphenicol resistance gene; bp: base pairs.

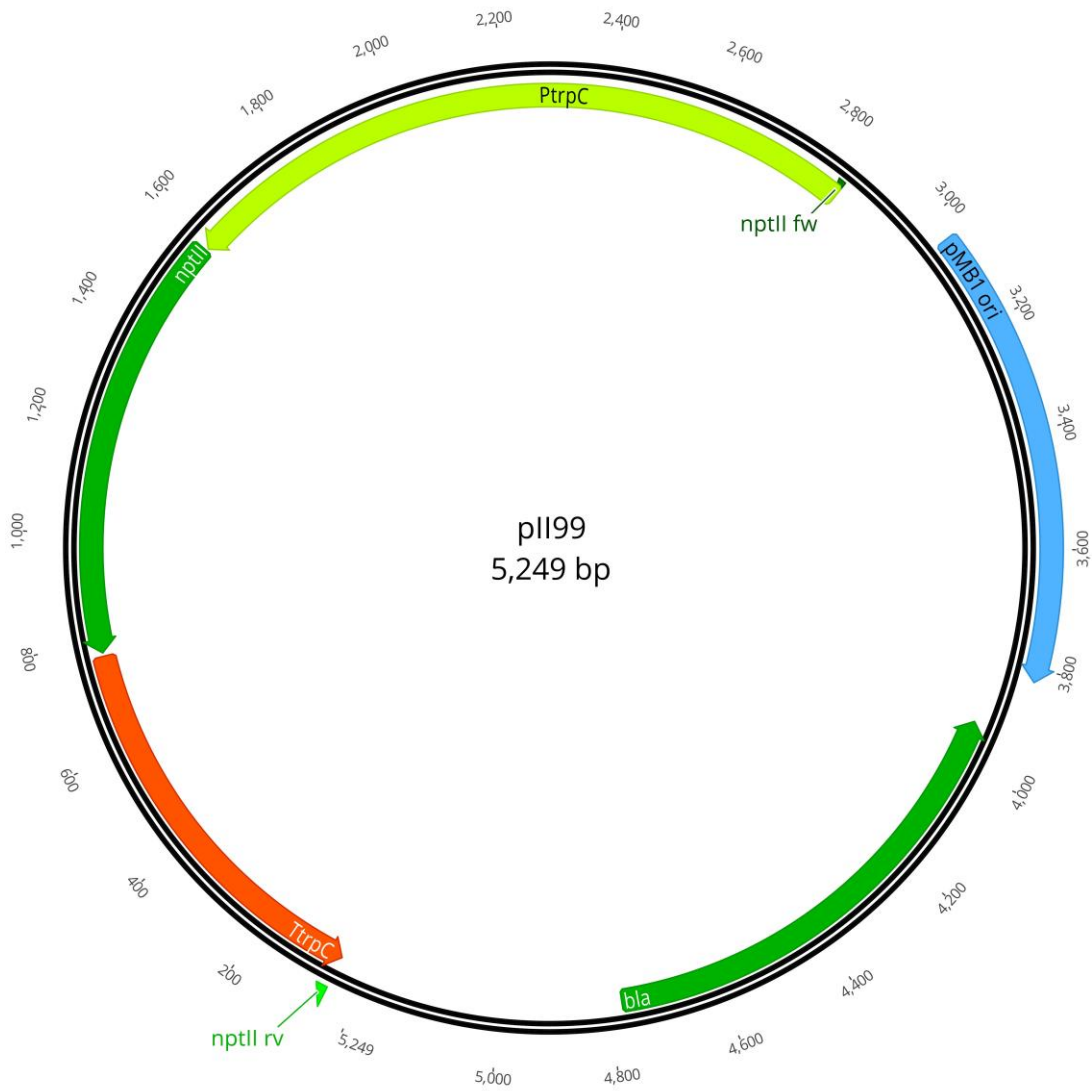


Figure A.26. Schematic of pII99 (Lara-Ortiz et al., 2003). Key features: nptII forward (fw) and reverse (rv) primers from *nptII* cassette (*PtrpC*-*nptII*-*TtrpC*). The *nptII* cassette confers resistance to geneticin and was used as a selective marker for *Dothistroma septosporum* CRISPR/Cas9 transformation. bp: base pairs.

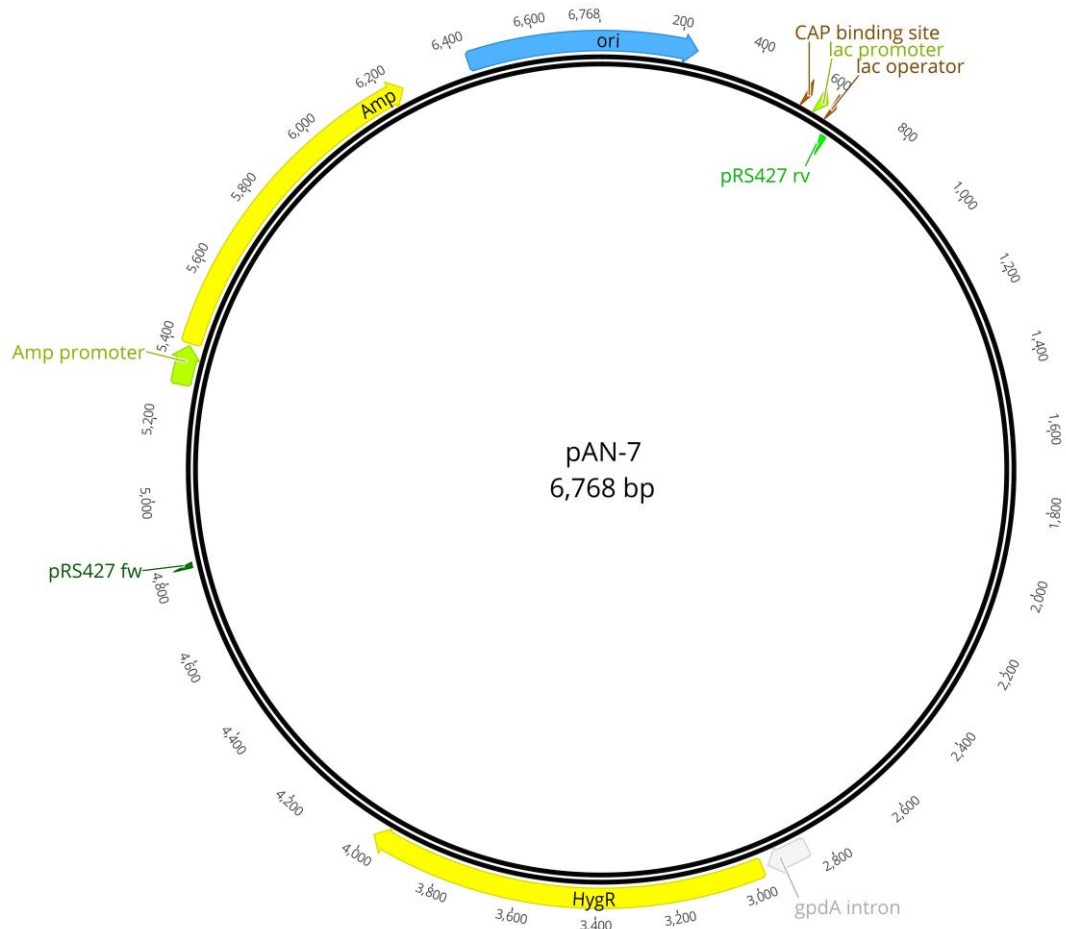


Figure A.27. Schematic of pAN-7 (Punt et al., 1990). Key features: *Amp*, ampicillin resistance gene; pRS427 forward (fw) and reverse (rv) primers for amplification of the Gibson-compatible backbone to be used to generate the donor DNA (consisting of a resistance selection marker flanked by the candidate gene sequence) that was used for *Dothistroma septosporum* CRISPR/Cas9 transformation; bp: base pairs.

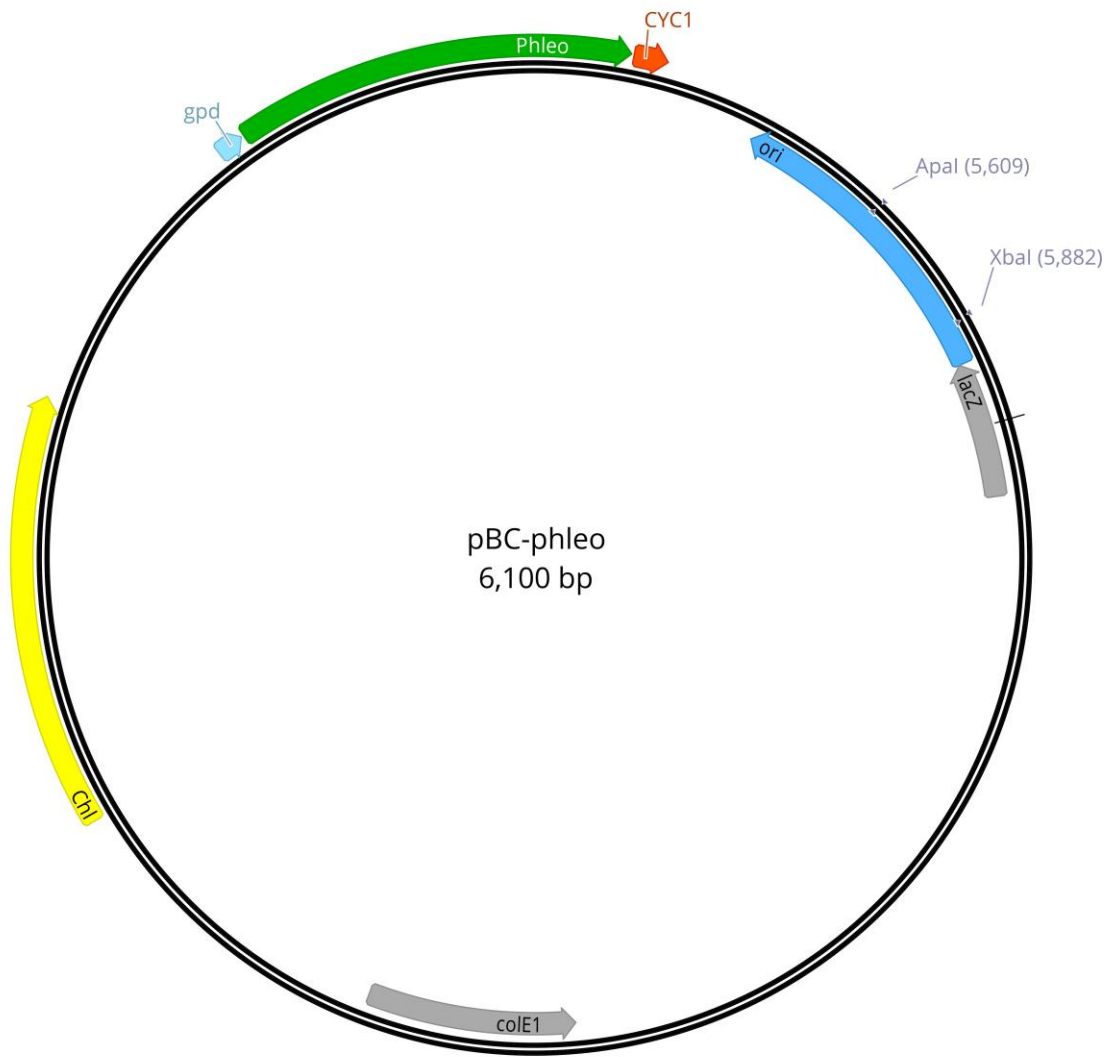


Figure A.28. Schematic of pBC-phleo (Silar, 1995). Key features: *Chl*, chloramphenicol resistance gene; *Phleo*, phleomycin resistance gene; the sequence used for complementation is ligated into restriction sites (Apal and Xbal) that allow in-frame directional cloning; bp, base pairs.

>NZFS110 Cmv8527
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WSTSGNPVIPDCSAVSLHREFV*

>NZFS725 Cmv5485
MKS AVFASILAFAASSALAQYDAQSPPFYLQLSDNSTLNGSYLASCHTGAAIESLCLYSSTDDSDHETYNLNTTDPYVANATGYLTWVL
HGGNFNESEPLTFDYDATSNVAMPVFEPSETGQGIAFDCEDNAYISSYLNYSTSPPSYEDAGDNGQQFYNNWWFCHTNFAGYTYETLV
WSTSGNPVIPDCSAVSLHREFV*

>NZFS759 Cms163
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>NZFS809 Cms2492
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VTAGEPVTDPDCSAVDLHRV FV*

>NZFS1800 Cmv2944
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WSTSGNPVIPDCSAVSLHREFV*

>NZFS3276 Cmv4855
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>NZFS3305 Cmn4577
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GEPVIPDCSAISLHREFV*

>NZFS3325_Cmn4351
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GEPVIPDCSAISLHREFV*

>NZFS110 Cmv scf53
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PTEDEMTDVDDDCWTFWWSGEGEEGLGTNCIEDPSTGECGEDSDGTFVAGSDSCT*

>NZFS725 Cmv scf36
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PTEDEMTDVDDDCWTFWWSGEGEEGLGTNCIEDPSTGECGEDSDGTFVAGSDSCT*

>NZFS759 Cms394
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PTEDEMTDVDEDCWTFWWSGEGEESGVTNCIEDPTTGECGEDSSGTFIAGSDSCT*

>NZFS809 Cms2721
MASLLKSLSLLLAATLVSANVDSCSSNTKGKCPGTLKCSASASSSAIQAAEC SHNTRTSETQTF AVFVTDHEYDSSHGAPYGTCEAYTCTP
PTEDEMTDVDEDCWTFWWSGEGEESGVTNCIEDPTTGECGEDSSGTFIAGSDSCT*

>NZFS1800 Cmv scf13
MPSLLKSLSLLLAATLVSANIDACSSNTKGKCPGTLNCSASASSSAIQAAEC SHNTRTSETQTF AVFVTDHEYDSVYGAPYGTCEAYTCTA
PTEDEMTDVDDDCWTFWWSGEGEEGLGTNCIEDPSTGECGEDSDGTFVAGSDSCT*

>NZFS3276 Cmv1489*
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PTEDEMTDVDDDCWTFWWSGEGEEGLGTNCIEDPSTGECGEDSDGTFVAGSDSCT*

Figure A.29. Legend on the next page.

>NZFS3305 Cmn7980*
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>NZFS3325 Cmn7756*
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 TAPTSDEMTDVEDDCWTFWWSGEGEESGVGTNCIEDPGTGECGEDSDGTDFIDGSSSCT*

>NZFS110 Cmv7855
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>NZFS725 Cmv9704
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 YNDTLAGYAATVAAKCVFAHDQTEGGGGYGQNIASFGETGDVERLGAEAMLAGAVTDMWYNGELGLFLPSFYGEANPDMTDFDKW
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 NIASFGETGDVEALGASSMLAGAITDMWYNGELGLFLPEYGEANPDFTNFEGWGHFSQLVWKNSNQVCASQFCPQSTGLDFDGDG
 WFTVCNYPGAGNMGGEYGDNIMKPLGHPSITA*

>NZFS809 Cms8840
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 NIASFGETGDVEALGASSMLAGAITDMWYNGELGLFLPEYGEANPDFTNFEGWGHFSQLVWKNSNQVCASQFCPQSTGLDFDGDG
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 GHFSQLVWKDSTEVEGCATQYCPAGGLPFTFAGWYTVCNYPGPNMGGEYGDNILEPLGHPSISA*

>NZFS3276 Cmv7190
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 HDQTEGGGGYGQNIASFGETGDVERLGAEAMLAGAVTDMWYNGELGLFLPSFYGEANPDMTDFDKWGHFSQLVWKDSTEVEGCAT
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>NZFS3325 Cmn6003
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 GNMGGEYGDNIMEPLGHPSISS*

Figure A.29. Amino acid sequences of homologues of Cms2492, Cms2721 and Cms8840 from *Cyclaneusma minus* NZFS809 in other *C. minus* isolates. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. The asterisk * following protein ID indicates the protein was wrongly predicted in the original annotation and the correct sequence was used. Some proteins were not originally predicted, so scf refers to the scaffold in which the predicted protein sequence can be found in the genome.

>NZFS110 Cmv937

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GFSDLVAPLGCTEDCIGFDMALEWISAASPPGAYEYD*

>NZFS110 Cmv2795

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VSSPHGPYSNGFGVYKGDGASFDILNFTLANTFQACPSKKGGKPYQVFLDVPFGKDHVVPBGKADCLPFEIAVSSYKTGAGAYQYE
*

>NZFS110 Cmv5316

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TTASMNSAVPGGQTIYVSPTGALSFTSPNRSIIPAGSYTSGFTIGCEQPYPPCESPTWNFTGGWATGFYACPLNDPAVYADPYGRDFPY
KVFLDVEGWWVPSGNMSDCLPFSVAVSPDMYSDBGVAWGAYEYV*

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AVPGSQNLVYAESGALQFTEPHAEGAYPAGSYTSGFNEMYGAANIDPTVNFTGGGATAFWACPPNDPAENSNEPTGIDDPYKIFLD
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>NZFS110 Cmv6323

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>NZFS110 Cmv9109

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VAGGQQLYIAASGAIEFTQPHSAAMPEGSVTGGWGAYTSENGIDMITNSAGTGFLACPTDLTSYQVFADIEGIPNSAVPSGNTDDCLG
FDIAASVVNDATAYEYL*

>NZFS725 Cmv1064

MCAATLLMGFLSALAAASPLIQAQRRETPYLASSFISVGTPTNTPIYFDTISAANSSFWIGASTRTYCPSSSPENFTNCSAFHNSTSLVYNTS
TTASMNSAVPGGQTIYVSPTGALSFTSPNRSIIPAGSYTSGFTIGCEQPYPPCESPTWNFTGGWATGFYACPLNDPAVYADPYGRDFPY
KVFLDVEGWWVPSGNMSDCLPFSVAVSPDMYSDBGVAWGAYEYV*

>NZFS725 Cmv1065*

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AVPGSQNLVYAESGALQFTEPHAEGAYPAGSYTSGFNEMYGAANIDPTVNFTGGGATAFWACPPNDPAENSNEPTGIDDPYKIFLD
VEGWNSVVPVSGNISHCLPFNIATKQDAYGDGEVHGAYQYI*

>NZFS725 Cmv1388

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*

>NZFS725 Cmv3556

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VINMQAIQEQMVVDATGALSFTTAHEEDITPPGSYVSGGFMVEEESVSLQFDQGGASQGWVACPITSDVDENGQPLGPFQVFASVV
GFSDLVAPLGCTEDCIGFDMALEWISAASPPGAYEYD*

>NZFS725 Cmv5591

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FDIAASVVNDATAYEYL*

>NZFS725 Cmv7620

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SSNVPGGQNVFVAKSGALSFTEPHQEYVFPDGSYTRGFEIANNGHGVDLLNFTGGGATTFTACPTTPSSTAGVYPYQVFLDVKGFNDS
VVPSGSVADCLPFGIAIYNSTGAGAFQYE*

Figure A.30. Legend on page 310.

>NZFS759 Cms3365
MFTPTLLTALLPALAAASPIFKRDDYVPPYTTSLLSLRASPIHFSAINASHGHFSIGEPTSYYCPVSDCSGFPNITLISIYEASGTAYMSTNVP
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CLPFGIAINNYTTDAGAFQYE*

>NZFS759 Cms4265
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GDSVPSGCTEDCIGIDIALAWESVTVPPSAYAYD*

>NZFS759 Cms4412
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DGVVPSGNTSDCIPFGVATNQDAYDDGEVHGAYQYI*

>NZFS759 Cms4413
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>NZFS759 Cms9202
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VAGGQQLFVAADGALEFTVVHSAIPEGAFFVGGWGAYTTENGVDMITFSSGGDQGGFLACPMKDSDEVYQIFADIEGIPNSAVPSGNT
ADCLGFDIAASKVDSPTAYQYD*

>NZFS809 Cms835
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GGQNVYVAESGALSFTTEPHQEYVFPDGSYTTGFAVSNNNGNGVDLLNFTQANTFTACPPCTGGEFPYQVFLDVPGFNDSSVPSGSTAD
CLPFGIAINNYTTDAGAFQYE*

>NZFS809 Cms3871
MLSTLLAAFLPALTLASSLSLLPRQDSHQSENAGMIALHSGSVIHFSSTINASDSIFYIGIPTGTYPDTPVGCSTGLTNTTVIHPYNPDGKV
YMGGINVQQLYVEPTGALAFSTVHEEDIFPPGAYTSSGDFTVDEESSVSLLEFGAGSSQGWVACAITNDLDESGQLLGPWQVFAEVEGF
GDSVPSGCTEDCIGIDIALAWESVTVPPSAYAYD*

>NZFS809 Cms4058
MFTAAILTLLSALFILSKASPLTTRQDGYTTSLLSIGRTPSWIFFEPVNATSSLFWIGAPTDAACPTPYDCSGFSNTTVIRVDRDPSQASAF
MDSPVAGGQMLYVTESGALSFMQPHCGSCGITNADSLTGFGINYNNGTGSDDLSEFVDRVAKGFFACPTHINVTNGNEEFYQVFL
DVKGWNDSSVPSGRTVDCFGFEIAVHDMGSDAMEGASEYV*

>NZFS809 Cms4059
MITTTILITLLSTLRSSLLQNKQETSFLATGLTAIGNLNTPVYGQTLNANSSSHFWLHTTTTRTSCPTFSNNFTCAIYPNDTSILVYNSSSTAY
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DGVVPSGNTSDCIPFGVATNQDAYDDGEVHGAYQYI*

>NZFS809 Cms9849
MYAAALAAFLPALAAASPLAKRNITFPYSAEWTALRSGSEYIHYQSLNANSQLIWIGKETNVDCPIGGCESYSNTTSITLSSGNAYMNDV
VAGGQQLFVAADGALEFTVVHSAIPEGAFFVGGWGAYTTENGVDMITFSSGGDQGGFLACPMKDSDEVYQIFADIEGIPNSAVPSGNT
ADCLGFDIAASKVDSPTAYQYD*

>NZFS1800 Cmv116
MLFTPKLLAVLLPALAAASTIPKRTDSGMSGDKSAAMSPETFGAAPVPVLGDKTPDNSGDKYPVSGAGSSNIFGAAPVPVSGDKSDTPY
TTGLVAIRSGSDFQYASVNASSGLFYIGESTKTGCAAVANCGNYPNITLITIHETEGTAYLDADVAGQVVFVAQSGALSFAHPHQSAF
VSSPHGPYSNGFGVYNKGDGASFDILNFTLANTFQACPSKKGKPYQVFLDVPFGKDHVVPVGKADCLPFEIAVSSYKTGAGAYQYE
*

>NZFS1800 Cmv2836
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VAGGQQLYIAASGAIEFTQPHSAAMPEGSVTGGWGAYTSENGIDMITNSAGTGFLACPTTDLTSYQVFADIEGIPNSAVPSGNTDDCLG
FDIAASVVNDATAYEYL*

Figure A.30. Legend on page 310.

>NZFS1800 Cmv4618
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 SSNPVGGQNVFVAKSGALSFTPHQEYVFPDGSYTRGFEIANNHGVDLLNFTGGGATTFTACPTTPSSTAGVYPYQVFLDVKGFNDS
 VVPSGSVADCLPFGIAIYNSTGAGAFQYE*

>NZFS1800 Cmv5684
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 VINMQAIQEQMVVDATGALSFTTAHEEDITPPGSYVSGGFMVEESSVLLQFDQGASQGWVACPITSDVDENGQPLGPFQVFASVV
 GFSDLVAPLGCTEDCIGFDMALEWISAASPPGAYEYD*

>NZFS1800 Cmv9352
 MSAAIFFLLAFISLCSASPLTTLSPPPFLATSLVAIGPLNTPIYTQPIVANSTFFWIGTTTTRTSCPHFSNNFTCAIYPNDTSLIYPSSSAYMNS
 AVPGSQNLVYAESGALQFTEPHAEGAYPAGSYTSGFNVEMYGAANIDPTVNFTGGGATAFWACPPNDPAENSNEPGTGIDDPYKIFLD
 VEGWNSDVVPSGNISHCLPFNIATKQDAYGDGEVHGAYQYI*

>NZFS1800 Cmv9353
 MCAATLLMGFLSALAAASPLIQAQRRETPYLASSFISVGTNPNTPIYFDTISAANSSFWIGASTRTYCPSSSPENFTNCSAFHNSTSLVYNTS
 TTASMNSAVPGGQTIYVSPTGALSFTSPNRSIIPAGSYTSGFTIGCEQPYYPPECSPTWNFTGGWATGFYACPLNDPAVYADPYGRDFPY
 KVFLDVEGWVVPNGMSDCLPFSVAVSPDMYGDGVAWGAYEYV*

>NZFS3276 Cmv92
 MLSATLLAVLLPALTCAASPLNLLPRQNDTHPDESAGMIALASGTVIHFGTVNASDNAFYIGRPTDTCSTPDICGGLLNITMIHPYDTG
 VINMQAIQEQMVVDATGALSFTTAHEEDITPPGSYVSGGFMVEESSVLLQFDQGASQGWVACPITSDVDENGQPLGPFQVFASVV
 GFSDLVAPLGCTEDCIGFDMALEWISAASPPGAYEYD*

>NZFS3276 Cmv2968
 MSAAIFFLLAFISLCSASPLTTLSPPPFLATSLVAIGPLNTPIYTQPIVANSTFFWIGTTTTRTSCPHFSNNFTCAIYPNDTSLIYPSSSAYMNS
 AVPGSQNLVYAESGALQFTEPHAEGAYPAGSYTSGFNVEMYGAANIDPTVNFTGGGATAFWACPPNDPAENSNEPGTGIDDPYKIFLD
 VEGWNSDVVPSGNISHCLPFNIATKQDAYGDGEVHGAYQYI*

>NZFS3276 Cmv2969
 MCAATLLMGFLSALAAASPLIQAQRRETPYLASSFISVGTNPNTPIYFDTISAANSSFWIGASTRTYCPSSSPENFTNCSAFHNSTSLVYNTS
 TTASMNSAVPGGQTIYVSPTGALSFTSPNRSIIPAGSYTSGFTIGCEQPYYPPECSPTWNFTGGWATGFYACPLNDPAVYADPYGRDFPY
 KVFLDVEGWVVPNGMSDCLPFSVAVSPDMYGDGVAWGAYEYV*

>NZFS3276 Cmv3467
 MLFTPKLLAVLLPALAAASTIPKRTDSGMSGDKSAAMSPDTFGAAPVPVLGDKTPDNISGDKYPVSGAGSSNIFGAAPVPVSGDKSDTPY
 TTGLVAIRSGSDFQYASVNASSGLFYIGESTKTGCAAVANCGNYPNITLITIHETEGTAYLDADVAGQQVFVAQSGALSFAHPHQSAF
 VSSPHGPYSNGFGVYNKGDGASFILNFTLANTFQACPSKKGGKPYQVFLDVPFGKDHVPSGKVADCLPFEIAVSSYKTGAGAYQYE
 *

>NZFS3276 Cmv3921
 MVATAAVLATLLPALAAASPLKQRAFLADYPYTTGLEALRSASPIHFSAVNASLGHHFFIGKATNTYCPVSDCSGFPNITVISIYEASGTAYM
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 VVPSGSIADCLPFGIAIYNSTGAGAFQYE*

>NZFS3276 Cmv4749
 MHAVAAIVAILPALAAASPLAKRAITFPYSAEWTALRSASPVHFQSLNANSSTIWIWIGKETATYCDPVADCESYINTTSITLRSGNAYMNVD
 VAGGQQLYIAASGAIEFTQPHSAAMPEGSVTGGWGAYTSENGIDMITNSAGTGFLACPTTDLTSYQVFADIEGIPNSAVPSGNTDDCLG
 FDIAASVVNDATAYEYL*

>NZFS3305 Cmn3003
 MYAAIIAAVLPALAVAGPIAERAITFPYSATLTLRSGSDIQYAAINANSQAFWIGKETDYDCPIGGCESYSNTTSITISGQSATMNSVVA
 GGQAVYVTLSGAIEFTQAHSAKPEGAITGGWGASTTANGVDIITFTGGGATTFLACPASTGGSPYQVFANVEGMVNSGVPNGVADCL
 LDISIAGSVVEGATAYQYD*

>NZFS3305 Cmn5237
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 GGQDVFAESGALSFTPHQEFSFPDGYTTGFAVTNNGNGVDLLNFTAEESFTACPVTPGEAPYQVFLTVPGFNDSVVPSGSTADCLG
 FGIYINSTGKGAQYQYE*

Figure A.30. Legend on page 310.

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>NZFS3305 Cmn7338
MLAPTVLAALLPILTSASPLDFLVPRQGTGATHSNQPAALAALDSGSPHYQSINASDNQFYIGLPTDVYCPLEPLCAGFPNITAVYPDD
GGRDGAGMDAVAPGGQDVFAESGALMFTTAHEEDDYPPNSYTDGGFEVQEYEDGALSELAFAGDASSWLACPITDDVDDSGMQ
LGPWQVFADVVGFNDVSVVPLGCVDECLGFDMLLQWIDVDAVEAAFEV*

>NZFS3325 Cmn2808
MYAAIIAAVLPALAVAGPIAERAITFPYSATLTSLRSGSDIQYAAINANSQAFWIGKETDYDCPIGGCESYSNTTSITISGQSATMNSVVA
GGQAVYVTLGAIEFTQAHSAAKPEGAITGGWGASTTANGVDIITFTGGGATTFLACPASTGGSPYQVFANVEGMVNSGVPSGNVADC
LDISIAGSVVEGATAYQYD*

>NZFS3325 Cmn5078
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FGIGIYNSTGKGAFQYE*

>NZFS3325 Cmn6869
MLAPTVLAALLPILTSASPLDFLVPRQGTGATHSNQPAALAALDSGSPHYQSINASDNQFYIGLPTDVYCPLEPLCAGFPNITAVYPDD
GGRDGAGMDAVAPGGQDVFAESGALMFTTAHEEDDYPPNSYTDGGFEVQEYEDGALSELAFAGDASSWLACPITDDVDDSGMQ
LGPWQVFADVVGFNDVSVVPLGCVDECLGFDMLLQWIDVDAVEAAFEV*

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Figure A.30. Amino acid sequences of *Cyclaneusma minus* Ecp32 family members in eight isolates. Cms: *C. minus* morphotype simile; Cmv: *C. minus* morphotype verum; Cmn: *C. minus* morphotype novus. The asterisk * following protein ID indicates the protein was wrongly predicted in the original annotation and the correct sequence was used.

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